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Title

An intrinsically disordered radish vacuolar calcium-binding protein (RVCaB) showed cryoprotective activity for lactate dehydrogenase with its hydrophobic region.

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Highlights

1. A protein with cryoprotective activity was purified from radish.
2. The cryoprotective protein was identified as a vacuolar calcium-binding protein.
3. The protein's hydrophobic area was involved in its cryoprotective activity.

Abstract

A soluble protein fraction from radish (*Raphanus sativus* L.) taproot had cryoprotective activity for lactate dehydrogenase (LDH). The activity was found mainly in the heat-stable fractions of soluble proteins. The cryoprotective protein, whose molecular mass was 43 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis, was obtained by successive chromatographies on TOYOPEARL SuperQ and TOYOPEARL DEAE. MALDI-TOF MS/MS analysis indicated that the purified protein was a radish vacuolar calcium-binding protein (RVCaB), which is reportedly related to calcium storage in the vacuoles of radish taproot. The purified RVCaB inhibited the cryoinactivation, cryodenaturation, and cryoaggregation of LDH. RVCaB had greater cryoprotective activity than general cryoprotectants. When RVCaB was divided into 15 segments (Seg01

to Seg15, 15 amino acids each), Seg03, which had a high hydrophobicity scale, showed remarkable cryoprotective activity. This indicated that RVCaB protected LDH from freezing and thawing damage presumably through a specific hydrophobic area (i.e., Seg03).

Keywords Cryoprotection; Dehydrin; Intrinsically disordered proteins; *Raphanus sativus* L.; Vacuolar calcium-binding protein

1. Introduction

The cold tolerance of plants is a crucial factor in determining plant production, vegetation formation, seed longevity, and so on [1, 2]. Knowledge of the molecular basis of cold tolerance has been applied to various technologies, such as postharvest storage and seed banks [3, 4]. The production of cryoprotectants, which can prevent damage to cells and biomolecules due to freezing, is involved in the cold responses of plants [5]. Compatible solutes such as betaine, proline, and sugars can act as cryoprotectants [6]. In addition, late embryogenesis abundant (LEA) proteins and cryoprotectins are known to be proteinous cryoprotectants [7, 8].

LEA proteins were produced in the late stage of seed maturation and in growing plants exposed to various stresses, including cold [9-11]. Genetic analyses and transgenic studies demonstrated that the accumulation of LEA proteins was correlated with seed longevity and stress tolerance in plants [9-12]. Although LEA proteins have been known to prevent the freeze denaturation of cold-sensitive enzymes [13], there are few reports of plant proteins other than LEA proteins showing cryoprotective activities for such enzymes.

Group 2 LEA proteins, also called dehydrins, are plant-specific and intrinsically disordered [9, 10]. Dehydrins are among the most-characterized LEA proteins. It has been repeatedly demonstrated that dehydrins are multifunctional: they protect freezing-sensitive enzymes, and bind to various molecules (such as lipids, water, ions, and nucleic acids) [14-16]. Among these functions, cryoprotective activity is thought to be a major role of dehydrins, as many studies have previously reported the issue [e.g., 17-20]. Genetic and transgenic approaches have found that the expression of dehydrin genes reduced cold damage to plants [21-25]. The *in vitro* and *in vivo* evidence supported that dehydrins are related to establishing cold tolerance in plants.

Previously, we found that taproot of radish (*Raphanus sativus* L.) contained dehydrin, which was detected by an antibody against a KS-type dehydrin of *Arabidopsis thaliana* (AtHIRD11) [26]. The radish dehydrin existed mostly in the high-salt extract of cell debris that had been pelleted by centrifugation of radish taproot homogenates. After purification, the radish dehydrin designated as RsDHN (*R. sativus* dehydrin) showed cryoprotective activity for malate dehydrogenase [27]. Thereafter, it was revealed that the soluble fraction of the taproot homogenate had considerable cryoprotective activity, whereas the soluble fraction contained a small quantity of antigens for the anti-AtHIRD11 antibody. This suggested that one or more cryoprotective proteins that are likely different from dehydrins might exist in the soluble fraction of the taproot homogenate.

In this paper, we report the purification of a cryoprotective protein from the soluble fraction of the taproot homogenate. The purified protein was identified as a radish vacuolar calcium-binding protein (RVCaB), which was proposed to be related to the sequestration of calcium in the vacuole. The protein was previously isolated from the vacuolar membranes of the radish taproot via ion exchange chromatography and gel

filtration chromatography [28]. Here, we proposed a simple purification protocol with a higher yield of RVCaB. In addition, we found that the cryoprotective site was located near the N-terminus of RVCaB. The putative mechanisms underlying the cryoprotective activity of RVCaB and its physiological roles in radish were discussed.

2. Materials and methods

2.1. Chemicals

Anion exchange resins, TOYOPEARL SuperQ-650M and TOYOPEARL DEAE-650M, were purchased from Tosoh (Tokyo, Japan). Lactate dehydrogenase (LDH, rabbit muscle, recombinant) and nicotine adenine dinucleotide (NADH) were obtained from Oriental Yeast (Tokyo, Japan). 8-Anilino-1-naphthalene sulfonic acid (ANS) and dithiothreitol (DTT) were purchased from Sigma (Tokyo, Japan) and Wako (Osaka, Japan), respectively.

2.2. Peptides

An automated solid phase peptide synthesizer (Tetras, Advanced ChemTech, Louisville, KY, USA) was used to prepare peptides (Seg01 to Seg15). The peptides were purified by an ultrafast liquid chromatograph (UFLC-20AB, Shimadzu, Kyoto, Japan) with a C18 reversed-phase column (Altima^{TM} 4.6 x 250 mm). A linear gradient of acetonitrile (5-95%) in 0.05% trifluoroacetic acid solution was performed for 25 min. The synthesized peptides were identified by mass spectrometry (LCMS-2020, Shimadzu) and lyophilized.

When the peptides were dissolved in solution for use, the peptide concentrations were determined from the dry weight.

