3. Results

3.1. Expression vector

Expression vector named pJTS1 witch carries dehydrin genes was obtained from Jan Svensson (Uppsala University – Sweden). This expression vector possesses a multicloning site with the following restriction cleavage sites: *Nco*I, *Bam*HI, *Sph*I, *Kpn*I, *Sma*I/*Xma*I, *Sal*I and *Pst*I.
Vector was designed in following way (Svensson et al., 2000): PCR amplification was used to amplify the entire coding regions for the dehydrin genes LTI29, COR47, ERD14 and RAB18. In all amplifications, the sense primers introduced an *Nco*I restriction site at the translational start codon and the antisense primers, a *Pst*I restriction site after the stop codon. Amplified PCR fragments were digested and thereafter ligated into *Nco*I and *Pst*I digested pJTS1.

3.2. Transformation and checking by colony PCR

To insert obtained plasmids the *E. coli* host M15 strain was transformed. Screening for positive clones was performed with PCR using constructed primers for each gene individually.
Previously the PCR program was standardized (suitable annealing temperature) for all analyzed genes (Material and methods Tab.2.4.).

The annealing temperatures were established experimentally : (*)

(*)LTI29 56 °C
ERD14 50 °C
COR47 63 °C
RAB18 63 °C

PCR products were analysed by agarose gel electrophoresis.

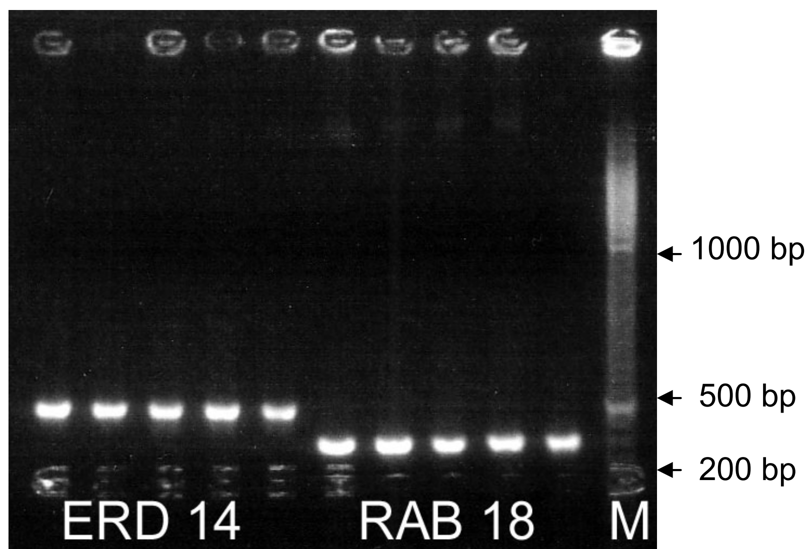


Fig. 3.1. PCR products for RAB 18 and ERD 14 genes.
Five transformants for each analysed protein were checked by colony PCR. Figure shows PCR products for ERD14 (558 bp) and RAB18 (508 bp) dehydrins genes. PCR products for LTI 29 and COR 47 were 822bp and 884bp. M-DNA marker GeneRuler™ 100bp DNA Ladders Plus

3.3. Dehydrins expression

Maximal production of soluble dehydrins was obtained by inducing expression of the cloned dehydrin genes with IPTG when the cells reached an OD_{600} of 0.5-0.7. Overproduction of recombinant proteins in bacteria often results in formation of insoluble inclusion bodies. To study if this was the case for the proteins produced in this study, cells were lysed and soluble proteins were separated from insoluble proteins. Pelleted proteins were resuspended in 20 mM NaH_2PO_4, pH 6.0, and the supernatants and the pellets were thereafter analyzed on SDS-PAGE (Fig. 3.2.). From this analysis it was clear that the recombinant proteins were localized in the supernatant and did not form inclusion bodies.

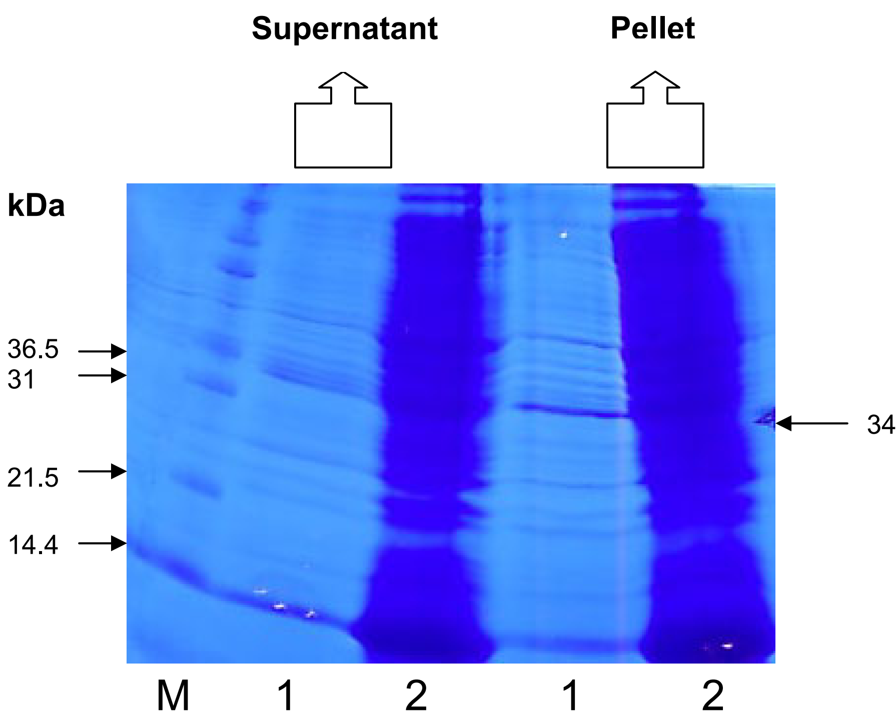


Fig. 3.2. SDS-PAGE analysis for protein ERD 14.
Picture shows presence of ERD 14 dehydrin in supernatant after centrifugation. ERD 14 is at the position of 34 KDa and it is soluble protein.
The similar was shown for other analysed dehydrin proteins.

M - Protein marker – "Mark 12 ™ Unstaind Standard"
1 - Supernatant
2 - Pellet

After resuspention step, cells were first treated by lysozyme treatment and sonication. For heat fractination lysates (6 ml) were placed in a 75 °C water bath for 10 min. Heat precipitated proteins were pelleted by centifugation and supernatants were stored at -20 °C. Samples were analyzed on SDS-PAGE before and after heat treatment. Approximately 55%-70% of the contaminating proteins precipitated during the heat fractionation (Fig. 3.3; 3.4.). Last step was concentration with centrifugal concentrator (Microsep centrifugal concentrator (Pall Filtron, Northborough, MA). Using concentration filters final concentration was doubled (Fig.3.3 5 6).

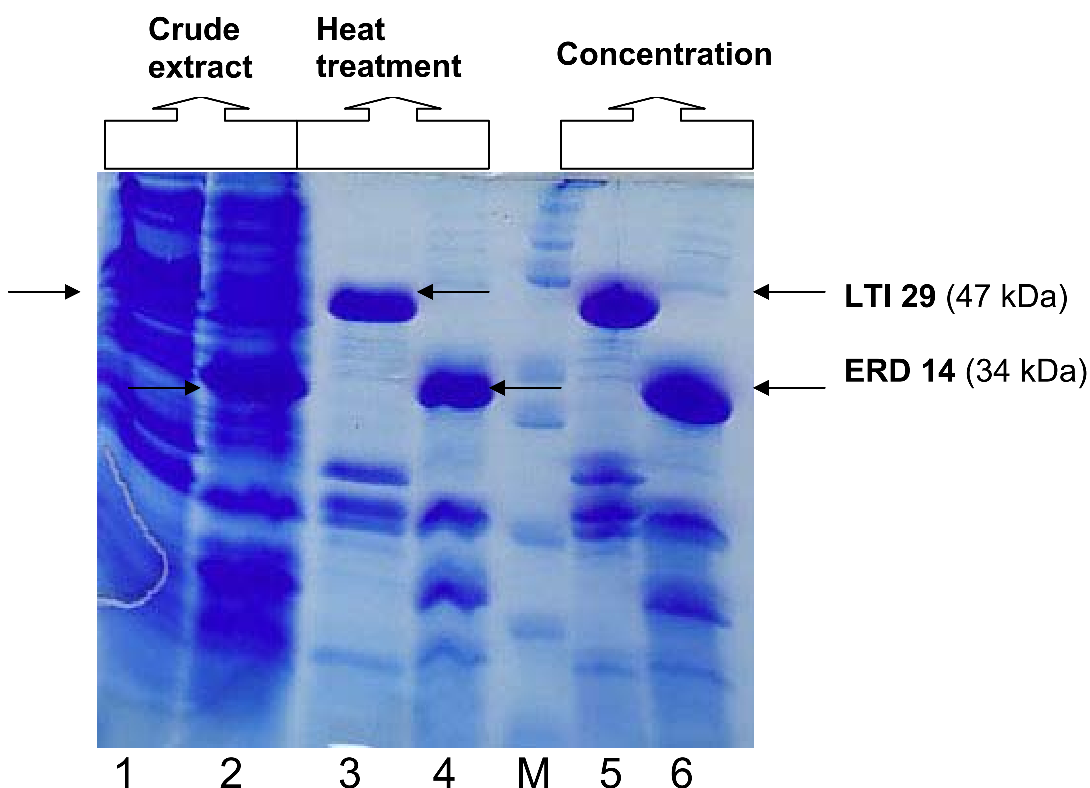


Fig. 3.3. SDS-PAGE analysis for dehydrins LTI29 and ERD 14. Crude bacterial extract was heat treated and concentrated
1,2 -SDS-PAGE analyzes for bacterial crude extract, for proteins LTI29 and ERD 14 respectively.
3,4 -SDS-PAGE analyses for bacterial extract after boiling treatment (on 75°C) ,for proteins LTI29 and ERD 14.
M - Protein marker “Mark 12 TM Unstaind Standard”
5,6 - SDS-PAGE analyses for bacterial extract after concentration using Filtron centrifugal concentrators, for proteins LTI29 and ERD 14 respectively.

The apparent molecular weights of the purified recombinant proteins were analyzed by SDS-PAGE (Laemmli et al., 1970). MW also was determined by analytical gel filtration under native conditions (Svensson et al., 2000).

Tab. 3.1. demonstrate a difference among calculated MW (kDa) and MW on SDS-PAGE.

Tab. 3.1. Molecular weight analyses of recombinant proteins: calculated MW and SDS-PAGE MW.

Recombinant dehydrin	Calculated MW(kDa)	SDS-PAGE MW (kDa)
LTI 29	**29.417**	**47**
ERD 14	**20.783**	**34**
COR 47	**29.718**	**54**
RAB 18	**18.333**	**25**

Proteins were concentrated by precipitation with 80% $(NH_4)_2SO_4$ and pelleted by centrifugation.
For further usage proteins were resuspended in Probe buffer (10 mM sucrose) containing 1mM Ca^{2+} and 1mM Mn^{2+} ions. Resuspended proteins were desalted on Sephadex G-25 F in prepacked PD-10 columns (Pharmacia) using stated buffers.

3.4. Protein yield

Recombinant proteins were purified from 0.6-0.8 l of bacterial suspension as described under Materials and Methods. The results of the purifications are summarized in Table 3.2.
From 800 ml of LB culture OD_{600} 0.6-0.7 reached yield for analysed proteins was different. The best expression was noticed for protein COR47, similar for proteins LTI 29 and ERD 14 and the lowest for RAB18 protein.
The reason for this low production of RAB18 could be due to the fact that cells expressing RAB 18 grew slower compared to the other constructs (data not shown)

Yield of purified protein in mg per 800 ml of *E. coli* culture was determined by Bradford method of protein concentration measurement.

Tab. 3.2. Purification of recombinant dehydrin proteins from 800 ml of *E.coli* suspension.

