4. Discussion

4.1. Dehydrins expression from *E.coli*

Expression of recombinant dehydrins was performed in *E.coli* under the control of an IPTG-inducible promoter. We expressed dehydrin proteins with maximum expression levels established by Svensson et al. (Svensson et al., 2000). Total protein yield for analyzed dehydrins (LTI29, COR47 and ERD14) per 800 ml *E.coli* suspension was from 24 to 36 mg. Only for protein RAB 18 the protein yield was reduced (10,5 mg). The reason for this low production of RAB 18 could be due to the fact that cells expressing RAB 18 grew slower compared to the other constructs.
Overproduction of recombinant proteins in bacteria often results in formation of insoluble inclusion bodies (Choi et al., 1999). To study if this was the case for the proteins analyzed in this study, cells were lysed and soluble proteins were separated from insoluble proteins (Figure 4.2.). From these analyses it was clear that the recombinant proteins were localized in the supernatant and did not form inclusion bodies. This is in agreement with previous studies as no recombinant dehydrins have been reported to form inclusion bodies in studies before (Ismail et al., 1999; Houde et al., 1992).

Close *et al.* (1996) demonstrated that dehydrins remain soluble upon boiling, a property that can be used as an initial step in the purification of dehydrins.
Jepson and Close (Jepson and Close, 1995) reported that approximately 95% of the contaminating proteins precipitated during the heat fractionation. Using this treatment in our experiments approximately 55%-70% of the contaminating proteins precipitated, this was lower than what was reported by these authors during purifying a maize dehydrin in *E. coli*.
We decided to use as low temperature as possible, for the heat fractionation step as undesired chemical modifications such as deamidation, hydrolysis, and oxidation are more common at high temperatures.
It was by (Svensson et al., 2000) recommended precipitation in 80% of ammonium sulphate because of high hidrophility of these proteins. Ammonium sulphate has been removed by gel filtration.

Further SDS-PAGE analyses of expressed dehydrins demonstrate anomalous migration on SDS-PAGE (Figure 4.3.). As a result there is a difference among calculated MW (kDa) and MW detected on the gel (Tab. 4.1.).
This can be a consequence of predominantly hydrophilic regions of these proteins which have less binding capacity to SDS.

4.2. Freeze-thaw injury and cryoprotection of thylakoids membranes

With a view to determine possible cryoprotective activity of analyzed dehydrins "freezing test" was used. This test is based on ability of cryoprotective active substances to protect thylakoids during freeze-thaw cycle (Hincha and Schmitt, 1992a; 1992b).

It was shown that in plant's tissue during the freezing process ice first crystallizes in extra cellular space. Since the water potential of ice is lower at the same temperature than that of liquid water, cellular water diffuses from the cells to the extra cellular ice crystals. As a consequence of this passive diffusion the cells dehydrated, until equilibrium is reached. Therefore, the freezing process in plant tissues is somehow process of dehydration. Under equilibrium conditions, the osmolality of a solution coexisting with ice is a function of the temperature. On constant temperature, the volume of the unfrozen phase is therefore determined by initial solute concentration of the unfrozen solution.
Similarly thylakoids in freeze-thaw cycle are exposed to process of losing water during freezing until equilibrium is reached. Opposite, during the thawing, the water potential gradients are reversed and water diffuses back to the thylakoids. During this process thylakoids collapsed. There were two possible reasons why this physical (mechanical) thylakoids injury accrued:

a) Hincha et al. (Hincha et al., 1986) points that the ability of thylakoids to expand during thawing is reduced. While unfrozen thylakoids responded as osmometers when subjected in a wide range of sucrose concentrations, the frozen-thawed thylakoids vesicles ruptured when suspended in dilute solutions before freezing. Capacity of thylakoids to re-expand was less than that of unfrozen controls.

b) Thylakoids take up external solutes during freezing. This has been reported for sucrose (Hincha 1986) and sorbitol (Wiliams and Meryman, 1970).