2.3. Purification of RVCaB

European red radish (*R. sativus* L.), obtained from a local food market in Shizuoka, Japan, was used for the purification of RVCaB. Radish taproot (26 g fresh weight) was ground by a food grater on ice. The squeezed juice, obtained by passing the ground root through double gauze, was centrifuged at 10,000 g for 10 min at 4 ℃. The supernatant was collected in a 50-ml centrifuge tube (TPP, Trasadingen, Switzerland) and then DTT was added to reach a concentration of 1 mM. This sample was considered the crude extract (20.5 mL). The crude extract was placed in an aluminum block heater (dry thermo unit DTU-1B, TAITEC, Saitama, Japan) which was controlled at 100 ℃ for 40 min. After cooling for 10 min on ice, the formed aggregates were precipitated by centrifugation (at 10,000 g for 10 min at 4 ℃). The supernatant (20 mL, heat-stable fraction) was loaded onto the TOYOPEARL SuperQ-650M column (45 mm × 1.5 mm ID) at a flow rate of 1.2 mL min^{-1}. The column was washed with 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT (running buffer). Bound proteins were eluted with a linear gradient of NaCl (0 to 500 mM) in the running buffer by an Econo Gradient pump (Bio-Rad, Tokyo, Japan) at 1.5 mL min^{-1} for 25 min. The fraction size was approximately 3.3 mL. The cryoprotective fractions were combined (9.9 mL) and desalted by a gel filtration column (NAP-25, GE Healthcare, Tokyo, Japan) equilibrated with the running buffer. The sample was applied to the TOYOPEARL DEAE-650M column (40 mm × 1.5 mm ID) at a flow rate of 1.2 mL min^{-1}. After the column was washed with the running buffer, linear gradient

145 elution was performed as described above except that the change in NaCl concentration
146 was from 0 to 250 mM at a flow rate of 1.0 mL min^{-1}. The active fractions were combined,
147 desalted, and stored at -20 ℃ until use. The cryoprotective activity of the purified protein
148 was stable under this storage condition.
149 The amount of protein was determined from the band intensities in sodium dodecyl
150 sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with
151 Coomassie brilliant blue (Bio-Safe, Bio-Rad). The data for the electrophoretogram were
152 obtained by the Fusion Solo S imaging system (Vilber Lourmat, Collégien, France).
153 ImageJ software (https://imagej.nih.gov/ij/) was applied to quantify the protein bands.
154 Bovine serum albumin was used as a standard.
155
156 *2.4. Protein identification*
157
158 The purified protein was identified by using matrix-assisted laser desorption ionization
159 time-of-flight mass spectrometry tandem mass spectrometry (MALDI-TOF MS/MS) with
160 the oMALDI-Qq-TOF MS/MS QSTAR Pulsar i system (Applied Biosystems, Foster, CA,
161 USA). PEAKS *de novo* sequencing software was applied to predict the amino acid
162 sequence of the protein's fragments. A peak whose m/z was 2302 was matched to
163 ATADVEQVTPAAAEHVEVTPPK (acetylated at the N-terminus). A Mascot search
164 (http://www.matrixscience.com) and a BLAST analysis
165 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that the sequence was matched only to
166 a radish vacuolar calcium-binding protein (RVCaB, accession AB035900).
167
168 *2.5. Cryoprotective activities*

169

170 Cryoprotective activities were evaluated by the inhibition of cryoinactivation of LDH.
171 In some cases, the inhibition of cryodenaturation and the inhibition of cryoaggregation of
172 the enzyme were also tested. The following experiments were performed according to our
173 previous methods [29] with some modifications.

174 The inhibitory activity of LDH cryoinactivation was determined as follows. In brief,
175 the test solutions (30 μL) were mixed with the LDH solution (20 μL, 0.34 μM as a
176 monomer in 10 mM Tris-HCl buffer pH 7.5) in 1.5-mL plastic tubes. In the case of
177 peptides (Seg01-Seg15), the test solutions contained the peptides at a concentration of
178 275 μg mL^{-1}. Thus, the peptide concentration in all the tubes was 165 μg mL^{-1}. The tubes
179 were immersed in liquid N_2 for 1 min and then in a water bath at 25±2ºC for 3 min. This
180 freezing and thawing process was done three times, and then the LDH activity was
181 measured. The samples (4 μL) were added to the reaction solutions (196 μL), i.e., 9.5 mM
182 Tris-HCl pH 7.5, 0.58 mM sodium pyruvate, and 60 μM NADH, in a 96-well microplate.
183 Changes in absorbance at 340 nm were recorded at 25 ºC by using a microplate reader
184 (Varioskan Flash, Thermo Fisher Scientific, Tokyo, Japan). In most cases, the LDH
185 activities were decreased to approximately 20% of the initial activities by the freezing
186 and thawing treatment. To evaluate the cryoprotective activity, a value of relative
187 cryoprotective activity was used. The range of decrease in LDH activity after freezing
188 and thawing was standardized as 100% of relative cryoinactivation. For example, if a
189 sample recovered 70% of relative cryoinactivation, the relative cryoprotection value was
190 70%. This type of data representation was applied to Fig. 1C, Fig. 2B, and Fig. 5. Besides
191 that, when the inhibition of LDH cryoinactivation by different concentrations of RVCaB
192 was tested, the initial enzymatic activity of LDH (before freezing and thawing) was

193 relativized to 100%. This representation was done in Fig. 4A and Fig. 6A.

194 LDH cryodenaturation was assessed by an ANS fluorescence probe, which can
195 quantify hydrophobicity on the protein surface. Mixtures containing ANS (10 μM), LDH
196 (4 μM), RVCaB (corresponding concentrations), and 10 mM sodium phosphate buffer pH
197 7.0 were prepared in a total volume of 250 μL in 1.5-ml plastic tubes. After the three
198 freezing and thawing cycles, fluorescence was detected in the 96-well plates (Ex 350 nm
199 and Em 470 nm, Varioskan Flash). The increment of fluorescence enhanced by freezing
200 and thawing in the sample containing no RVCaB was standardized as 100% (Fig. 4B and
201 Fig. 6B).