Purification step	**Volume** (ml)	**Total protein** (mg/ml)	**Protein yield** (%)
	LTI 29		
Cell lysate	22 ml	7.2	100
Heat treatment	19 ml	3.6	43.2
$(NH_4)_2SO_4$ /PD10	6 ml	4.2	15
Purification step	**Volume** (ml)	**Total protein** (mg/ml)	
	ERD 14		
Cell lysate	22 ml	6.6	100
Heat treatment	18 ml	3.0	37.2
$(NH_4)_2SO_4$ /PD10	6.4 ml	4.0	17.6
Purification step	**Volume** (ml)	**Total protein** (mg/ml)	
	COR 47		
Cell lysate	22 ml	7.6	100
Heat treatment	18.5 ml	5.9	65.2
$(NH_4)_2SO_4$ /PD10	6 ml	6.4	22.9
Purification step	**Volume** (ml)	**Total protein** (mg/ml)	
	RAB 18		
Cell lysate	22 ml	5.2	100
Heat treatment	20 ml	2.4	41.9
$(NH_4)_2SO_4$ /PD10	4.8ml	2.2	9.2

SDS-PAGE shows a difference in protein expression among analysed proteins. It can bee seen that dehydrin RAB 18 has the lowest yield.

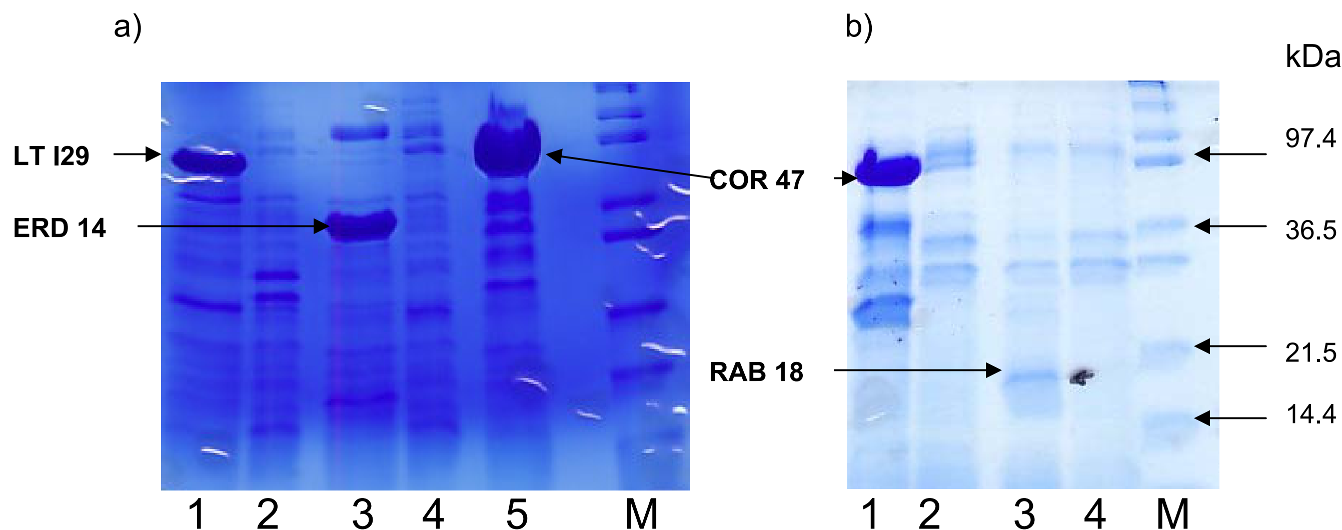


Fig. 3.4.

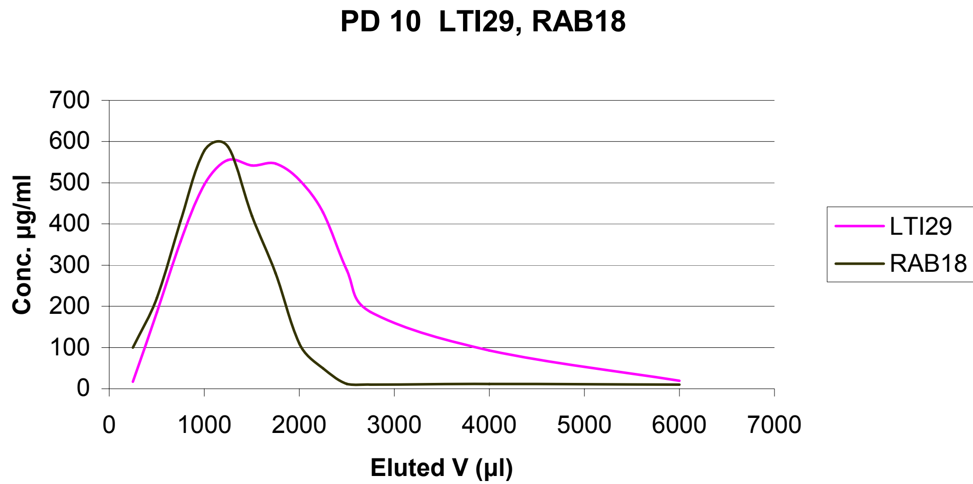
a) SDS - PAGE analyses of three dehydrins (LTI29,ERD14 and COR47) after heat treatment and concentration. From the gel the difference in the relation between expression yields of analysed proteins is visible.
1 – LTI29 induced; 2-LTI29 no induced; 3-ERD14 induced; 4-ERD14 no induced; 5-COR47 induced; M- Protein marker "Mark 12 TM Unstaind Standard"

b) SDS - PAGE analyses of two dehydrins (COR47 and RAB18) after heat treatment and concentration. From the gel the difference in the relation between expression yields of analysed proteins is visible.
1-COR47 induced; 2-COR47 no induced; 3-RAB18 induced; 4-RAB18 no induced; M- Protein marker "Mark 12 TM Unstaind Standard"

3.5. Protein desalting

To check whether desalting columns PD10 are suitable for used proteins and to find out in which range during elution the protein concentration is highest, protein concentration in small aliquots (50 μl) was measured. From these results graphs were constructed for two analysed proteins LTI 29 and RAB 18.

It was shown that protein LTI 29 flow through column after 1, 5 ml of eluting buffer and the highest concentration of protein was reached at 2, 5 – 3,5 ml. The similar elution kinetic was found for other dehydrins except RAB 18. Elution kinetics for protein RAB 18 is different because of smaller size of this protein.



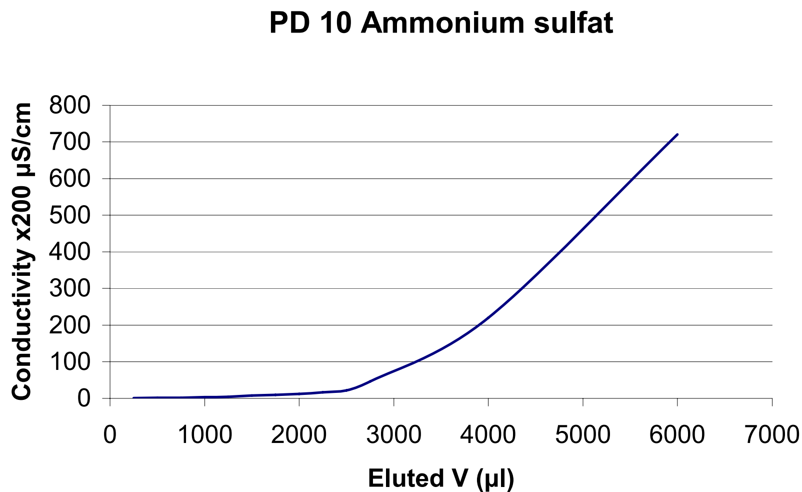


Fig. 3.5. Protein desalting using desalting columns PD 10.
Using desalting columns ammonium sulphate was removed from solution containing analysed proteins. It was shown that proteins LTI 29 and RAB 18 have different elution volumes because of different dimensions of analysed proteins.

According to these results in the next experiments dehydrins were eluted in the range from 0,5 to 3 ml.

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

It was shown (Thomashow, 1999) that dehydrins are expressed during the period of plant acclimation to low temperatures, which points to possible cryoprotective activity of these proteins. A freezing test based on measuring the ability of proteins to protect thylakoids during a freeze-thaw cycle has been used. Thylakoid membrane vesicles are isolated from spinach (*Spinacia oleracea* L.) for this test.
The results showed that three of four dehydrins had cryoprotective activity (Fig.3.6.). In addition to these results dehydrin RAB18 had a small percentage of cryoprotective activity over the level of the negative control. As negative control preparations from non induced crude bacterial extract were used which show to no cryoprotective activity. As positive control a crude extract of cold hardened *Brassica oleracea* containing cryoprotectin (CPP is not member of the dehydrin gene family) was used (Hincha et al., 1996).
These were the first results which confirm that dehydrins (LTI29, ERD14, and COR47) from *Arabidopsis thaliana* have cryoprotective activity.

Tab. 3.2. Cryoprotective activity of four analyzed dehydrins, cryoprotectin protein from cabbage (CPP) and control are shown in percentage (%).
AV- average values; SD- standard deviation

	1	2	3	4	SD	**AV**
ERD 14	47,4	43,6	56,8	53,1	5,89	**49,3**
RAB 18	7,6	15,2	3,8	5,7	4,99	**8,1**
LTI 29	47,4	36	43,6	45,5	4,9	**43,1**
COR 47	41,7	45,5	43,6	30,3	6,8	**40,2**
Cont. 0°C	115,6	94,8	96,7	92,9	10,5	**100**
Cont - 20°C	0.0	1.9	0.0	1.9	1.55	**0.95**
CPP	59.4	55	62	60.8	4.94	**59,3**

Percentage difference in cryoprotective activity for four analyzed dehydrins can very well be caused by the difference in protein's functioning mechanism, by different protein structures, or by the differences in binding ability of the proteins to the thylakoid membranes used in this test.

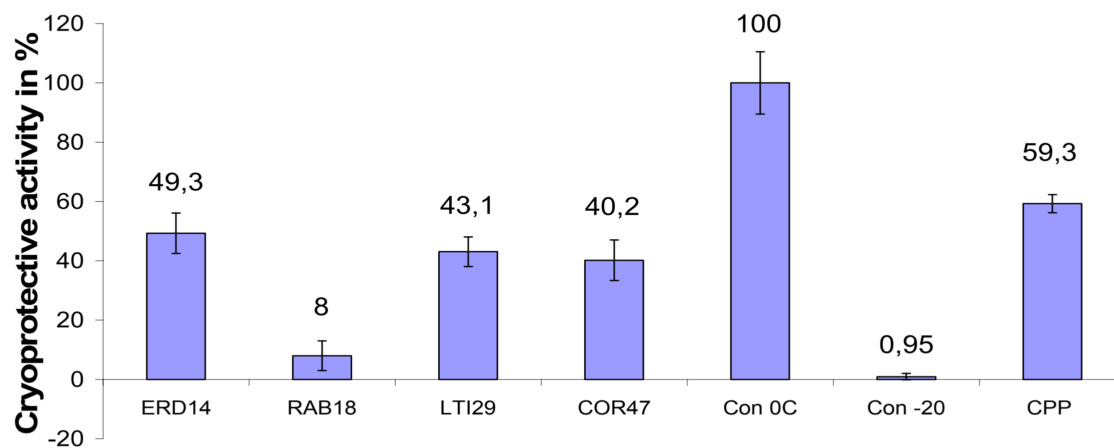


Fig. 3.6. Cryoprotective activity of four analyzed dehydrins shown in %.
Using "freezing test" the results show that four dehydrins had different cryoprotective activity. It was also revealed that dehydrin RAB18 had small percentage of cryoprotective activity. A crude extract of cold hardened *Brassica oleracea* containing cryoprotectin (Hincha et al., 1996 a) was used as a positive control (CPP). Non induced crude bacterial extract was tested as negative control. Proteins concentration in assay 290 µg/ml

3.7. Protein concentration and cryoprotective activity

The concentration of proteins is a relevant factor for the level of cryoprotective activity of the analyzed proteins (Fig. 3.7.).

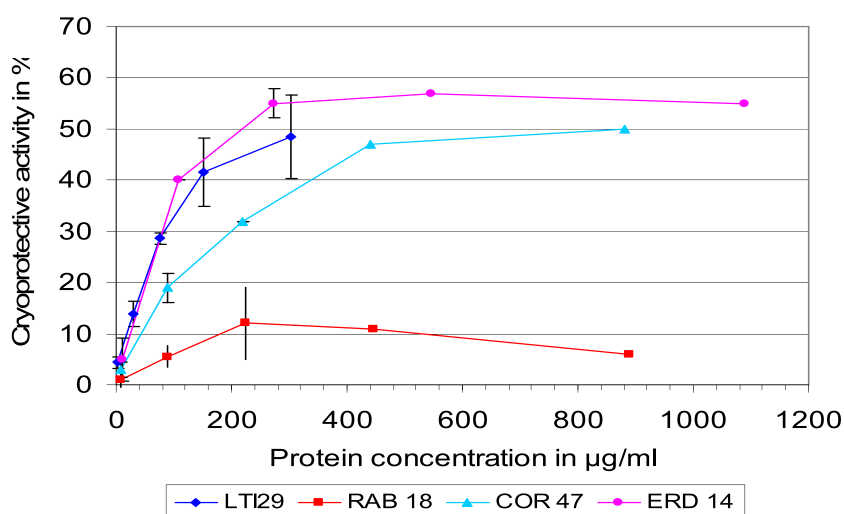


Fig. 3.7. Cryoprotective activity is dependent on protein concentration.