During freeze-thaw cycle in presence of cryoprotective substances (cryoprotective proteins or sugars) thylakoids remain intact.
The mechanism of cryoprotective activity of dehydrins in this system was of largest interest for us. This system was also suitable for further analyses because of:

1. Thylakoids can be readily isolated in a functionally intact form.
2. It is much known about structure and biochemical activities of photosynthetic membranes.

4.3. Cryoprotective activity of dehydrins expressed in *E.coli*

Biochemical analyses of dehydrins have shown that spinach COR85, maize G50, wheat WSC120 and peach PCA60 have cryoprotective activity (Close, 1996; Houde *et al.*, 1995; Kazuoka and Oeda, 1994; Wisniewski *et al.*, 1999). The cryoprotective activity was measured in a lactate dehydrogenase freeze-thaw test (Carpenter and Crowe, 1988). Also PCA60 dehydrin from peach was demonstrated to posse anti-freeze activity (Wisniewski *et al.*, 1999).

Using a method established by Hincha and Schmitt (Hincha and Schmitt, 1992a; 1992b) the cryoprotective activity of four recombinant dehydrins was measured. This method is based on the ability of proteins to protect thylakoids during a freeze-thaw cycle. When thylakoids from non acclimated plants are frozen and thawed, they rupture and collapse. In presence of substances which can protect membranes during freeze-thaw cycle thylakoids remain firm. Collapsed thylakoids can be distinguished from non collapsed ones by a volumetric hematocrit centrifugation assay. Ratio of protected and ruptured thylakoids after freeze-thaw cycle makes a level of cryoprotective activity in percentage. The method is explained in details in chapter 2.
This method was used in the analyses of cabbage cryoprotectin (member of the nonspecific plant lipid transfer protein gene family). Method has been proved by detection of membrane rupture indicated by the release of electron-transport protein plastocyanin from the thylakoid lumen in vitro (Hincha et al. 1990). Namely, the substances that show high cryoprotective activity (cryoprotectants) in the freezing test also in the parallel experiment caused lessening of plastocyanin release from the thylakoid lumen.

Using freezing test three of four dehydrins according to our results have cryoprotective activity (Fig.3.6.).
Statistical analyses were done with a view to determine standard deviation. Percentage of cryoprotective activity for each analyzed protein was similar in several repetitions and the low value for standard deviation was found (Tab.3.2). These are the first results which confirm that dehydrins (LTI29, ERD14, COR47) from *Arabidopsis thaliana* have cryoprotective activity.

For dehydrin RAB 18 the analyses pointed out that this protein has low cryoprotective activity (Fig.3.6.).
If we take under consideration the results obtained from Svensson et al.,(Svensson et al., 2000) it can be seen that RAB 18 was ABA regulated and that cold acclimation has no influence on the level of this protein in the tissue. In contrast, LTI29 and COR 47 were accumulated primarily in response to low temperature. Furthermore, RAB 18 was the only dehydrin from *Arabidopsis thaliana* detected in seeds. This point out a different function of RAB 18 in comparison to other analyzed dehydrins.

Our results point (Fig.3.6.) that cryoprotective activity of analyzed dehydrins is lower in relation to positive control (CCP). This difference in cryoprotective activity between dehydrins and CPP can be caused by differences in protein's functioning mechanism, by different protein structures, or by the differences in binding ability of the proteins to the thylakoids membranes used in this test.

4.4. Additive effect (synergetic effect) between dehydrins and CPP

In aim to find out whether mutual interactions between dehydrins have influence on the total cryoprotective activity we tested combinations of dehydrins for additive/synergistic effect in the freezing test. Tab. 3.3. presents measured individually and mutual cryoprotective activity for dehydrin COR47 and cryoprotectin (CPP). Different concentrations of CPP in this test were used.