202 The cryoaggregation of LDH was measured by turbidity. Test solutions (250 μL)
203 consisting of 4 μM LDH, corresponding concentrations of RVCaB, and 10 mM Tris-HCl
204 buffer pH 7.5 were prepared in 1.5-ml plastic tubes. The solution was treated with the
205 three freezing−thawing cycles described above, after which turbidity was determined in
206 a 96-well microplate (415 nm, Bio-Rad iMark). The increment of absorbance by freezing
207 and thawing in the sample without RVCaB was standardized as 100% (Fig. 4C and Fig.
208 6C).

209

210 **3. Results**

211

212 Cryoprotective activities were determined by the inhibition of cryoinactivation of
213 lactate dehydrogenase (LDH), a model of cryosensitive enzymes [30]. After the
214 homogenates from the radish taproot were centrifuged, the soluble fraction was analyzed
215 by SDS-PAGE (Fig. 1A, lane Sf). This fraction contained various proteins and showed
216 apparent cryoprotective activity (Fig. 1C, Sf). Since cryoprotective proteins (e.g.,

dehydrins) were known to be highly hydrophilic and thus heat stable, we decided to prepare the heat-stable fraction from the soluble fraction to obtain the cryoprotective proteins. Consequently, most of cryoprotective activity was maintained (approximately 87% of the activity in the soluble fraction) in the heat-stable fraction (Fig. 1C, Hs). SDS-PAGE analysis indicated that the heat-stable fraction contained a major protein whose molecular mass was approximately 43 kDa (Fig. 1A, lane Hs, an arrowhead).

Subsequently, an anion exchange chromatography (TOYOPEARL SuperQ) was applied. Proteins were eluted with a linear gradient of NaCl (0.0 - 0.5 M) (Fig. 1B). Fraction number 7, which showed the highest cryoprotective activity among the eluate fractions (Fig. 1C), primarily contained the 43 kDa protein (Fig. 1B, an arrowhead). The 43 kDa protein was also found in the 6th and 8th fractions, in which considerable cryoprotective activities were detected. On the other hand, fraction numbers 4, 5, and 9-13 showed cryoprotective activities even though they did not contain the 43 kDa protein, suggesting that proteins other than the 43 kDa protein and/or nonprotein molecules might contribute to the cryoprotective activity of the heat-stable fraction. However, we focused on the 43 kDa protein because its presence was predominant and reproducibly observed in the cryoprotective fractions. Fraction numbers 6, 7, and 8 were combined for the subsequent chromatography (TOYOPEARL DEAE). The results demonstrated that the amounts of the 43 kDa protein were well correlated with the cryoprotective activities through the eluate fractions (Fig. 2). The purity of the 43 kDa protein was 94.7%, and the yield on the basis of cryoprotective activity was 10% (Table 1).

A MALDI-TOF MS/MS analysis with a *de novo* sequencing method revealed that the 43 kDa protein contained the amino acid sequence ATADVEQVTPAAAEHVEVTPPK. According to a BLAST search, the sequence totally matched that of the corresponding

241 site (A_2TADVEQVTPAAAEHVEVTPPK$_{23}$) of a radish vacuolar calcium-binding
242 protein (RVCaB, accession AB035900) (Fig. 3). Considering a previous report that
243 detected RVCaB at the size of 43 kDa in SDS-PAGE [28], we confirmed that RVCaB was
244 the cryoprotective protein purified from radish taproot. RVCaB was first isolated from
245 the vacuoles of radish taproot [28]. The theoretical molecular weight of RVCaB was
246 27094 and the isoelectric point was calculated as 4.1 (Supplementary Fig. 1A). A previous
247 report described that RVCaB in SDS-PAGE (43 kDa) was larger than the theoretical
248 molecular mass (27 kDa), because generally acidic proteins slowly migrated in SDS-
249 PAGE [28]. The protein had small amounts of hydrophobic residues and neither aromatic
250 nor cysteine residues (Supplementary Fig. 1B). Analysis by using secondary structure
251 prediction software suggested that RVCaB had a primarily coiled structure
252 (Supplementary Fig. 1C). Indeed, RVCaB was demonstrated to be an intrinsically
253 disordered protein (IDP) in the previous study [31].
254 The concentration dependence of the cryoprotective activities of RVCaB was analyzed
255 (Fig. 4). Although the LDH activity was reduced to approximately 20% of the initial
256 activity after the freezing and thawing treatment, this reduction was mitigated by RVCaB
257 in a concentration-dependent manner (Fig. 4A). The protection dose 50% (PD_{50}) value
258 was 1.8 μM. Cryodenaturation was recorded by the fluorescence of ANS, which is a
259 hydrophobicity indicator during the denaturation process of proteins. Cryoaggregation
260 was determined by the turbidity of the protein solution. RVCaB inhibited the
261 cryodenaturation and cryoaggregation of LDH (Fig. 4B, C). The PD_{50} values for
262 cryodenaturation and cryoaggregation were 1.5 and 1.4 μM, respectively.
263 Finally, cryoprotective sites of RVCaB were investigated. RVCaB had two repeat
264 sequences, from E91 to E120 and from E121 to E150, which were totally identical (Fig.

3, represented by yellow highlights). In this work, the sequence was divided into 15 segments designated Seg01 to Seg15 (15 amino acids each). Seg07 and Seg08 covered the repeat sequences. The inhibitory activities of these segments for the cryoinactivation of LDH were determined (Fig. 5). As a result, four segments, Seg02, Seg03, Seg06, and Seg14, showed cryoprotective activities, with Seg03 being the most potent. This suggests that Seg03 was a major cryoprotective site of RVCaB. Seg03 inhibited the cryoinactivation, cryodenaturation, and cryoaggregation of LDH (Fig. 6). However, higher concentrations were needed in Seg03 than in RVCaB in order to examine the cryoprotective activities. The PD_{50} values for cryoinactivation, cryodenaturation, and cryoaggregation were 21, 17, and 21 μM, respectively.