The effect of dehydrin protein concentration on the level of cryoprotective activity. For dehydrin cryoprotective proteins it was shown that increasing the protein concentration of dehydrins the cryoprotective activity was also increased.

During increasing of the initial protein concentration, the cryoprotective activity was also increased. The saturation was reached at 50% - 60% of activity.

3.8. Cryoprotective activity and storage stability

It was shown that cryoprotective activity of analyzed proteins decreases during storage time in solution. For protein LTI29 and CPP data indicates that cryoprotective activity goes to half activity after two days of incubation at +4 °C in probe buffer (10 mM sucrose, 1mM $MnCl_2$, 1mM $CaCl_2$).

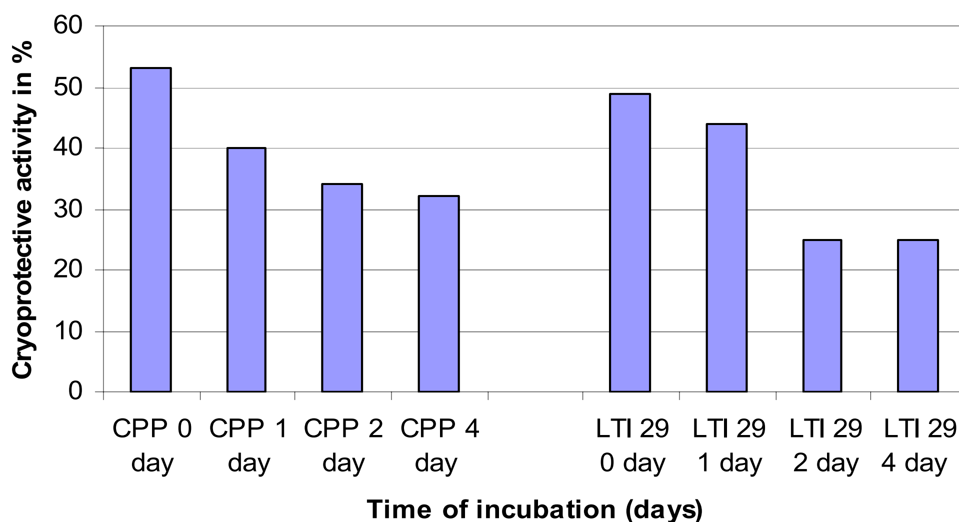


Fig. 3.8. Cryoprotective activity of LTI 29 dehydrin and CPP after several days of incubation at +4 °C in Probe buffer (10 mM sucrose, 1mM $MnCl_2$, 1mM $CaCl_2$).

It can be seen that that cryoprotective activity goes to the approximately half of activity after two days of incubation for both analyzed proteins. Final protein concentration for both proteins were 280 μg/ml

3.9. Additive effect (synergetic effect)

When two cryoprotective proteins are present into the suspension of thylakoids and are further analyzed in the freezing test they can together show a higher degree of cryoprotective activity in comparison to the case where thy have been examined separately.

If this is the case, then there is a synergic effect between dehydrins. As well it can accrue that two proteins have inhibitory effect on their activity. Our analyses have shown that dehydrins and CPP are partially additive.

Protein combinations COR47 /CPP	CPP (cryoprotective activity in %)	COR47(cryoprotective activity in %)	Additive cryoprotective activity in %
COR47/CPP (50μl:50μl)	**36**	**28**	**-**
COR47+CPP (50μl:50μl)	**-**	**-**	**56**
COR47/CPP (50μl:25μl)	**30**	**28**	**-**
COR47+CPP (50μl:25μl)	**-**	**-**	**42**
COR47/CPP (50μl:12,5μl)	**19**	**28**	**-**
COR47+CPP (50μl:12,5μl)	**-**	**-**	**33**

Tab. 3.3. Measured cryoprotective activity shown in percentage for dehydrin COR 47 and CPP.

The individually and mutual cryoprotective activity for dehydrin COR47 and CPP was measured in order to examine whether exist synergetic activity between these two proteins.

Tab 3.3. presents measured individually and mutual cryoprotective activity for dehydrin COR47 and CPP. Different concentrations of CPP in this test were used.

On the Fig. 3.9 can be seen a percentage ratio of individually calculated and measured cryoprotective activity for these two proteins. COR47/CPP presents a percentage of assembled individually cryoprotective activity of dehydrin COR47 and CPP protein separately. COR47+CPP present a percentage of cryoprotective activity when the proteins are together in solution.
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 (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 (Fig. 3.11.). RAB 18 in the combination to other dehydrin proteins or CPP decrease mutual cryoprotective activity.

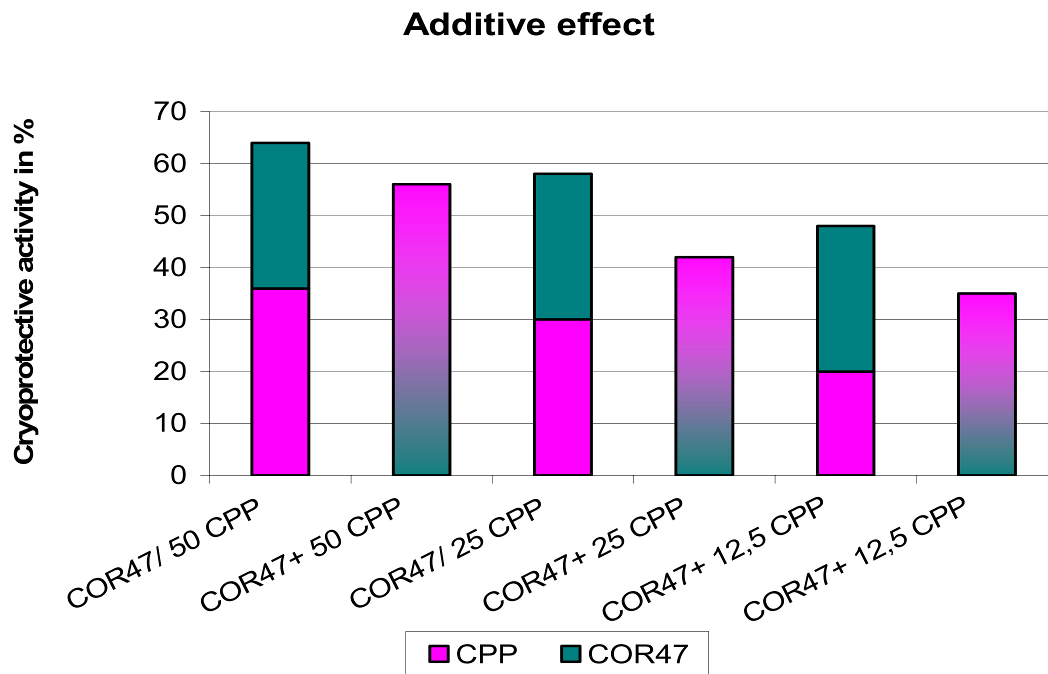


Fig. 3.9. Additive effect between CPP (Cabbage cryoprotectin) and dehydrin COR 47 (Arabidopsis thaliana).

Percentage ratio of individually calculated and measured cryoprotective activity for dehydrin COR 47 and CPP. COR 47/CPP present a percentage of assembled individually cryoprotective activity of dehydrin COR 47 and CPP protein separately. COR 47+CPP present a percentage of cryoprotective activity when proteins are together in solution. Final protein concentration of 50µl for both proteins in assay is 180 µg/ml

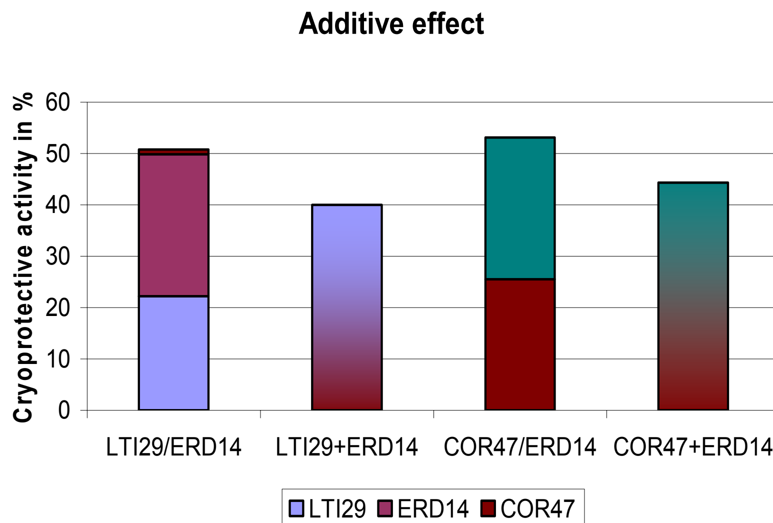


Fig. 3.10. Additive effect between two dehydrin proteins (LTI 29 and ERD 14)

Percentage ratio of individually calculated and measured cryoprotective activity for dehydrin proteins LTI 29 and ERD 14.
On the Fig. 3.10. LTI 29/ERD 14 present a percentage of assembled individually cryoprotective activity of dehydrin LTI 29 and ERD 14 separately. LTI 29 + ERD 14 present a percentage of cryoprotective activity when proteins are together in solution. Final protein concentration in assay is 160 μg/ml

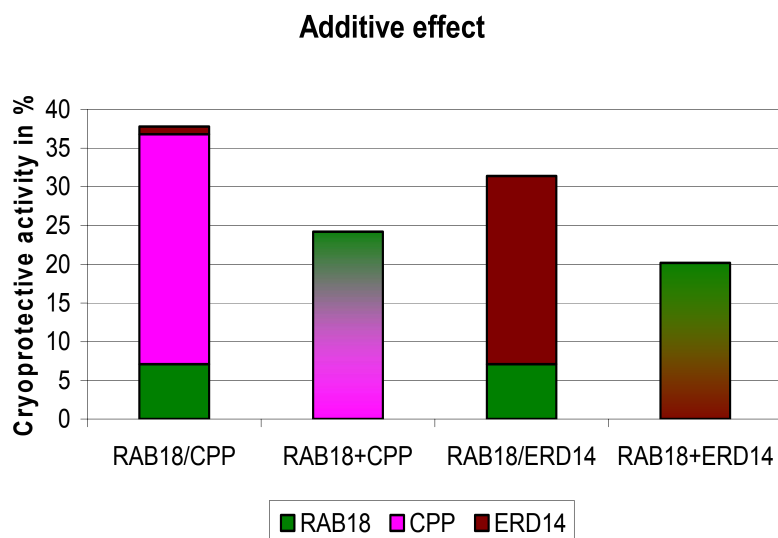


Fig. 3.11. Additive effect between dehydrin RAB 18 and CPP and between RAB 18 and ERD 14 respectively.

RAB 18 in combination with CPP or dehydrin proteins decreases cryoprotective activity.
Final protein concentration in assay is 180 μg/ml

%	LTI29	ERD14	COR47	RAB18	CPP
LTI29		40.2	42.1	24.5	45.4
ERD14	50		44.3	20.2	42.6
COR47	56.2	54.1		25.6	44
RAB18	34.6	31.3	35.4		24.2
CPP	57.2	54	58	37	

- Calculated values
- Measured values

Tab. 3.4. Calculated and measured values of cryoprotective activity for analysed dehydrins combinations. There are 10 possible combinations.

The cryoprotective activity was measured for each analysed protein separately. Parallel cryoprotective activity was measured when proteins are together in solution.
These values were compared and expressed in percentage.

Relation between measured and calculated values of cryoprotective activity expressed in percentage gives a degree of mutual cryoprotective activity for all analyzed dehydrin proteins.

Protein combination	%
LTI29/ERD14	80
LTI29/COR47	75
LTI29RAB18	70
LTI29/CPP	80
ERD14/COR47	82
ERD14/RAB18	63
ERD14/CPP	85
COR47/RAB18	73
COR47/CPP	76
CPP/RAB18	65

Tab. 3.5. Relation between measured and calculated values of cryoprotective activity for each analyzed dehydrin combinations expressed in percentage.