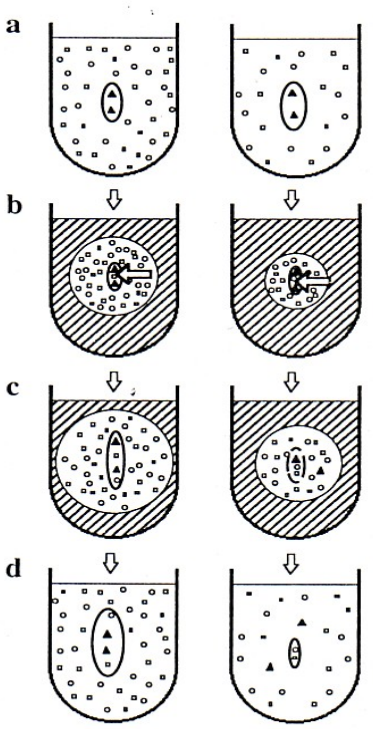
According to the results these two analyzed proteins can have a cooperative effect. Namely, if they are present in the solution together, the final cryoprotective activity increases compared to cryoprotective activity measured for these two proteins separately.

On the Fig. 3.10. can be seen a percentage ratio of individually calculated and measured cryoprotective activity for these two proteins. From these results it could be concluded that dehydrin proteins did not inhibit the activity of CPP and conversely.

Additional experiments have shown similar results when other analyzed cryoprotective dehydrins were combined. In fact, the cryoprotective dehydrins (LTI29, COR47 and ERD14) act cooperatively according to our results. (Fig.3.10.).
When the cryoprotective activity of one of the cryoprotective proteins is measured in the combination to RAB 18 protein, different result was obtained. RAB 18 in the combination to other dehydrin proteins or CPP decreased cryoprotective activity.

4.5. Boyle van't Hoff plot – analyzing of thylakoid membrane permeability in presence of added dehydrin proteins.

Freezing test used for determining the dehydrins cryoprotective activity is based on ability of cryoprotectant to prevent thylakoids vesicles rupturing after freeze-thaw cycle. The cryoprotectivity degree depends on cryoprotectant concentration.



If the thylakoids are subjected in different concentrations of cryoprotective substance (sucrose), their ability to stay protected varies. Hincha and Schmitt explain the process that is happening during the freeze-thaw cycle when the thylakoids are subjected in high and low concentrations of sucrose, separately (Fig.4.1.). Influx of water, during thawing, is dependent on the concentration gradient between both sides of the thylakoid membrane. Because of the difference in the concentration inside and outside of thylakoid vesicle during thawing vesicle swell osmotically, and water permeate membrane by diffusion. When thylakoids are subjected in the high initial solute concentration they swell but have enough osmotic support by the medium, to that rupture is prevented (Figure 4.1.d.left). Thylakoids that have initially been suspended in dilute solutions will swell excessively and finally rupture (Figure 4.1.d.right).

Figure 4.1. Schematic representation of an in vitro freeze-thaw process.

a) Thylakoid membrane vesicles (open ovals) are suspended in solutions of high (left column) and low (right column) solute concentrations.
b) Freezing removes water to give pure ice (hatched area). Membranes and solutes are concentrated in a small volume. Driven by the steep gradient, solutes permeate the membranes.
c) During thawing solute concentrations is decreased by the melting ice. The thylakoids swell osmotically and may rupture (second column).
d) After thawing, thylakoids have either increased in volume due to solute influx (first column) or, after rupturing, resealed and decreased in volume (second column).

This figure is assumed from D.K. Hincha and J.Schmitt paper. (Hincha and Schmitt, 1992).