4. Discussion

Here we report that a cryoprotective protein was purified from radish taproot and identified as RVCaB, a radish vacuolar calcium-binding protein. The protein was obtained from the soluble fraction of taproot via heat treatment and two-step anion exchange chromatographies. The purity of the final sample was approximately 95% (Table 1). Previous purification procedures required the isolation of vacuolar membranes, ion exchange chromatography, and gel filtration chromatography [28], indicating that the present purification method was simpler than the previous one. In a previous report, 100 μg of RVCaB was prepared from 2 kg of taproot [28]. In our method, on the other hand, 11 mg of RVCaB was purified from 26 g of taproot. This indicates that the purification efficiency of the present method was at least 4000 times higher than that of the previous method. Radish is a Brassicaceae vegetable that has been widely produced in Asia and

289 Europe [32]. Thus, using our purification procedure, RVCaB can be prepared as a
290 cryoprotectant from excessively produced radish. Circular dichroism (CD) analysis
291 previously determined that the RVCaB structure was disordered [31]. RVCaB purified in
292 the present study was also shown to be disordered by CD (Supplementary Fig. 2A),
293 suggesting that our purification procedures did not affect the structural characteristics of
294 RVCaB.

295 A comparison of our results against those of previous studies reveals that the
296 cryoprotective characteristics of RVCaB were similar to those of dehydrins, except that
297 the two amino acid sequences were totally distinct from one another. It is worth
298 mentioning that both RVCaB and dehydrins are IDPs [16, 31], which are highly
299 disordered in solution and hence heat stable. Both proteins were rich in hydrophilic amino
300 acids, although some hydrophobic amino acids were found. RVCaB and dehydrins
301 inhibited the cryoinactivation of LDH. Figure 4A shows that the protection dose 50%
302 (PD_{50}) value of RVCaB was approximately 1.8 μM (49 mg L^{-1}, calculated from a
303 molecular weight of 27110). On the other hand, the corresponding value of AtHIRD11
304 (an *Arabidopsis* dehydrin) was approximately 2.6 μM (28 mg L^{-1}, calculated from a
305 molecular weight 10796) [27], indicating that the two proteins inhibited the LDH
306 cryoinactivation to similar degrees. Moreover, their cryoprotective activities were
307 remarkably high, because the cryoprotection of common cryoprotectants such as
308 trehalose, proline, and glycine betaine occurred at concentrations of around 10 g L^{-1} [26].
309 Taken together, the present and previous results suggest that similar mechanisms, which
310 might be related to the length of the disordered region, underlie the cryoprotective
311 activities of RVCaB and dehydrins.

312 We found that Seg03 (V_{31}AAAVVADSAPAPVT_{45}) was the major cryoprotective site

313 of RVCaB (Fig. 5). Although the whole range of the amino acid sequence of RVCaB was
314 predicted to be highly disordered (Supplementary Fig. 3A, IUpred2A software [33]),
315 some hydrophobic areas were localized in the sequence (Supplementary Fig. 3B,
316 ProtScale software [34]). Intriguingly, the region corresponding to Seg03 was predicted
317 to be the most hydrophobic area through the sequence. The CD analysis demonstrated
318 that the structure of Seg03 was disordered (Supplementary Fig. 2B), suggesting that the
319 hydrophobic amino acids of Seg03 were likely to have been exposed to the solution.
320 Seg02, Seg06, and Seg14, which had low but significant cryoprotective activities, tended
321 to show hydrophobicity. Also, AtHIRD11 (98 amino acids in length) had two
322 cryoprotective segments, AtHIRD11_NK1 (M_1AGLINKIGDALHIG_{15}) and
323 AtHIRD11_Kseg (H_{41}KEGIVDKIKDKIHG_{55}), in which most hydrophobic amino acids
324 of the dehydrin were located [29]. AtHIRD11_Kseg was AtHIRD11's K-segment, which
325 is a conserved sequence in all dehydrins. It was demonstrated that hydrophobic amino
326 acids were required for the cryoprotective activity of the K-segment [35]. Thus,
327 hydrophobic amino acids may be related to the cryoprotective activities of RVCaB and
328 dehydrins.
329 Here, a putative mechanism for the cryoprotection of LDH by RVCaB was represented
330 (Fig. 7). It has been reported that, during the freezing and thawing process, hydrophobic
331 areas (i.e., hydrophobic patches) were exposed on the surface of LDH. The hydrophobic
332 patches may also have been formed during the deconstruction of tetrameric LDH due to
333 freezing and thawing. After that, LDH aggregated via the hydrophobic patches [36] (Fig.
334 7). The hydrophobic Seg03 of RVCaB may hinder the hydrophobic self-association with
335 cryo-damaged LDH. However, it is likely that the hydrophobic effect by Seg03 was not
336 the only factor that determined the cryoprotective activity of RVCaB, because the PD_{50}

337 value of Seg03 (21 μM) was approximately 12 times higher than that of RVCaB. A large
338 hydrodynamic radius due to the disordered nature of the structure might contribute to the
339 cryoprotective activity of RVCaB. On this point, RVCaB might stabilize LDH on the basis
340 of a preferential exclusion mechanism [37, 38] and an extended molecular shield
341 mechanism [39, 40] as well, both of which have been established as mechanisms of
342 protein protection. In the case of dehydrins, the cryoprotective activities were attributed
343 basically to the large hydrodynamic radius [41, 42], whereas transient hydrophobic
344 interaction without binding is needed to facilitate the cryoprotective activities [29]. In
345 addition, LEA proteins and small heat shock proteins, both of which prevent protein
346 denaturation, have been known to possess disordered regions [43]. Taken together, the
347 previous and present results suggested that the disordered nature is a crucial factor for
348 protective IDPs, including RVCaB.
349 Finally, a physiological role of RVCaB in radish was discussed. RVCaB was found
350 mainly in the taproot of radish and was little detected in leaves [44]. Since RVCaB has
351 been found in the vacuolar lumen of the taproot, it has been suggested that RVCaB
352 contributed to the sequestration of calcium ion to the interior of vacuoles [28, 45]. Here,
353 we added cryoprotective activity to RVCaB's functions. Generally, radish is grown in
354 autumn and harvested in winter. Since the radish taproot mainly consists of parenchymal
355 cells whose interiors are filled with vacuoles, preventing damage to vacuoles is important
356 for the taproot. Moreover, taproot is a crucial organ for the storage of nutrients for the
357 following spring. The cryoprotective protein RVCaB may be produced to reduce damage
358 to taproot from the cold of winter.
359
360 **Funding**