It can be seen that cryoprotective proteins are partially additive. In case of RAB 18 dehydrin values in percentage are lower what means that RAB 18 in combination to other dehydrin proteins decreases mutual cryoprotective activity.

3.10. Binding ability of dehydrins to the thylakoid membrane

In this investigation the binding of dehydrins LTI29 and RAB 18 to thylakoid membranes was studied as a possible mechanism of cryoprotection.

Sror (Sror et al., 2003) showed that the protective effect of CPP (cryoprotectin from Cabbage) is, at least in part, based on the binding of CPP with the thylakoid membrane. Therefore in this project it was important to examine a capability of dehydrins to bind with the thylakoid membrane. Procedure is described in 2.2.16.

To investigate the possible binding of dehydrins with the thylakoid membranes, thylakoids from an nonhardened spinach leaves were incubated with dehydrins LTI29 and RAB18 and then subjected to three rounds of centrifugation and resuspension in protein free solution.
Western blot analysis of supernatant after three washing steps is shown in Fig. 4.12.

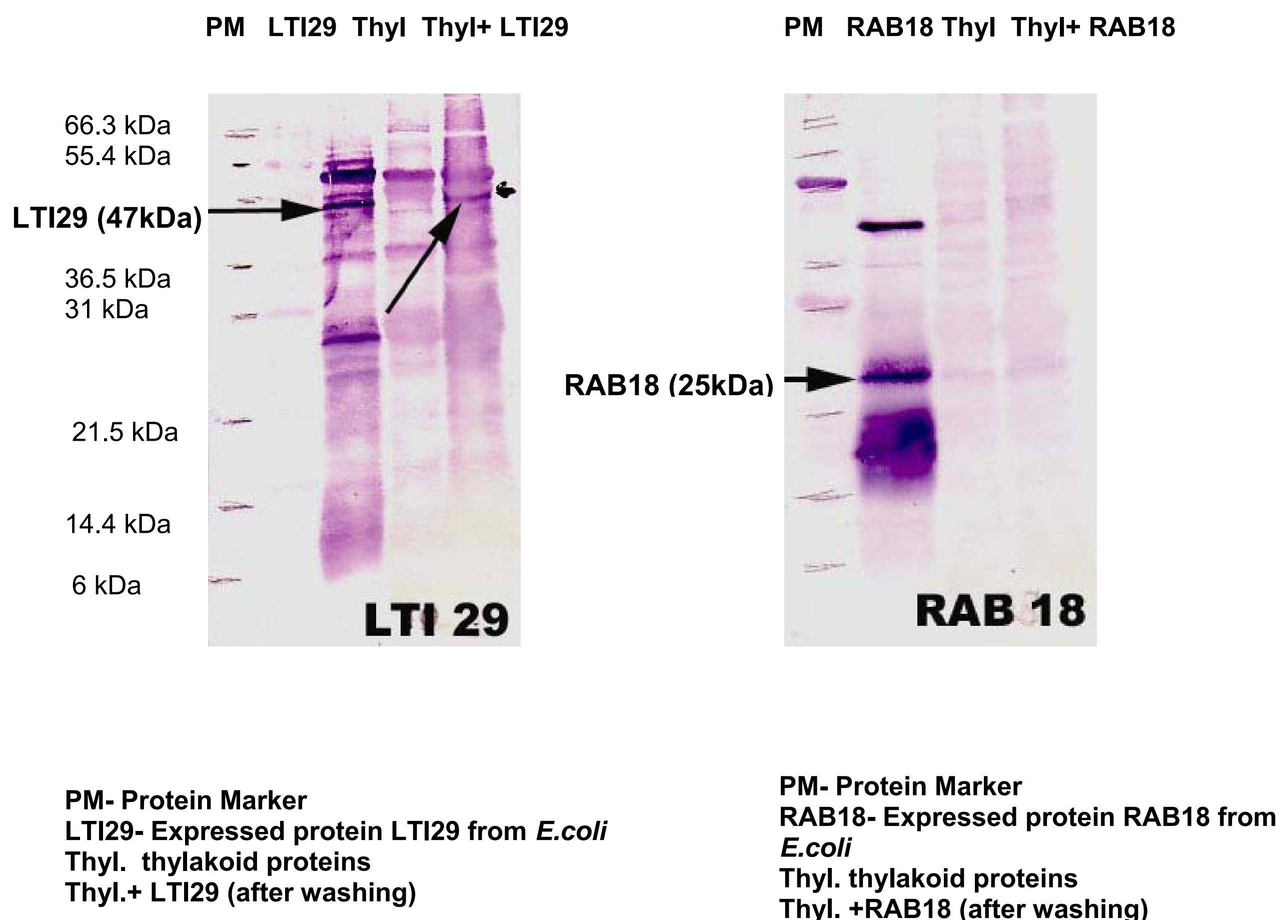


Fig. 3.12. Western blot analyses of supernatant after three washing steps

To investigate the possible binding of dehydrins to thylakoid membranes, thylakoids were incubated with dehydrins LTI29 and RAB18 and then subjected to three rounds of centrifugation and resuspension in protein free solution.
It can be observed that after several washing steps, the supernatant was essentially free of dehydrins. This experiment shows ability of binding LTI 29 to thylakoid membranes which can be linked to cryoprotective activity of this protein. The opposite was observed for protein RAB 18.

It can be seen from Western blot that in line thylakoid-protein even after washing is still detected protein LTI29 (47 kDa). In parallel experiment with RAB 18 (25 kDa) (no cryoprotection) protein is not visible. That means, protein LTI29 is still bounded to thylakoid membranes after several washing steps, RAB 18 is not present, and this protein was removed by washing.
This experiment shows ability of binding LTI 29 to thylakoid membranes which can be linked to cryoprotective activity of this protein.
Western analysis of thylakoid membranes before addition of dehydrins shows that there was no protein present reacting with the antibody.

3.11. Boyle-van't Hoff type analysis

To obtain information about the mechanism by which cryoprotective dehydrins protect the membranes from freezing damage, a Boyle-van't Hoff analysis was used.
Vesicles surrounded by semipermeable membranes react with a change in volume to a change in the osmolality of the external solution. Such changes can be analyzed using the Boyle-van't Hoff equation (volumes as a function of external osmotic pressure)

V-b=(1/p) RTn

V- total vesicle volume ; b- apparent non-osmotic volume; p- osmotic pressure of the suspending medium in osmol/kg H_2O; R- gas constant; T- absolute temperature; n- number of moles of solute within a unit volume V-b.

Boyle-van't Hoff plot graphs vesicle volume as the dependent variable and reciprocal of external osmolality as the independent variable.
The higher the osmolality of the external solution, the more water will osmotically leave the vesicle and the more the vesicle will shrink in size to a smaller vesicle volume. A vesicle can be exposed to several osmolalities and the resulting vesicle volumes can be measured.

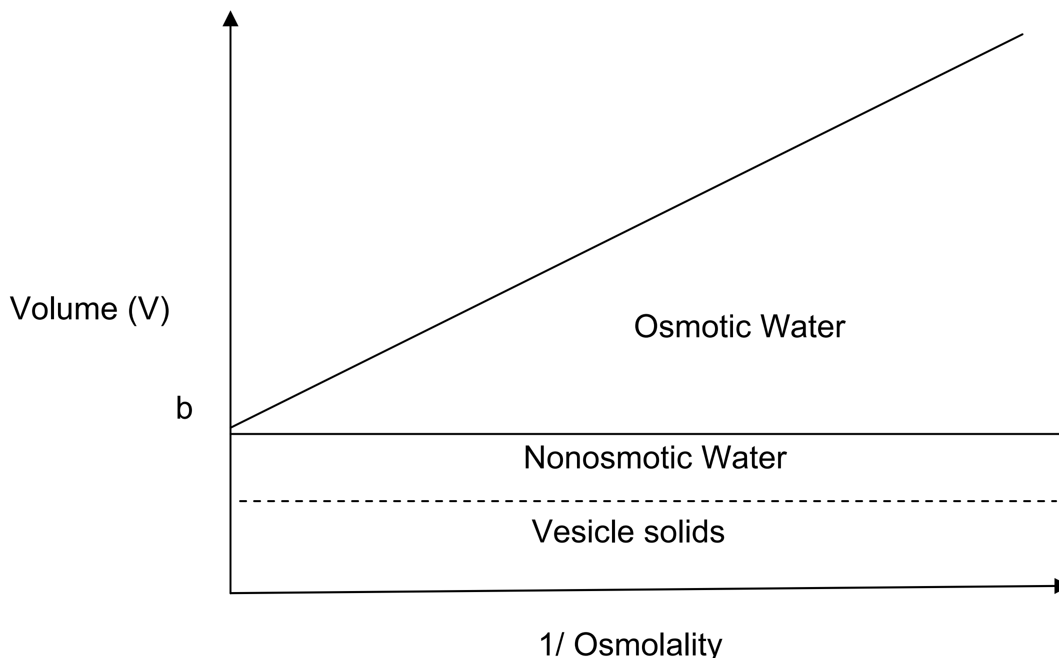


Fig. 3.13. Boyle-van't Hoff type analysis.

A plot of the vesicle volume (V) as a function of the reciprocal osmolality of the suspending solution will yield a straight line with the slope RTn and an intercept with the ordinate at b (non osmotic volume approximately 10-15 %).This variability was probably due to differences in the starch content of the leaves since starch granules sediment along with the membranes during centrifugation and were thus not removed by our washing procedure. If the volume measurements are taken at constant temperature and pressure, the slope of the straight line in a Boyle-van't Hoff plot is only a function of the amount and osmotic activity of the solutes (n) inside vesicles.

When these values are placed on a Boyle-van't Hoff plot they should lie approximately on a straight line (the Boyle-van't Hoff equation) because the relationship between vesicle volume and external osmolality is linear when osmolality is not too low (Boyle-van't Hoff correlation) Extrapolating the line to the vertical axis gives the osmotically inactive vesicle volume, corresponding to an infinite external osmolality and a vesicle that has shrunken from the loss of all of its vesicle water.

3.11.1. Analyzing of dehydrin proteins in Boyle van't Hoff analyses

In this study the function of reciprocal osmolality of the sucrose solutions used to suspend the membranes and the volume of packed thylakoids was plotted. Thylakoids were incubated for 3 h at 0°C or -20°C in solutions containing 20 to 500 mM sucrose with and without added dehydrins.
After the samples were thawed, the volume of the membrane vesicles was measured.

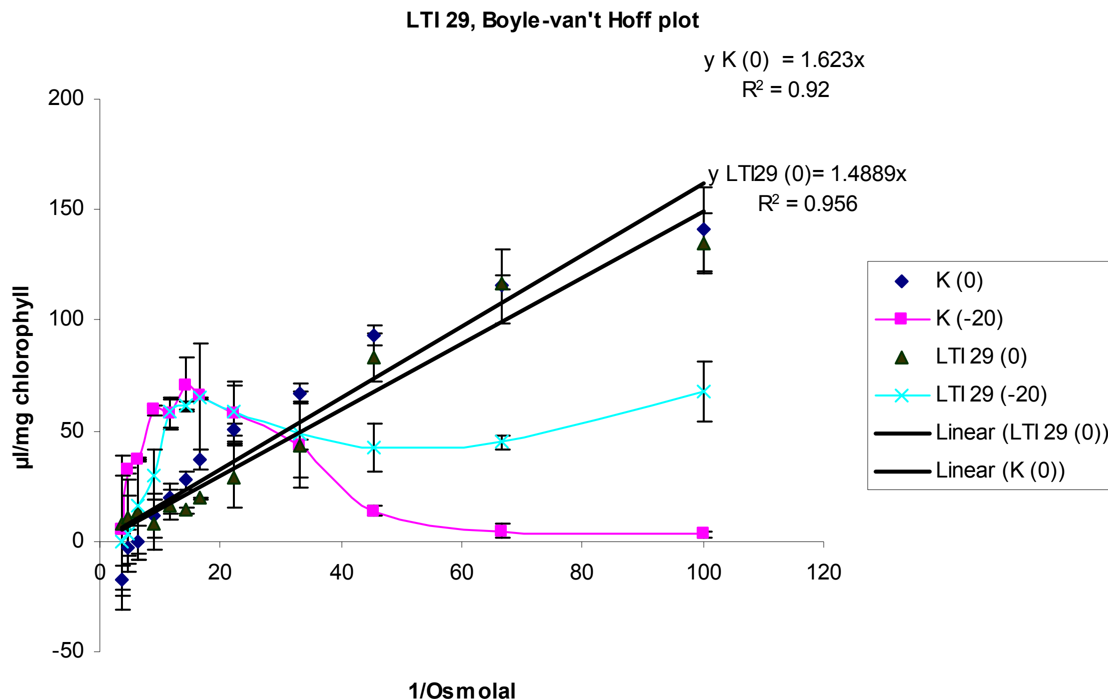


Fig. 3.14. Boyle van't Hoff -type analysis of the freeze-thaw behavior of thylakoid membranes. Thylakoids were washed in 5 mM NaCl. All samples contained 2.5 mM NaCl, 7 to 167 mM Sucrose, 0.3 mM $MnCl_2$, 0.3 mM $CaCl_2$. Values from 0°C controls with protective protein (LTI 29 (0°C)) and without protein (K(0°C) have the coefficient of regression ($R^2_{K(0)}$ = 0.92; R^2_{LTI29}(0)=0.96).
K(0) - 3h at 0°C, no protein present ; LTI29(0) - 3h at 0°C, protein present; K(-20) - 3h at -20°C, no protein present; LTI29(-20°C) - 3h at - 20°C, protein present. K- control. Final protein concentration in assay 290 µg/ml.