By constant pressure and temperature the process of diffusion explicitly depends on concentration on both sides of the thylakoid membrane. In those conditions thylakoid vesicles react with a change in volume to a change in the osmolality of the external solution (sucrose). By this, a volume of measured thylakoids depended only on the concentration of sucrose in which they were subjected. A plot of the vesicle volume as a function of the reciprocal osmolality of the suspending solution will yield straight line with the slope RTn according to Boyle-van't Hoff equation (results).
If the presence of dehydrin proteins changes the thylakoid's membrane permeability then the correlation between measured thylakoid volumes after

thawing and reciprocal osmolality of the suspending solution will be different to control obtained after freezing-thaw cycle (without protein).

Therefore we used Boyle-van't Hoff plot in order to see whether presence of dehydrin proteins can have an effect on the membrane permeability during swelling of thylakoids (by thawing).

Fig. 3.14. shows measured osmotic response of thylakoids kept at 0°C and -20°C in solutions containing different sucrose concentrations, with and without cryoprotective dehydrin LTI29.
In unfrozen control samples packed thylakoid volume increases with increasing reciprocal osmolality over the whole sucrose concentration range.

In frozen-thawed samples, this increase in volume was only evident at high sucrose concentrations. Furthermore under these conditions, (high sucrose concentrations) thylakoid volume was higher after a freeze-thaw cycle than in unfrozen controls. The increase of slope can be attributed to an increase in the amount of osmotically active solutes inside the vesicles during freezing.
When thylakoid membranes are suspended in solutions of low osmolality external osmotic support is insufficient and the loaded vesicles rupture during thawing, resulting in a loss of internal solutes and reduced volume (thylakoids rupture).

According to our results this decreasing in volume of thylakoids is prevented at low sucrose concentrations with the presence of cryoprotective dehydrin LTI29.
This points out to the conclusion that cryoprotective dehydrins protect thylakoids also in the case when the sucrose concentration is not sufficient to protect thylakoids.
From the Fig. 3.14. can be also seen that at 100 1/Osm (conditions of freezing test) dehydrins protect membrane in a range of 50 % in relation to 100% protection when thylakoids are incubated at 0°C (K (0°C).

In contrast, thylakoids frozen and thawed in absence of added cryoprotective dehydrin LTI29 at low sucrose concentrations (high 1/Osmolality) rapidly collapsed which is indicated by lower measured thylakoid volume.

Thylakoids frozen-thawed in the high sucrose solutions (low 1/Osmolality) in the presence of the protective dehydrins LTI29 show similar curve of osmolality versus volume as the frozen-thawed control (without added dehydrin) does. According to these results it wasn't possible to give any clear statement about the influence of these proteins on membrane permeability in the frozen state (Fig. 3.14.).

Therefore we focused our future work on statistical analyses of several experiments to check whether thylakoids behaved as the ideal osmometers in whole 1/Osm range.

In order to check this doubt, we calculated residual (distance of each single point from average regression line) in whole analyzed 1/Osmolal range (4 -100 1/Osm (milliosmol/kg^{-1}).
These analyses show that the deviation from regression line is not random (Fig. 3.17. b) but in the range of sucrose concentrations from 4 -15 1/Osm (milliosmol/kg^{-1}) the little deviation was noticed (Fig. 3.17. c.). This is confirmed by calculation of residual for this range which is shown on Fig. 3.17. d.

Little deviation from regression line means that only in this range thylakoids behaved as the ideal osmometers. The similar relation was found in presence of dehydrin LTI 29 on 0°C and -20°C.

Figure 3.18. shows Boyle van't Hoff plot which was obtained by analyzing high sucrose range in presence of dehydrins (4 -15 1/Osm (milliosmol/kg^{-1}). Namely, after freezing (at -20°C) and thawing, thylakoids expanded to larger volumes (K(-20) than in unfrozen controls (K(0)), (Figure 3.18 a.) indicating by this an influx of solutes during freezing. Also, under these conditions the volume of freeze-thawed thylakoids in the presence of cryoprotective dehydrin (LTI29(-20)) is measured as larger than those in the control (without protein K(-20)), what correlates with more uptake of solute.
Obtained results are indicating on an increase of permeability of the membranes in the presence of added dehydrin, which was not apparent in our previous experiments, in which we used a broader range of initial solute concentrations.
Thylakoids after freeze-thawing in high sucrose solutions in the presence of dehydrin RAB18 (RAB18(-20)) showed the similar curve as the frozen controls did indicating by this the unchanged solute permeability of the membranes under freezing conditions, in contrast to other analyzed dehydrins LTI29, COR47 and ERD14.