This work was supported by Grants-in-Aid (18H02222 and 19K22274) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

CRediT authorship contribution statement

H. Osuda: Experimentation and original manuscript writing. **Y. Sunano:** Experimentation. **M. Hara:** Supervision, conceptualization, and writing and editing of the manuscript.

Declaration of competing interest

All the authors declare no conflict of interest.

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534 **Figure legends**
535
536 **Fig. 1.** Separation of heat-stable proteins of radish taproot by an anion exchange chromatography
537 (TOYOPEARL SuperQ). (A) SDS-PAGE analysis of soluble fraction (Sf) and heat-stable fraction
538 (Hs) of radish. (B) SDS-PAGE analysis of elution fractions of TOYOPEARL SuperQ
539 chromatography. The heat-stable fraction was applied to chromatography, and bound proteins
540 were eluted with gradients of NaCl from 0 to 0.5 M. (C) Relative cryoprotective activities for
541 lactate dehydrogenase (LDH). Cryoprotective activities of samples were measured three times
542 (means ± SD). Bf means buffer blank. Arrowheads indicate the positions of the 43 kDa protein.

Fig. 2. Separation of heat-stable proteins of radish taproot by anion exchange chromatography (TOYOPEARL DEAE). (A) SDS-PAGE analysis of input sample (Is), unbound fraction (Ub), and elution fractions of TOYOPEARL DEAE chromatography. Bound proteins were eluted with gradients of NaCl from 0 to 0.25 M. Arrowhead indicates 43 kDa protein. (B) Relative cryoprotective activities for LDH. Cryoprotective activities of samples were measured three times (means ± SD). Bf means buffer blank.

Fig. 3. Amino acid sequence of RVCaB. The sequence of RVCaB (GenBank accession no. AB035900) [28] was divided into 15 segments (Seg01 to Seg15). An open bar indicates the position of a peptide fragment identified by MALDI-TOF MS/MS analysis with *de novo* sequencing. Yellow indicates the repeat sequences of Seg07 and Seg08.

Fig. 4. Cryoprotective activities of RVCaB. Cryoinactivation (A), cryodenaturation (B), and cryoaggregation (C) of LDH were inhibited by RVCaB. Relative enzymatic activity, relative fluorescence intensity, and relative turbidity are shown in cryoinactivation, cryodenaturation, and cryoaggregation, respectively. In the graphs, relative values after freezing and thawing (F/T) and before F/T are exhibited as broken lines. Data represent means ± SD (four experiments). Asterisks indicate significant differences ($p < 0.05$) from 0 μM RVCaB in the corresponding graphs.

Fig. 5. Relative cryoprotective activities of RVCaB segments for LDH. Cryoprotective activities of samples were measured four times (means ± SD). Bf means buffer blank. Asterisks indicate significant differences ($p < 0.05$) from Bf.

Fig. 6. Cryoprotective activities of Seg03. Cryoinactivation (A), cryodenaturation (B), and cryoaggregation (C) of LDH were inhibited by Seg03. Relative enzymatic activity, relative

569 fluorescence intensity, and relative turbidity are shown in cryoinactivation, cryodenaturation, and
570 cryoaggregation, respectively. In the graphs, relative values after freezing and thawing (F/T) and
571 before F/T are exhibited as broken lines. Data represent means ± SD (four experiments). Asterisks
572 indicate significant differences ($p < 0.05$) from 0 μM Seg03 in the corresponding graphs.
573
574 **Fig. 7.** Putative mechanism of cryoprotective activity of RVCaB for LDH. Native LDH (blue
575 ellipses) was damaged due to freezing and thawing (A). Although LDH is a tetrameric enzyme,
576 the scheme was simplified. During the freezing and thawing process, hydrophobic patches
577 (yellow ellipses) may form on the LDH surface [36]. LDH was then inactivated, denatured, and
578 aggregated (B). After RVCaB was added, RVCaB may have protected LDH via the hydrophobic
579 attractions between the hydrophobic areas (e.g., Seg03) of RVCaB and hydrophobic patches of
580 LDH (C).
581
582

Table 1 Purification of cryoprotective protein from radish taproot.

	Cryoprotective activity (U)*	Volume (mL)	Protein (mg)	Purity (%)	Purification factor (-fold)	Activity yield (%)
Crude soluble fraction	1,201	20.5	199.0	1.4	1	100
Heat-stable fraction	1,045	20.0	30.2	15.3	11	87
TOYOPEARL SuperQ	347	9.9	30.0	33.1	24	29
TOYOPEARL DEAE	119	9.9	10.7	94.7	67	10

*One U represents 2% of relative cryoprotective activity for LDH in 1 mL.