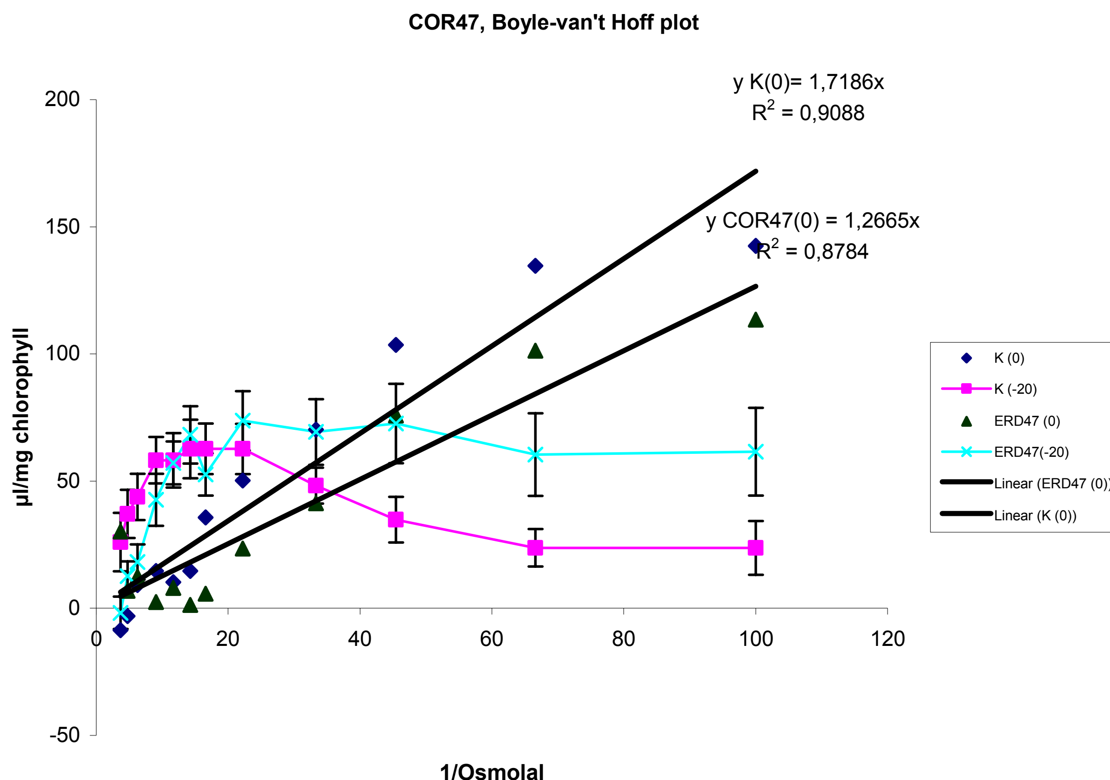


Fig. 3.15. Boyle van't Hoff -type analysis of the freeze-thaw behavior of thylakoid membranes. Thylakoids were washed in 5 mM NaCl. All samples contained 2.5 mM NaCl, 7 to 167 mM Sucrose and 0.3 mM $MnCl_2$, $CaCl_2$. Values from 0°C controls with protective protein (COR47 (0°C)) and without protein (K(0°C) have the coefficient of regression ($R^2_{K(0)}$ = 0.9; R^2_{COR47}(0)=0.88). K(0) - 3h at 0°C, no protein present ; COR47(0) - 3h at 0°C, protein present; K(-20) - 3h at -20°C, no protein present; COR47(-20°C) - 3h at - 20°C, protein present. K- control. Protein concentration in assay 280µg/ml.

It is shown that in the control experiment thylakoids maintained at 0°C behaved as osmometers: the volume increased with reciprocal osmolality (Fig. 3.14; 3.15; 3.16.- regression lines). At -20°C the curves with and without dehydrins (DHN(-20°C) and K(-20°C) respectively), are completely different (Fig 3.14.). After freezing and thawing, thylakoids suspended in moderate or high sucrose concentrations expanded to larger volumes than unfrozen controls, indicating a influx of solutes during freezing. In the controls (K(-20°C)) the rupture of thylakoids (as indicated by a decreasing volume) is increased at lower osmolality of the suspending medium.

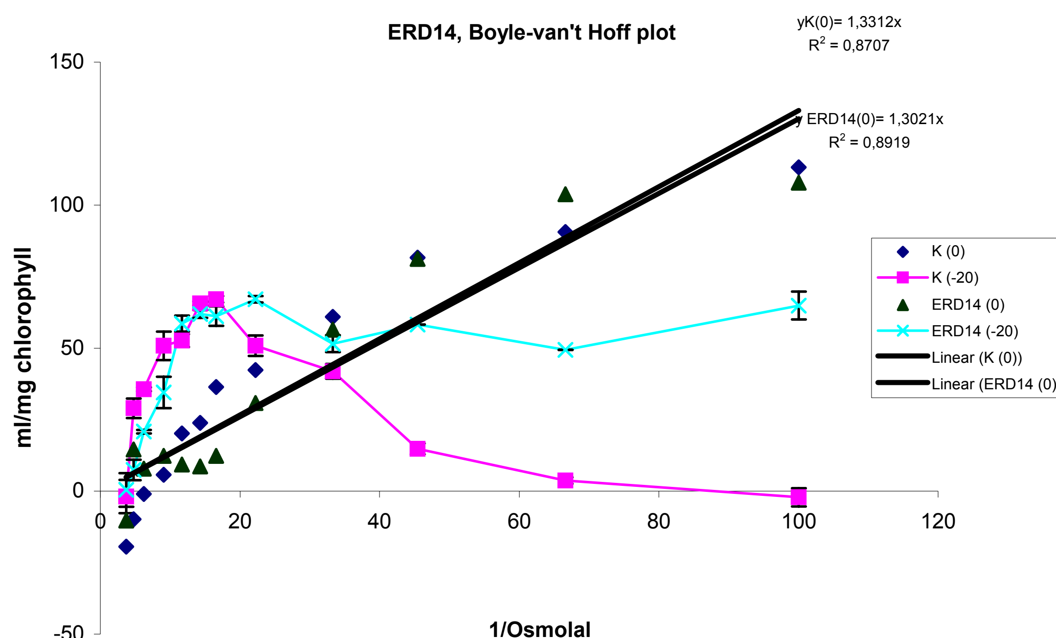


Fig. 3.16. Boyle van't Hoff -type analysis of the freeze-thaw behavior of thylakoid membranes. Thylakoids were washed in 5 mM NaCl. All samples contained 2.5 mM NaCl, 7 to 167 mM Sucrose and 0.3 mM $MnCl_2$, $CaCl_2$. Values from 0°C controls with protective protein (ERD14 (0°C)) and without protein (K(0°C) have the coefficient of regression ($R^2_{K(0)} = 0.87$; $R^2_{ERD14}(0)=0.89$). K(0) - 3h at 0°C, no protein present ; ERD14(0) - 3h at 0°C, protein present; K(-20) - 3h at -20°C, no protein present; ERD14(-20°C) - 3h at - 20°C, protein present. K- control. Protein concentration in assay 290µg/ml.

In contrast thylakoids after freeze-thawing in sucrose solutions in the presence of a protective concentration of proteins (DHN(-20°C)) are less collapsed even at the lowest concentrations of sucrose. Fig. 3.14. shows that dehydrin LTI 29 protected thylakoid membrane during freezing even at the lowest concentrations of sucrose (100 1/Osmolal).

It can be noticed that using the same conditions as used for the standard freezing test (lowest concentration of sucrose is 100 1/Osmolal) the cryoprotective activity is approximately 50% (Fig. 3.14.), which correspond to the previous results of aprox. 50% of cryoprotective activity of LTI 29 (Fig. 3.6.). Similar was shown for others analyzed cryoprotective dehydrins (Fig. 3.15.; 3.16.).

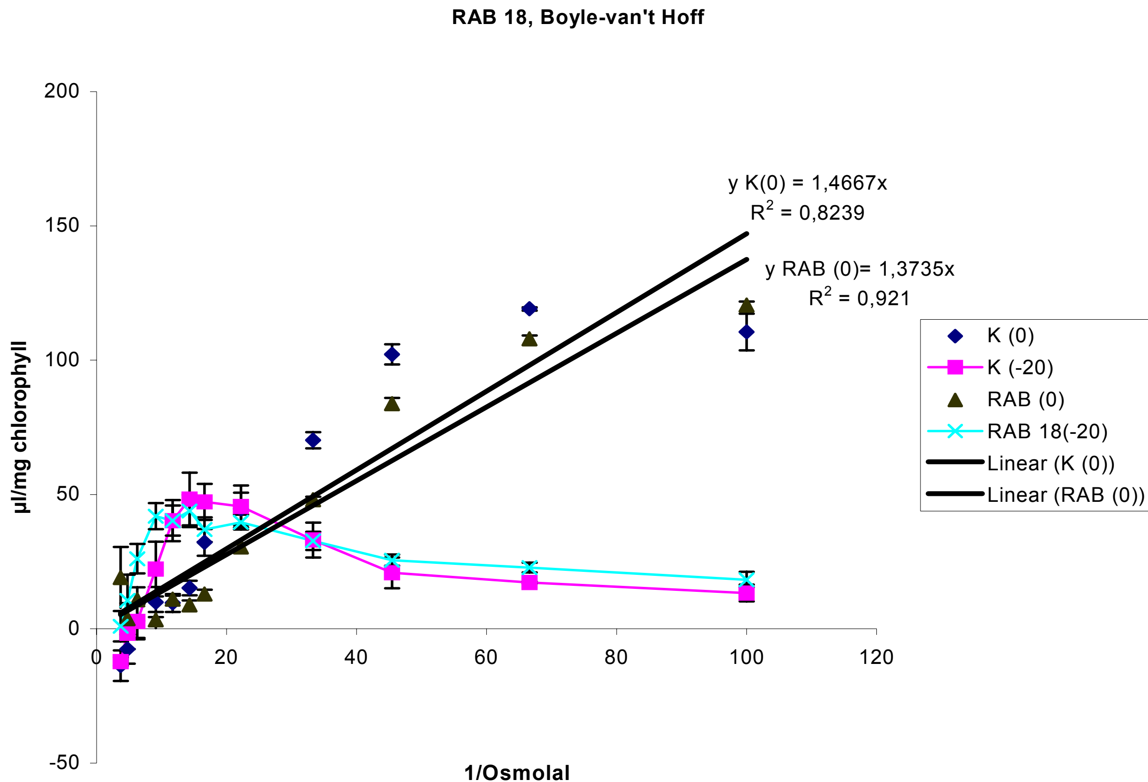


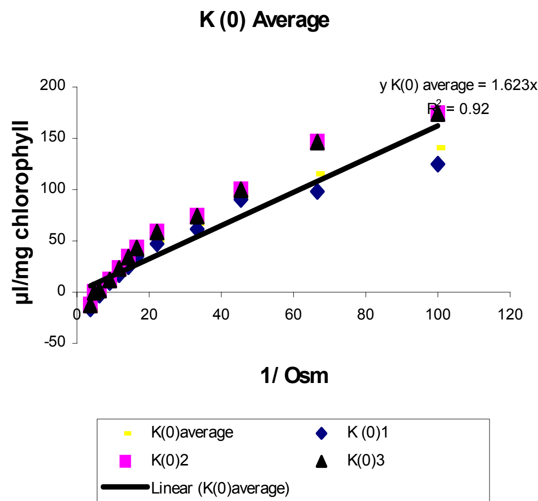
Fig. 3.14. Boyle van't Hoff -type analysis of the freeze-thaw behavior of thylakoid membranes. Thylakoids were washed in 5 mM NaCl. All samples contained 2.5 mM NaCl, 7 to 167 mM Sucrose, 0.3 mM $MnCl_2$, 0.3 mM $CaCl_2$. Values from 0°C controls with no protective protein (RAB 18 (0°C)) and without protein (K(0°C) have the coefficient of regression ($R^2_{K(0)}$ = 0.82; $R^2_{RAB\ 18}$(0)=0.92).
K(0) - 3h at 0°C, no protein present ; RAB 18(0) - 3h at 0°C, protein present; K(-20) - 3h at -20°C, no protein present; RAB 18(-20°C) - 3h at - 20°C, protein present. K-control. Protein concentration in assay 290µg/ml.