From these results we can conclude that the protective effect of dehydrins is maybe, at least partially, correlated with an increase of solute permeability of the membranes.

This is contrary to the results obtained for CPP protein which in the freeze-thaw cycle reduces the membrane permeability (Hincha et al., 1990).

If CPP and dehydrin proteins have different activity on the membrane permeability according to analyses by Boyle van't Hoff plot, then this difference can be expected also in the analyse during long time incubation at 0°C. Fig. 3.19. shows plotted slopes that were obtained from Boyle van't Hoff plots versus time in the presence of CPP and dehydrin LTI29. At long time incubation in the presence of CPP thylakoids collapse (decreased thylakoid volume was measured), as it was in the case of control.
Contrary to this, in the presence of added dehydrin LTI29 thylakoids volume remained unchanged. This clear difference in activity of these two cryoprotective proteins led to the conclusion about the different mechanism of cryoprotective activity. We assume that LTI29 stabilizes thylakoid membranes which can be one of the reasons for cryoprotective activity of those proteins.

Later analyses of the thylakoids sedimentation activity in the presence of added CPP and dehydrin LTI29 support this assumption. Namely, in the presence of added CPP the thylakoids sediment after few hours where in the presence of added LTI29 the thylakoid sedimentation is fully avoided (Fig. 3.36.). Also the degree of sedimentation level in the presence of CPP depends on presence of sucrose in the thylakoid suspension (Fig.3.36).

4.6. Binding of dehydrins with thylakoid membranes

Several studies suggested that dehydrins interacts with membranes in the interior of the cells and reduces dehydration induced damages (Ismail et al., 1999). Structural analysis of dehydrins suggest appearance of an α helical amphipathic domain responsible for the mechanism of binding to the membrane. Wheat dehydrin WCOR410 was found to associate to the plasma membrane and is suggested to stabilize plasma membrane during freezing (Danyluk et al., 1998). These authors suggest three possible modes:

a) by replacing water and thereby solvate membranes
b) prevent interactions between membrane bilayers, reducing fusion of membranes and lamellar to hexagonal II phase transition (Introduction) and
c) by forming salt bridges to ions preventing the damaging effect by increased ionic concentrations (Danyluk et al., 1998)

Investigating the interactions of proline, serine and leucine with isolated spinach thylakoids Popova (Popova et al., 1998) concludes that amino acid proline reduces osmotic membrane rupture during thawing. Likewise, this amino acid was bound to the thylakoid membrane in contrast to serine and leucine. Sror (Sror et al., 2003) showed that the protective effect of CPP (cryoprotection from Cabbage) is, at least in part, based on the binding of CPP to the thylakoid membrane.

In this work the binding of dehydrins with thylakoid membranes has been studied as a possible mechanism of cryoprotective activity by dehydrin proteins. According to our western blot analyses (Figure 3.12.) dehydrin LTI29 is bound to the thylakoid membrane. Antibodies raised against isolated LTI29 identified a 47 kDa protein in the western blots of thylakoid pellet proteins after several washing steps. Western blot for RAB18 dehydrin (with a low cryoprotective activity) showed absence of this protein in thylakoid pellet in the same procedure.
This suggests that cryoprotective dehydrins have binding ability with the thylakoid membrane which can be included in complex mechanism of their cryoprotective activity.
Newer studies point out the fact that dehydrin is present in the thylakoid´s stroma medium as well as that it can also be bound to thylakoid membrane (Mueller et al., 2003) what confirms the results obtained in this work.