583 Table 1. Osuda et al.
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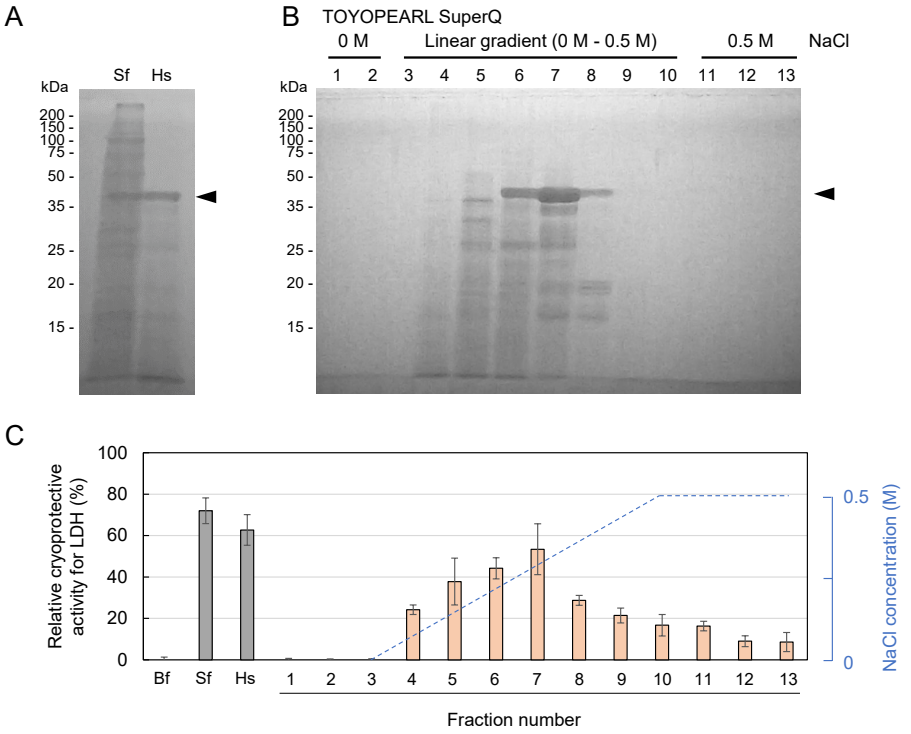

Fig. 1. Osuda et al.

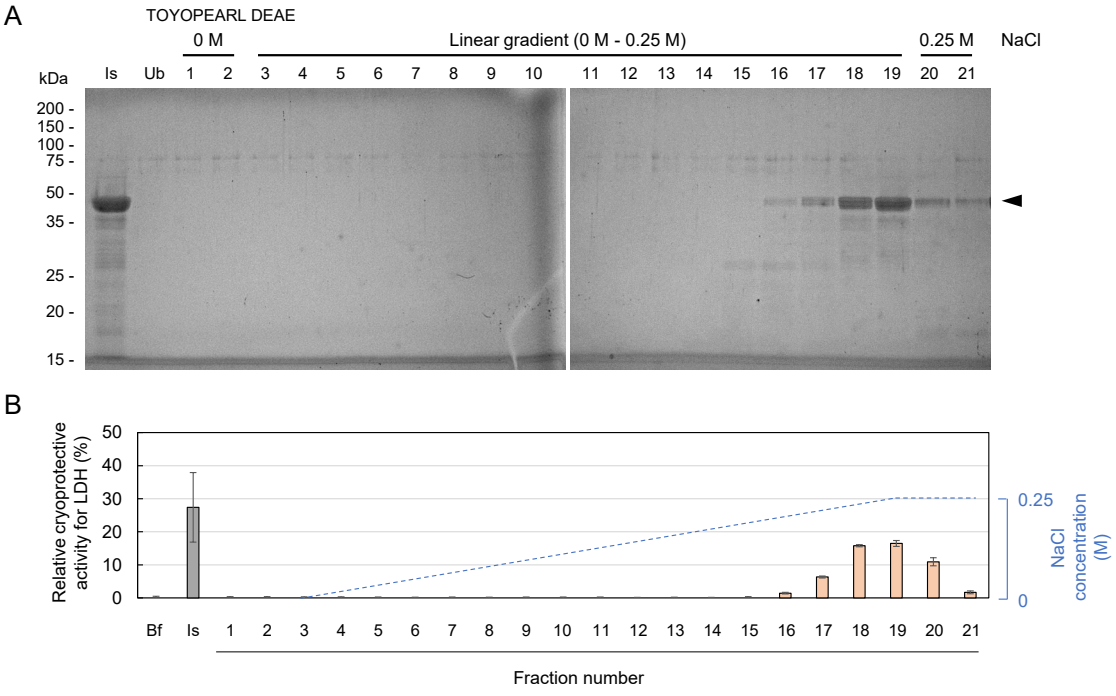

Fig. 2. Osuda et al.

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589 Fig. 3. Osuda et al.

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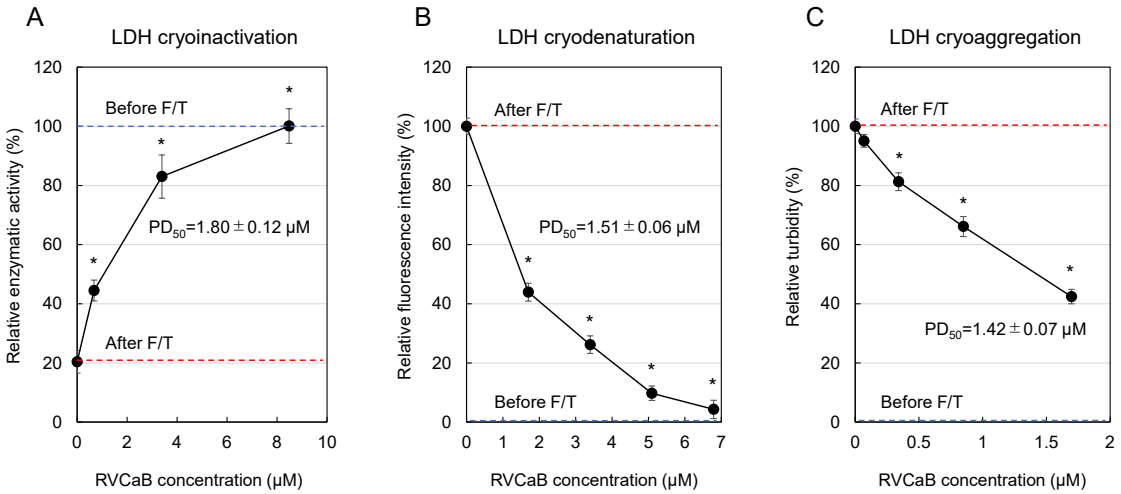