Observing the curves obtained in Boyle van't Hoff analyses at -20°C for dehydrin RAB 18 it can be seen that there is no significant increase in measured thylakoid volume at low sucrose concentrations relation to control at -20°C.
This correlate to the detected low cryoprotective activity of RAB 18 protein.

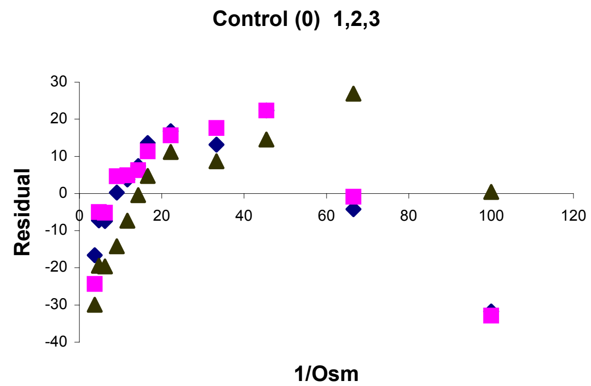
3.11.2. Calculating of residual (distance of each single point from an average regression line)

To test the suitability of the Boyle-van't Hoff method for our purpose a initial experiment using thylakoids at 0°C (Fig. 3.17) was made. To check whether

thylakoids behaved as ideal osmometers we calculated residual (distance of each single point from average regression line).
Apparently we can see that the deviation from regression line is not random (Fig. 3.17 b) but in the range of sucrose concentrations from 4 to 15 1/Osm (milliosmol/kg^{-1}) we noticed little deviation (Fig. 3.17 c). This is confirmed by calculation of residual for this range which is shown on Fig. 3.17. d.



a)



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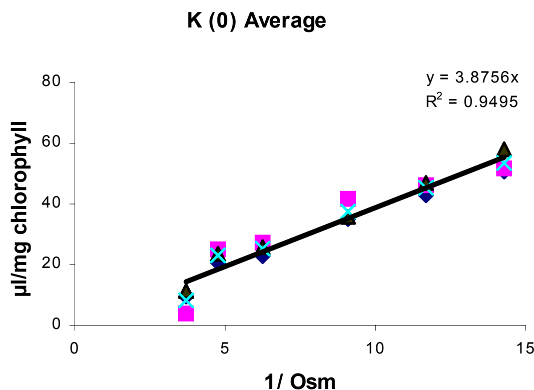
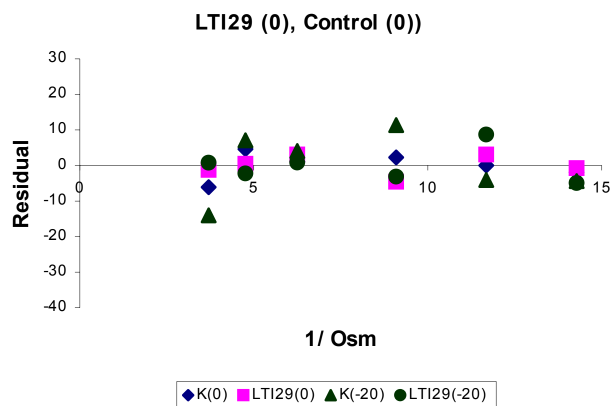


c)



d)

Fig. 3.17. Boyle van't Hoff -type analysis for control on 0°C.

a) Regression line from average control (K Average) from tree different experiments (broader range).
b) Residual-distance each single point from average regression line in whole analyzed range of sucrose concentrations.
c) Regression line from average control (K Average) in range from 4 to 15 1/Osm (milliosmol/kg^{-1}).
d) Residual- distances each single point from average regression line in range of high sucrose concentrations. Four regression lines were made in presence and absence of LTI29 on 0°C and -20°C. Residuals were calculated for each regression line named K (0); LTI29(0); K(-20); LTI29 (-20).Validity for this experiment was made in presence and absence of protein LTI 29.
All samples contained 2.5 mM NaCl, 7 to 167 mM Sucrose and 0.3 mM $MnCl_2$, $CaCl_2$.

Little deviation from regression line means that volume of thylakoids is dependent on reciprocal osmolality in a **linear relation** (in this range thylakoids behaved as a ideal osmometers).

The similar relation was found in presence of dehydrin LTI 29 on 0°C and -20°C. That means that in the range of the sucrose concentrations from **4 to 15 1/Osm** (milliosmol/kg^{-1}) the reliability of the values for the slopes of the Boyle van’t Hoff plots are most reliable.

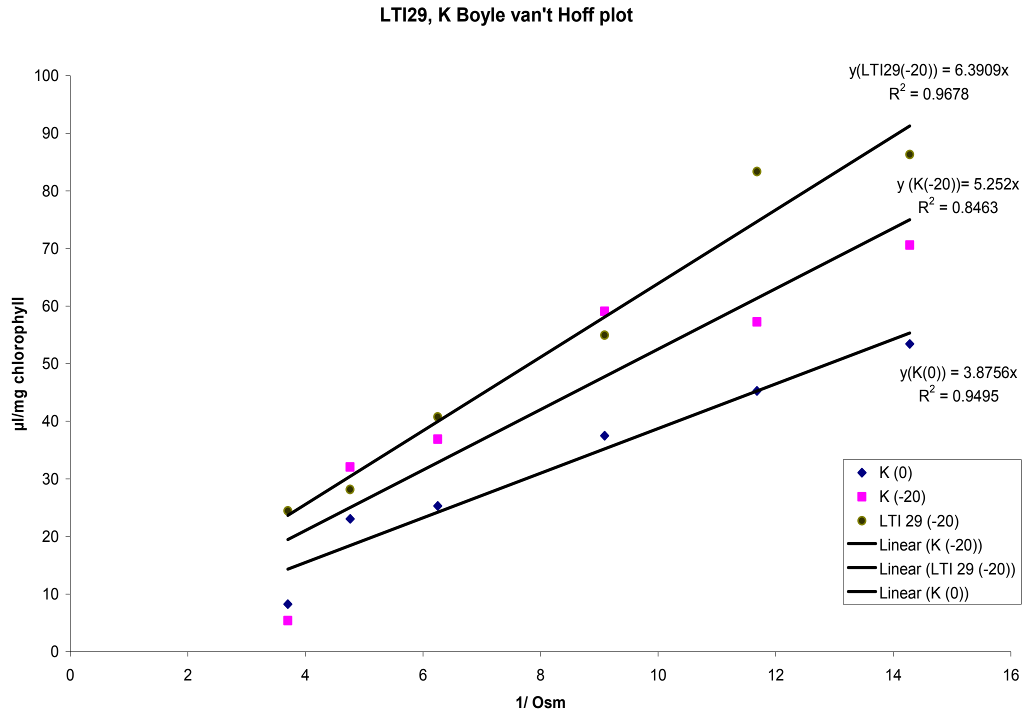
Therefore in the next experiments we analyzed this range of sucrose concentrations in Boyle van't Hoff type of analysis.

3.11.3. Analyzing of short 1/Osm range by Boyle van’t Hoff analyses

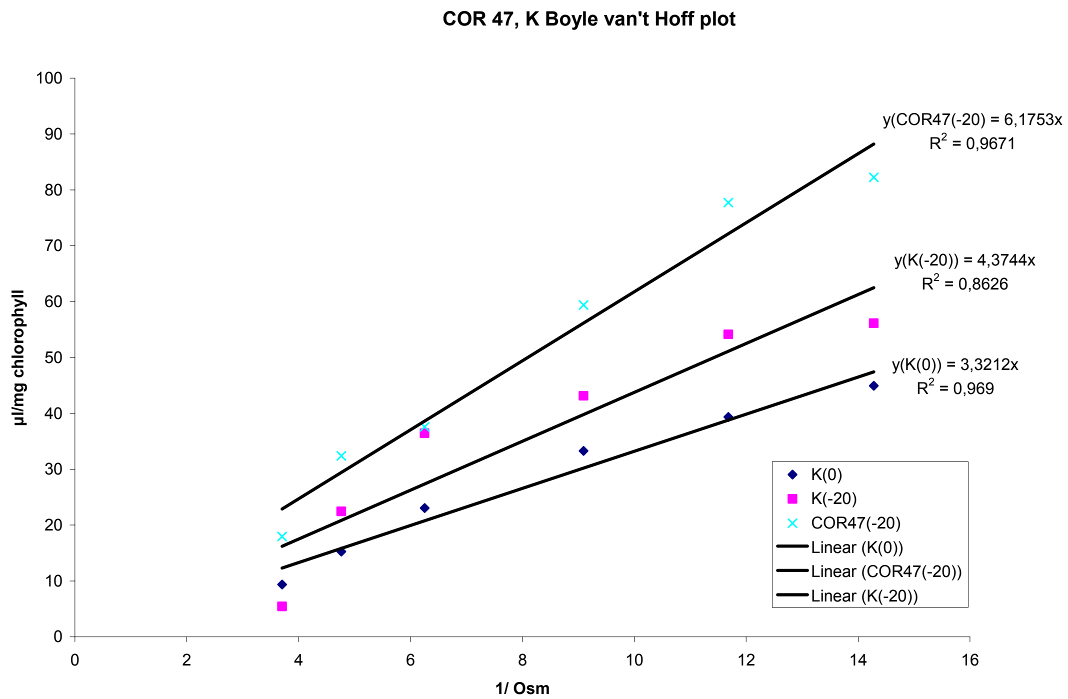
Analyzing this range we noticed that after freezing (at -20°C) and thawing, thylakoids expanded to larger volumes (K(-20) than unfrozen controls (K(0)), (Fig.3.18. a.b.c.) indicating a influx of solutes.
Thylakoids frozen-thawed in sucrose solutions in the presence of added dehydrin showed curve with a steep slope indicating uptake of solutes.

Thylakoids after freeze-thawing in high sucrose solutions in the presence of low cryoprotective dehydrin RAB18 (RAB18(-20)) showed the same curve of osmolalality versus volume as the frozen control (K(-20)),(Fig.3.18.d). That indicates less uptake of solute - in comparison to LTI 29.