4.7. Light Scattering Analyses (LSS) – analyzing of spinach thylakoid permeability membranes for glucose and sucrose

In order to better understand the results obtained in Boyle van´t Hoff analyses we analyzed the membrane's permeability by light-scattering technique. This technique was used to measure the volumetric changes associated with the efflux of water from thylakoid lumen after adding of osmotic substances such as sucrose and glucose in thylakoid suspension.
Bakaltcheva and Hincha (Bakaltcheva and Hincha, 1995) showed that concentrations of glycerol and glucose used in LSS technique have influence on membrane permeability. By increasing the concentrations of glycerol the membrane permeability (relative permeability coefficient) also increased but in the certain span from 150 mM to 1M (final concentration).
According to these results we have planned to test membrane permeability after adding glucose or sucrose in presence of dehydrin proteins and CPP.

4.7.1. Effect of sucrose or glucose to osmotic activity of thylakoids in Light scattering system

When highly concentrated glucose solution is added in the thylakoid suspension, the vesicles initially lose water and shrink, and than begin to swell when glucose and its associated water diffuse into vesicles, following the concentration gradient. These volume changes can be monitored using changes in light scattering properties of the suspension. Unlikely to this, by the presence of sucrose in membrane thylakoid suspension, vesicles initially lose water and shrink but oppositely, vesicles do not swell. Reason for this occasion is that sucrose can't permeate membrane, as would be expected for a larger molecule with more hydroxyl groups Figure 4.2.

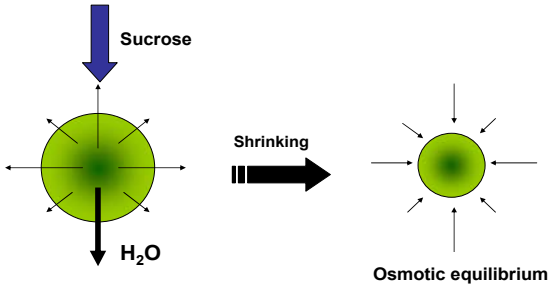


Fig. 4.2. Schematic representation of process of establishing the osmotic equilibrium after adding of sucrose in the thylakoid suspension (without added dehydrin protein).
Thylakoid membrane vesicles after adding of sucrose in solution initially lose water and solute and shrink until the osmotic equilibrium is reached. Sucrose can not permeate thylakoid membrane in absence of dehydrin LTI29.

In our light scattering analyses the difference in LSS signal after adding glucose and sucrose separately in thylakoid suspension was noticed (Figure 3.21.).
In the same time it was noticed that the amplitude of LSS is a function of concentration of added glucose (Figure 3.22.) as it was shown for glycerol previously (Bakaltcheva and Hincha, 1995).

4.8. Light Scattering Analyses (LSS) – analyzing of spinach thylakoid permeability membranes for glucose and sucrose in presence of added dehydrin protein LTI29 and cryoprotectin (CPP)

In further experiments in the thylakoid suspension a 60 µg/ml (final concentration) of cryoprotective LTI 29 protein were added. In the presence of added protein in thylakoid suspension, adding of sucrose (100 mM final concentration) causes the process during which thylakoid's vesicles initially lose water and shrink. Then very fast they begin to swell pointing to the influx of sucrose and its associated water into the vesicles, following the concentration gradient (Fig.4.3.). It would lead us to the conclusion that thylakoid membrane becomes more permeable for sucrose in the presence of dehydrin protein. This was confirmed by LSS recorded signal shown on Fig. 3.32.
The results obtained in sucrose permeation experiment indicate increased permeability of thylakoid membrane in the presence of dehydrin LTI29.

This is in correlation to the results obtained from Boyle van't Hoff analyses that membrane becomes more permeable after freeze-thaw cycle at high sucrose concentrations (low 1/osmolality) in presence of cryoprotective active dehydrins.