591 Fig. 4. Osuda et al.

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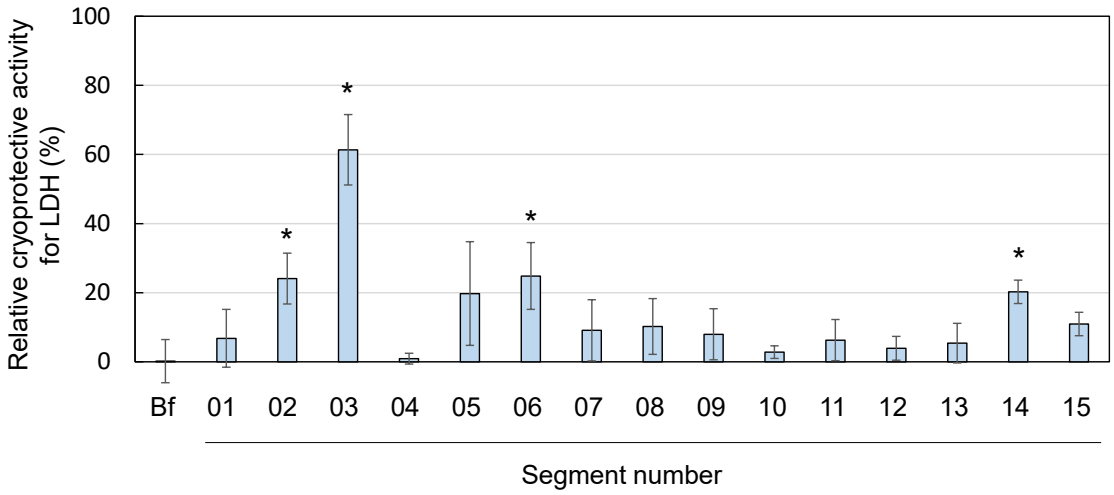

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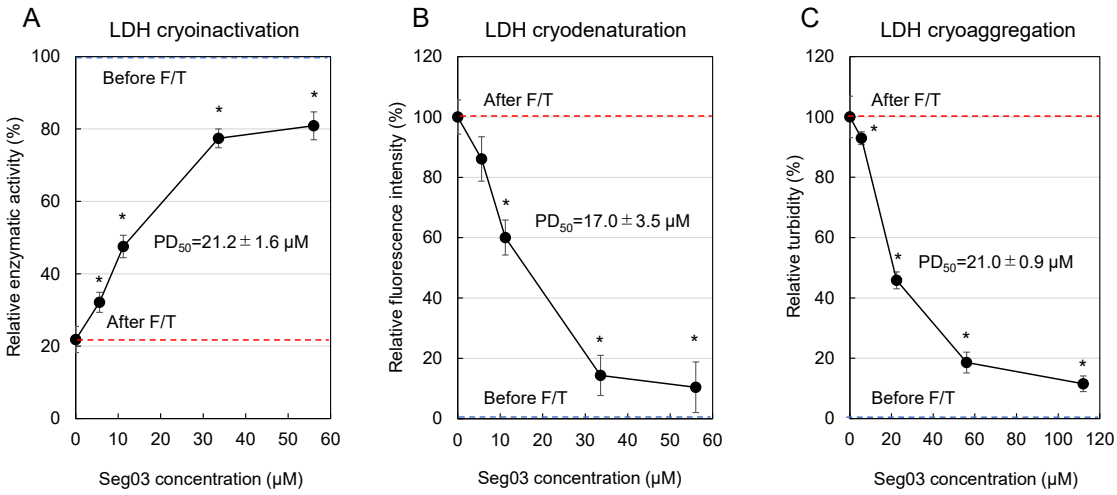