Obtained results are indicating on an increase of permeability of the membranes in the presence of added dehydrins, which was not apparent in our previous experiments, in which we used a broader range of initial solute concentrations.



a)



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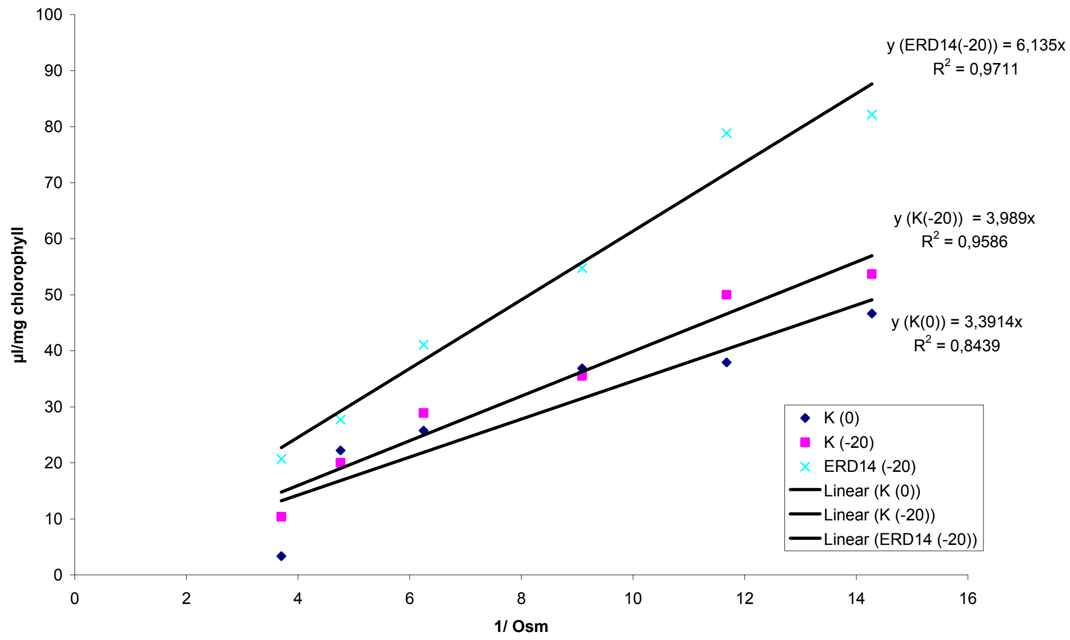

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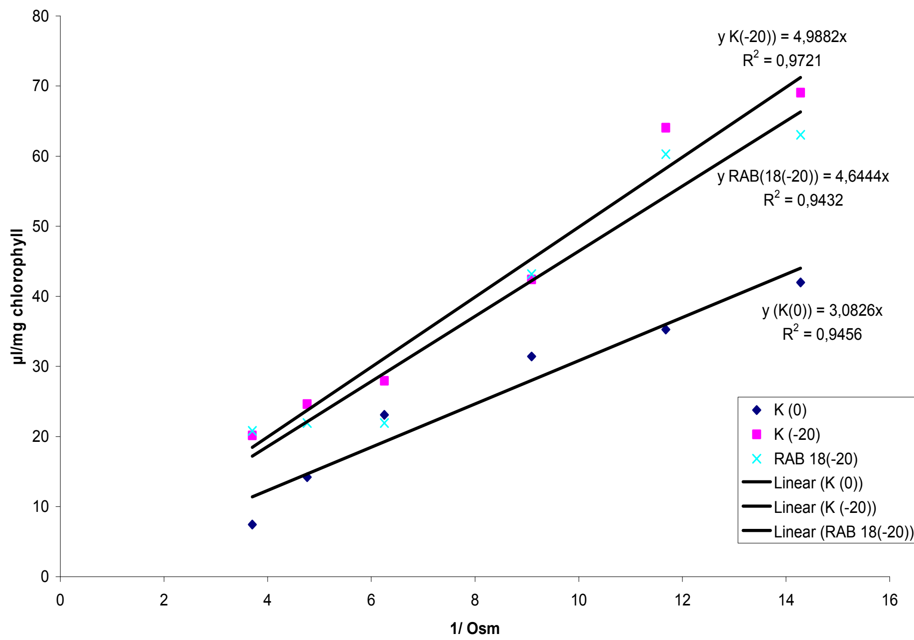


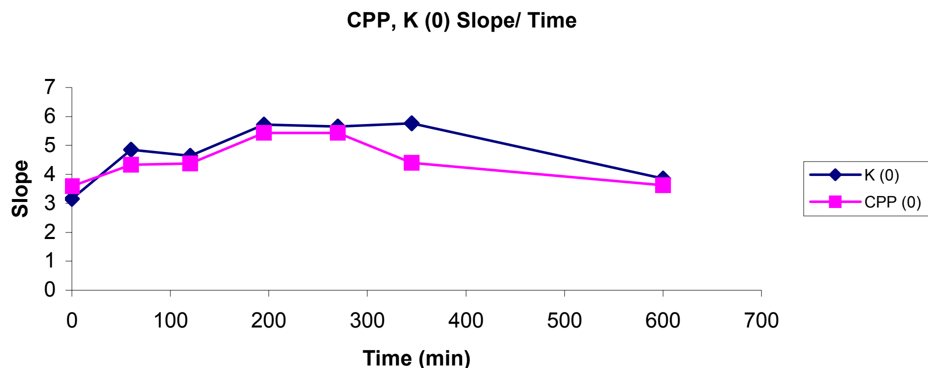
d)

Fig. 3.18. Boyle van't Hoff -type analysis of the freeze-thaw behavior of thylakoid membranes.

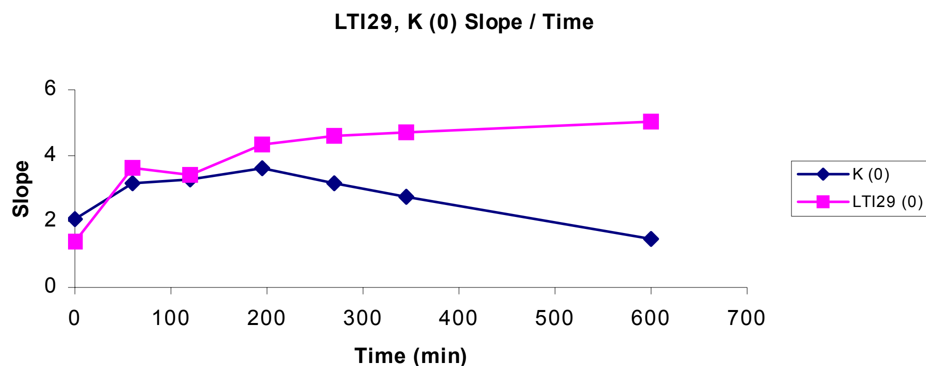
a) Volume of thylakoids in presence of cryoprotective dehydrin (LTI29(-20°C)) is larger in comparison to control (K(-20°C)). K(0) - 3h at 0°C, no protein present ; K(-20) - 3h at -20°C, no protein present; LTI29(-20°C) - 3h at - 20°C, protein present. K- control.
b) Volume of thylakoids in presence of cryoprotective dehydrin (CO47(-20°C)) is larger in comparison to control (K(-20°C)). K(0) - 3h at 0°C, no protein present ; K(-20) - 3h at -20°C, no protein present; CO47(-20°C) - 3h at - 20°C, protein present. K- control.
c) Volume of thylakoids in presence of cryoprotective dehydrin (ERD 14(-20°C)) is larger in comparison to control (K(-20°C)). K(0) - 3h at 0°C, no protein present ; K(-20) - 3h at -20°C, no protein present; ERD14(-20°C) - 3h at - 20°C, protein present. K- control.
d) Volume of thylakoids in presence of low cryoprotective dehydrin protein RAB18 (RAB18(-20°C)) shows no significant change in volume in comparison to control (K(-20°C)) K(0) - 3h at 0°C, no protein present ; K(-20) - 3h at -20°C, no protein present; RAB18 (-20°C) - 3h at - 20°C, protein present. K- control. Protein concentration in assay 300μg/ml (final concentration). All samples contained 2.5 mM NaCl; 7 to 170 mM Sucrose (final concentration) and 0.3mM $MnCl_2$, $CaCl_2$. Non osmotic volume was subtracted.

3.11.4. Influence of CPP and LTI29 on the thylakoids volume at long time incubation (10h)

Since CPP is known to decrease membrane permeability under freezing conditions (Hincha et al., 1989) it was of interest to see and compare influence of protein LTI 29 and CPP to membrane permeability at 0°C. Measurements of thylakoid volume (Boyle van't Hoff plots) was done in time interval from 0 min (time of making suspension thylakoids - protein) to 600 min (10 h).



a)



b)

Fig. 3.19. Influence of CPP and LTI29 on volume of thylakoids during a long time incubation (10h). Slope of Boyle - van't Hoff plots was plotted as a function of incubation time.

a)Influence of CPP on volume of thylakoids during a long time incubation (10h) in presence of different sucrose concentrations (from 7 to 170 mM -final concentration).

b)Influence of LTI29 on volume of thylakoids during a long time incubation (10h) in presence of different sucrose concentrations (from 7 to 170 mM -final concentration). All samples contained 2.5 mM NaCl, 7 to 167 mM Sucrose and 0.3 mM $MnCl_2$, $CaCl_2$.

As it is visible from Fig. 3.19. in first hours of incubation on 0°C (up to 200 min.) was noticed influx of solutes in vesicles in presence of LTI 29 and control (Fig 3 b). At long time of incubation (from 200 to 600 min) influence of LTI 29 and CPP on apparent thylakoid volume is different. While in presence of LTI 29 volume of thylakoids even after 10 h of incubation stays unchanged, in case of CPP and control measured volume becomes smaller (thylakoids collapsed).

This results points out to possible different mechanism of cryoprotective activity of CPP and dehydrins.

3.12. LSA - Light scattering analyses

For better understanding of results obtained in Boyle-van't Hoff analysis, the membrane permeability was analyzed by light-scattering technique (LSS-light scattering signal) in the presence of cryprotective proteins. Volumetric changes of the thylakoid vesicles were measured in response to added osmotically active substance. Osmotically active substance (sucrose or glucose) cause osmotic dehydration (shrinkage) of thylakoid vesicles which can be constant or temporal depending on:

1. Type of osmotically active substance added in thylakoids suspension and on
2. Changing in vesicle membrane permeability

According to the results obtained in Boyle-van't Hoff analysis thylakoids membrane permeability was changed in the presence of cryoprotective proteins (CPP and dehydrins LTI29, COR 47 and COR 14). To see whether these results can be reproduced in more sensitive system, this method was used.
In case where a change of membrane permeability is caused by the presence of cryoprotective active proteins a different LSS signal is being expected.

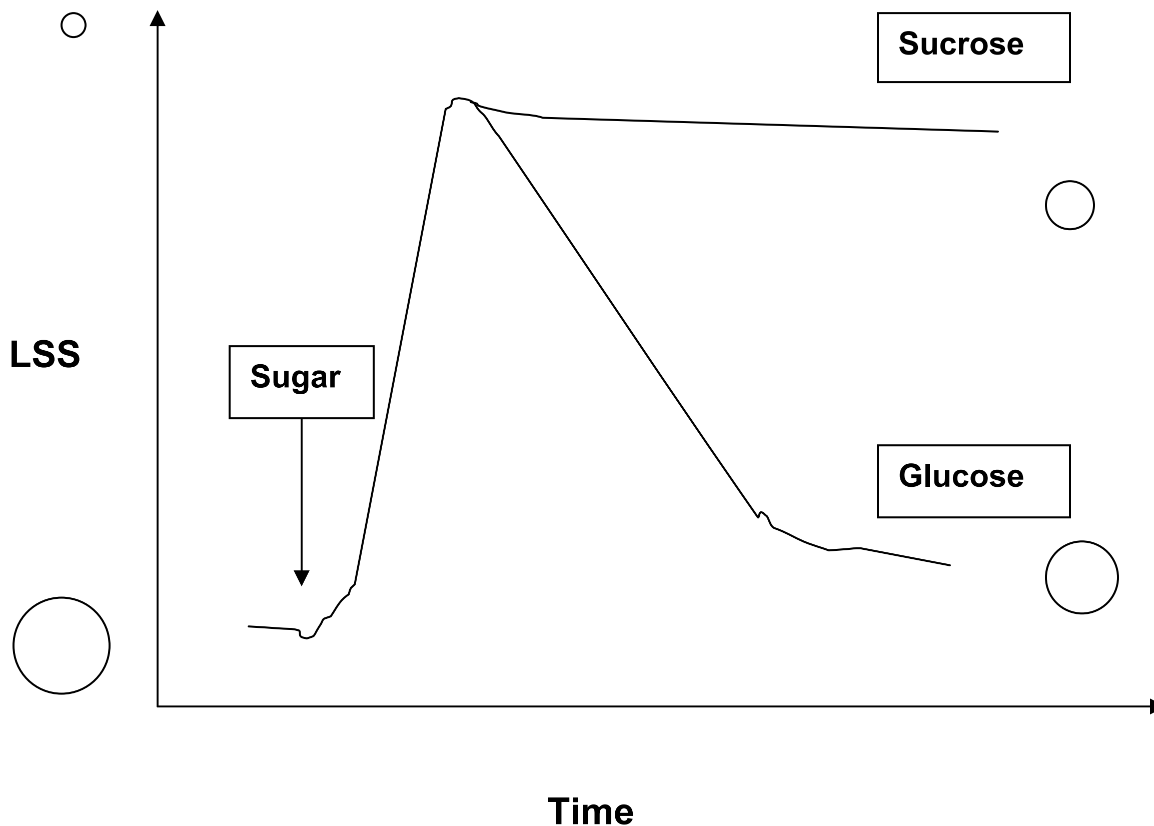
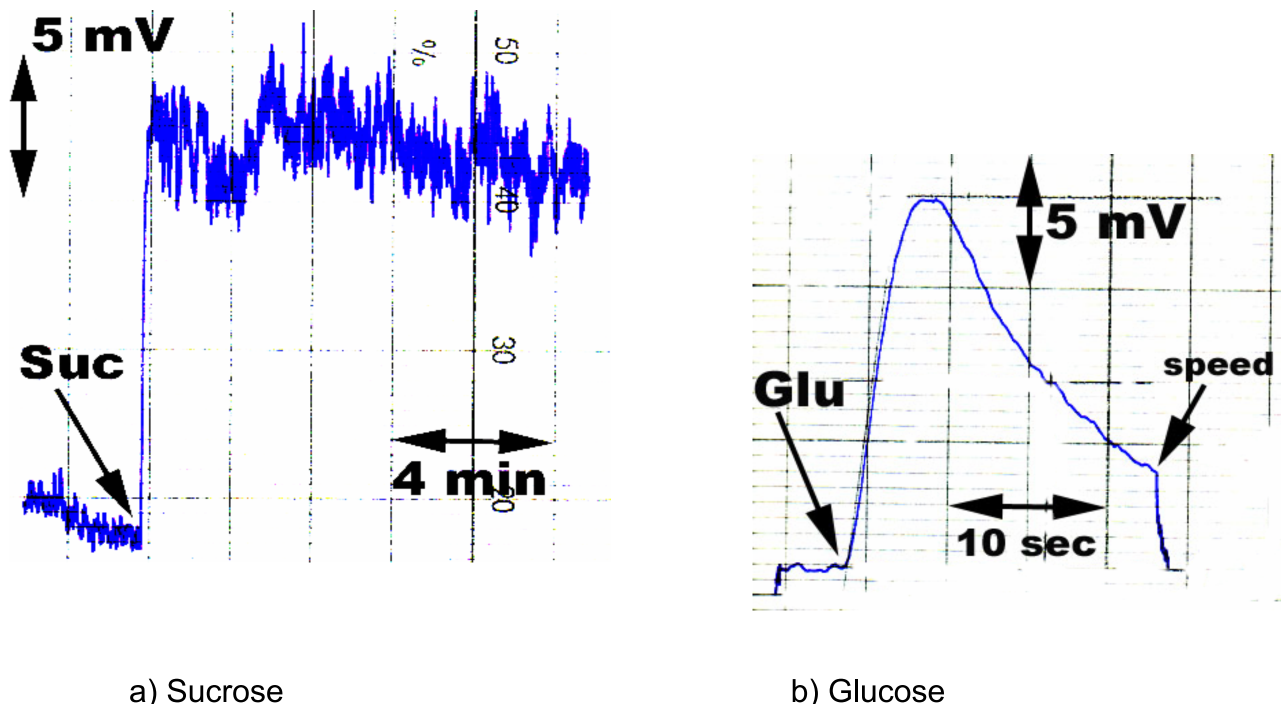