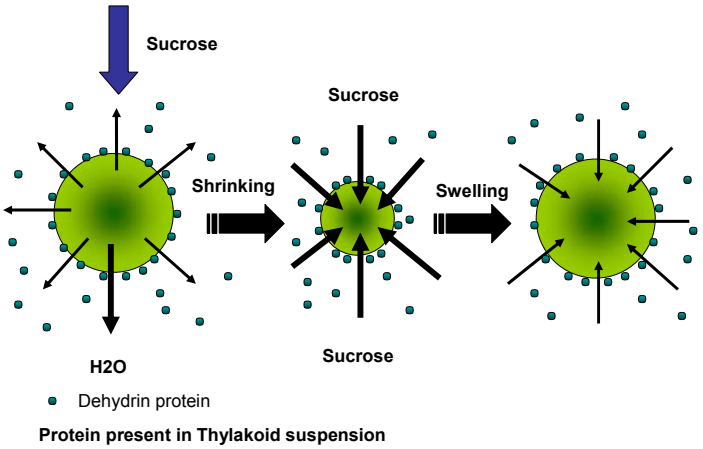


Fig.4.3. Schematic representation of process of the osmotic equilibrium establishing after adding of sucrose in the thylakoid suspension in the presence of added dehydrin LTI29 in thylakoid suspension.
Thylakoid membrane vesicles after adding of sucrose in solution initially lose water and shrink,and than begin to swell when sucrose and its associate diffuse into the vesicles, following the concentration gradient. These volume changes can be monitored by using changes in the light scattering properties of the suspension.

Further LSS analyses point out on new unknown effects recorded by LSS signal in the presence of analyzed proteins. In the presence of CPP and LTI29 the two different LSS signals were recorded. After adding only CPP in the thylakoid suspension the intensity of LSS increases (Fig.3.28.). In the case when only LTI29 was added the recorded LSS signal remains unchanged (Fig.3.32. a).
Also, the amplitude of increased LSS signal was dependent on the concentration of added CPP protein (Fig.3.25.).
We hypothesized that this increased LSS signal can be a consequence of forming thylakoid aggregations in presence of CPP. This was confirmed by light microscope analyses (Fig.3.34.) by which the clusters of thylakoids were noticed in the presence of CPP. The dimensions of clusters were in correlation to CPP concentration. In the presence of LTI29 the clusters were also noticed, but dimensions of clusters were much lesser.
These results can be attributed to the different activity of CPP and LTI29 on thylakoid membranes.

It is important to say that in previous analyses of the influence of CPP on the intensity of LSS signal, the difference in LSS signal amplitude was noticed in

case of glucose contrary to sucrose (compare Fig. 3.25. and Fig. 3.28.). Increasing of CPP concentration in thylakoid suspension and further adding of glucose causes decreasing of LSS signal amplitude (Fig. 3.25). Opposite, when sucrose was added in thylakoid suspension (CPP was already added) the amplitude of LSS signal was increased (Fig. 3.28).
Namely, the difference in LSS signal amplitude which accrues because of adding of different sort of sugar, could be the effect of different largeness of these two sugars molecule or the possibility of reacting with the membrane in the presence of cryoprotective proteins.
But all the above said possibilities remain still at the speculation level for only future analyses could bring further more reliable conclusions.

Hincha and Schmitt (Hincha and Schmitt, 1989) have shown that CPP decreases membrane permeability in freeze-thaw cycle, opposite to this, we have found that cryoprotective dehydrins increases membrane permeability in Boyle van't Hoff analyses. The difference in activity of those two proteins on the membrane permeability was also shown in presence of Ca^{2+} and Mn^{2+} ions in thylakoid suspension. Namely, according to our results the presence of divalent ions Ca^{2+} and Mn^{2+} in thylakoids suspension change activity of CPP on the membrane permeability, contrary to LTI29 where this effect misses (Fig.3.32.). For details see 4.8.