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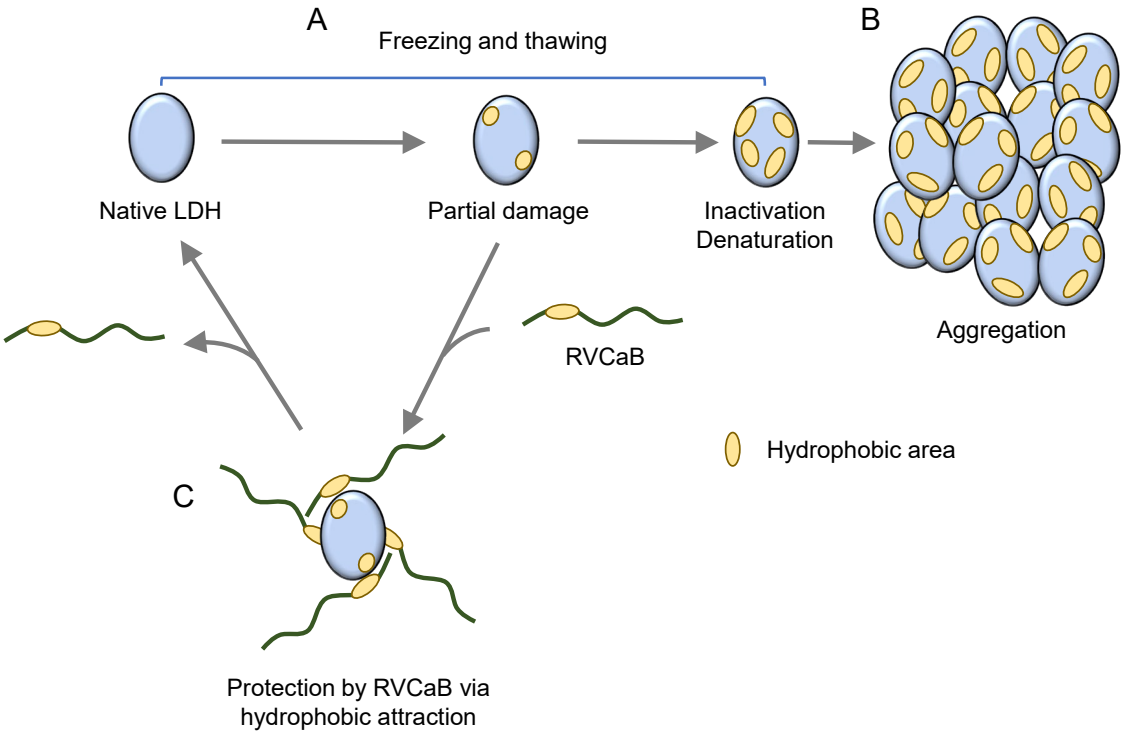
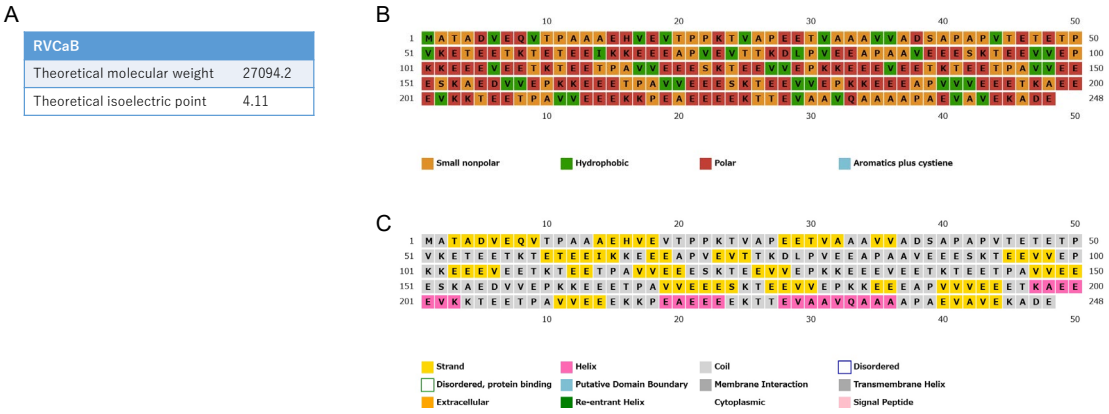


Fig. 7. Osuda et al.



Supplementary Fig. 1. Sequence properties of RVCaB. **A)** Theoretical molecular weight and theoretical isoelectric point. They were calculated by Compute pI/Mw (https://web.expasy.org/compute_pi/). **B)** Types of amino acids. It was produced by PSIPRED 4.0 (http://bioinf.cs.ucl.ac.uk/psipred/). **C)** Secondary structures predicted by PSIPRED 4.0.

Title: An intrinsically disordered radish vacuolar calcium-binding protein (RVCaB) showed cryoprotective activity for lactate dehydrogenase with its hydrophobic region.
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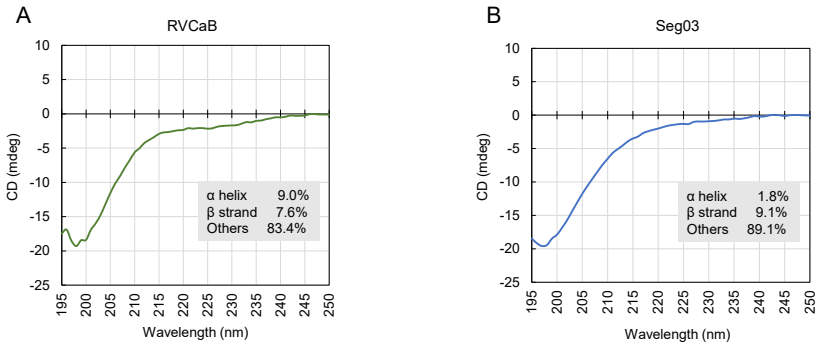

Supplementary Fig. 2. Circular dichroism (CD) spectra of purified RVCaB (**A**) and synthesized Seg03 peptide (**B**). Samples which were prepared in 10 mM Tris-HCl pH 7.5 at the concentration of 1.7 μM (46 mg L^{-1}) were analyzed by using a spectropolarimeter (J-820, Jasco, Tokyo, Japan). Scan range from 195 to 250 nm, scan speed 100 nm min^{-1}, resolution 1 nm, and cell width 2 mm. Secondary structures predicted from the CD data by K2D3 software (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/) are inserted in the graph.

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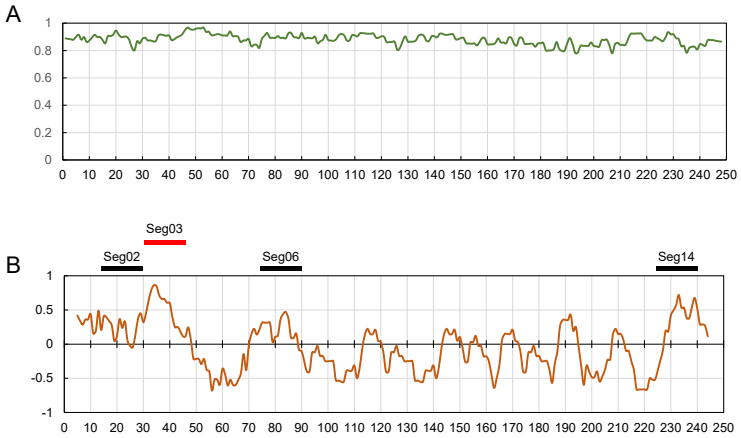

Supplementary Fig. 3. Disorder and hydrophobicity of RVCaB. (**A**) Disordered regions of RVCaB was predicted by IUpred2A (https://iupred2a.elte.hu/). (**B**) Hydrophobicity scale of RVCaB was produced by ProtScale (https://web.expasy.org/protscale/). Horizontal bars indicate the positions of segments which had cryoprotective activities (Seg02, Seg03, Seg06, and Seg14). The most potent cryoprotective segment was Seg03 (a red bar).

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