Furthermore the difference of activity among these two proteins was noticed in sedimentation experiments. These analyses show that the thylakoids in the presence of CPP or sucrose sediment faster. The presence of dehydrin LTI29 at long incubation time hinders the thylakoids sedimentation (Fig.3.35). This can be linked to the results obtained when slopes of Boyle van't Hoff plots were plotted as a function of incubation time (Fig.3.19.). According to these results apparent thylakoid volume in the presence of LTI29 remains unchanged at long incubation time (up to 10 h), what differs from results in the case of CPP present where the thylakoid volume became lower after 4 h approximately. All above said lead to the conclusion that LTI29 contrary to CPP protects thylakoids for longer period of time.

Fig. 3.29. shows the LSS signal kinetics after multiple adding of same amount of sucrose (200 μl 1M) in the thylakoid suspension. In this case the amplitude of LSS signal in the presence of CPP was higher after several adding of sucrose in the thylakoid suspension, contrary to the control.

4.9. Influence of Ca^{2+} and Mn^{2+} ions on LSS signal

Earlier works connected to thylakoid membrane's permeability show that the presence of Mn^{2+} and Ca^{2+} ions leads to the change of membrane permeability. Also it has been shown that the Mn^{2+} ions have defined binding places to the thylakoid's membrane (Takahashi and Asada, 1986).

In our results by adding of $MnCl_2$ and $CaCl_2$ in the thylakoid suspension the high increase of LSS signal occurres (Fig. 3.30. b.). In the presence of CPP this effect is even more discernible (Fig. 3.30. a).
At this moment it is important to say that the presence of Mn^{2+} and Ca^{2+} ions can also lead to the change in optical characteristics of analyzed suspension, which leads to the changes in LSS signal (Fig. 3.30.b) Adding of such a small amount of $MnCl_2$ or $CaCl_2$ (1 mM final concentration) can not be interpreted as a osmotic thylakoids volume change. There are two explanations whether presence of Ca^{2+} and Mn^{2+} ions changes spectroscopic properties of the membrane or their presence changes the membrane's permeability.

Analyzing and comparing the curves of LSS signals with and without the Mn^{2+} and Ca^{2+} ions in the presence of CPP the difference was noticed (Fig.3.31.).

Swelling and shrinking speed of thylakoid vesicles is in correlation to membrane's permeability. Fig. 3.31b shows that in the presence of Mn^{2+} and Ca^{2+} ions after the adding of sucrose, faster swelling and shrinking of thylakoids occurres, in comparison to (Fig.3.31a) where the Mn^{2+} and Ca^{2+} ions were not present. Those results point out to the increased permeability of membrane in the presence of Mn^{2+} and Ca^{2+} ions and in the presence of previously added CPP in thylakoid suspension. It is interesting to mention that in the similar experiment (Fig.3.32.) where the protein LTI29 was analyzed the differences of the LSS signal kinetics with and without Mn^{2+} and Ca^{2+} ions is hardly noticed. It is possible to conclude that the presence of Ca^{2+} and Mn^{2+} ions does not have a big influence on the LTI29 protein activity.

4.10. Influence of LTI29 concentration on the LSS signal kinetics

That the concentration of LTI29 influences the amplitude of LSS signal is shown on the Fig. 3.33. By increasing the LTI29 concentration in the thylakoid suspension, the amplitude of LSS signal after adding of sucrose proportionally decreases. In the case of higher LTI29 protein concentration in suspension (final concentration 100 μg/ml) LSS peak is not even noticed by such a high concentration (Fig.3.33. d). We assume that in this case the thylakoid membrane becomes permeable in such a high extent that after adding of sucrose, the osmotic activity of thylakoids is not present. By this it is again confirmed that the dehydrin LTI29 increases the thylakoid membrane's permeability as well as that the mechanism of its activity, at least partially, is based on this ability.