Fig 3.20. Schematic description of record of LSS signal

Volumetric changes of thylakoids were measured after adding of osmotically active sugars (sucrose or glucose) in thylakoids solution. Since water permeates membranes faster than sugar, the vesicles are osmotically dehydrated (thylakoids are shrinking). If membrane is enough permeable for sugar after short period of time the equilibrium will be reached (thylakoids swelling) to osmotically equivalent suspension. Sucrose in contrast to glucose can't penetrate thylakoid membrane (thylakoids remains shrunken). Changes of thylakoid volume are detected by changing in scattering light by the thylakoids.

3.12.1 Influence of different osmotically active substances (sucrose and glucose) to LSS signal

Depending on the type of added sugar (sucrose or glucose) in thylakoid suspension the two different LSS signals are noticed. While glucose can penetrate through the thylakoid membrane the osmotic gradient equalizes (thylakoids swelling) while in the event of sucrose thylakoids remain shrunken - sucrose can not penetrate membrane.



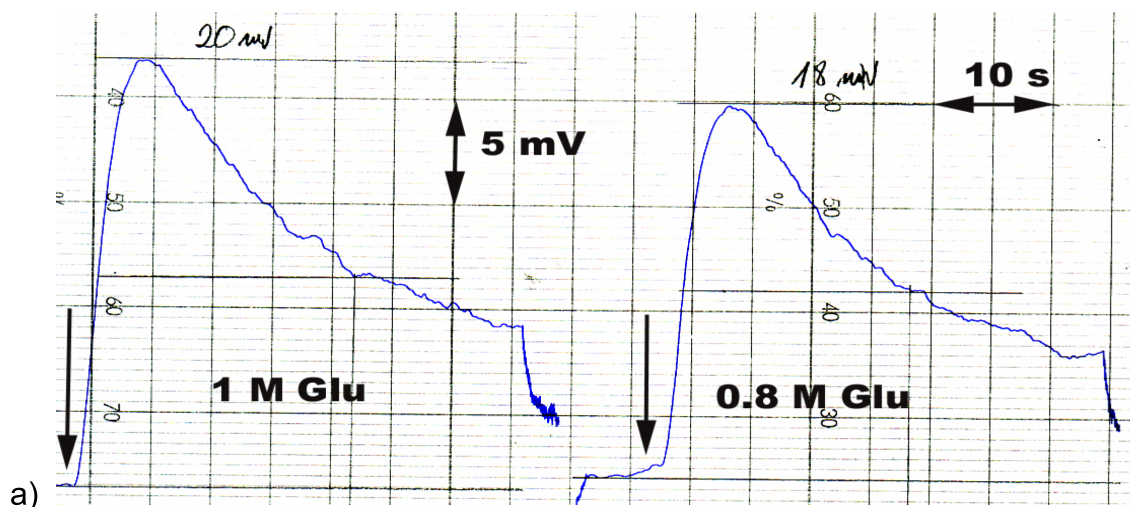
a) Sucrose b) Glucose

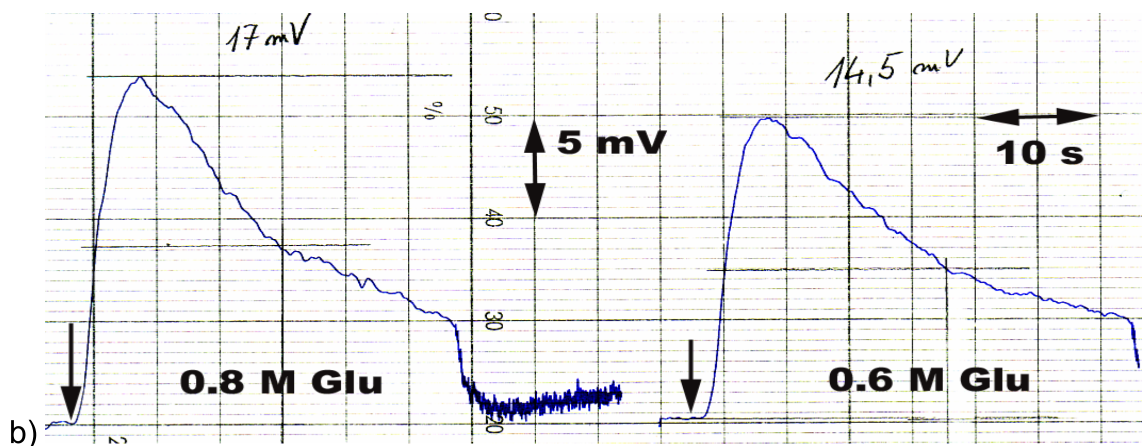
Fig. 3.21. Time course of the light scattering signal in sucrose and glucose permeation experiment. Light scattering of a thylakoid suspension was measured in a spectrofluorimeter. After a constant baseline was established, 200µl 1M sucrose (final conc. 100 mM) was added. Since water permeates membranes faster than sucrose or glucose, the vesicles are osmotically dehydrated. They shrink, leading to an increase in light scattering. Then external solute starts to enter the vesicles if it is permeable and they begin to swell again until they reach a new equilibrium. LSS(mV)-light scattering signal, base line was fit to 0 mV;
Different kinetics of LSS signal was observed in case of those two sugars. While glucose caused temporal change of thylakoid volume (after shrinking comes swelling) in case of sucrose as result

thylakoids become permanently shrunken. Difference in activity of those two sugars was caused by different membrane permeability for sucrose and glucose. Glucose easily pass thylakoid membrane in comparison to sucrose (glucose is smaller molecule in comparison to sucrose).
In 1800 μl thylakoid suspension (final chlorophyll concentration 90 μg/ ml) was added 200 μl (0.5 M) of glucose. Speed- changing of record speed 10 fold less.

3.12.2. Influence of different concentrations of added sugar (glucose) on the amplitude of LSS signal

In next step it was important to analyze influence of different concentrations of added sugar (glucose) on the amplitude of LSS signal. It was shown that by increasing of concentration of added glucose to the suspension the amplitude of measured LSS signal increases too.





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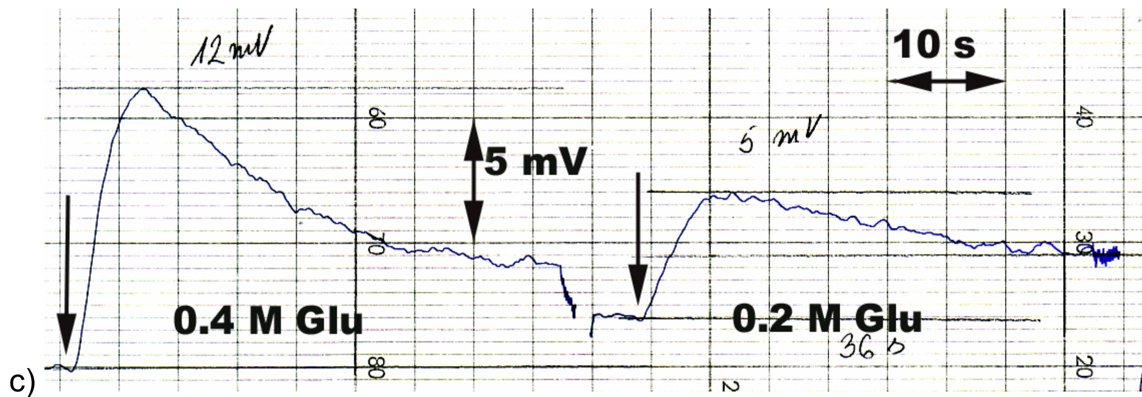


Fig. 3.22. Time course of the light scattering signal in glucose permeation experiment.
In 1800 µl thylakoid suspension (final chlorophyll concentration 60 µg/ ml) was added 200 µl of glucose of different molarities as indicated in the figure.

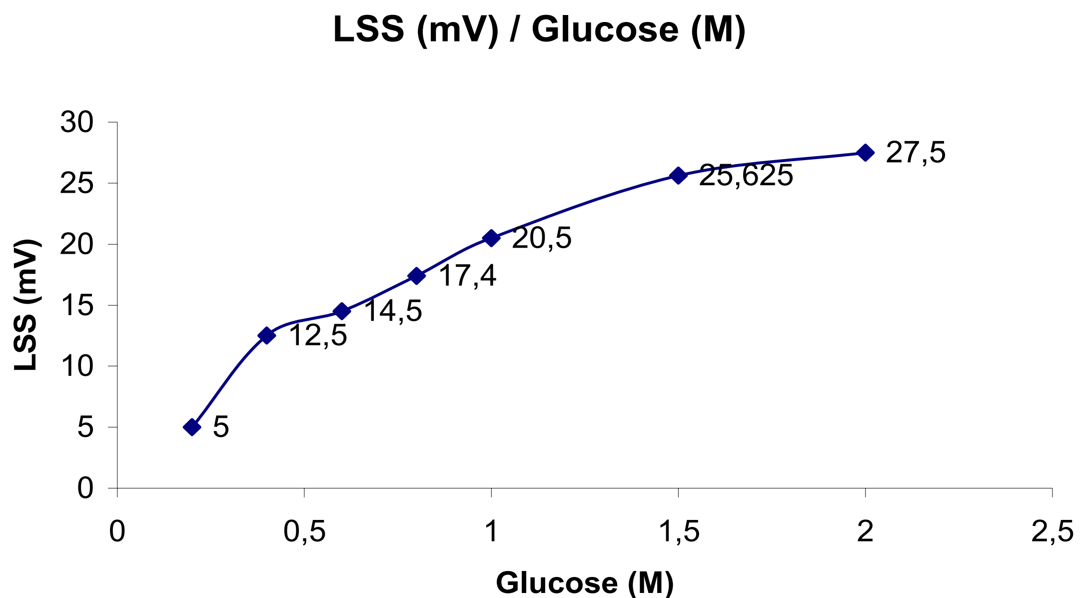


Fig 3.23. LSS signal intensity is dependent on added glucose concentration. In 1800 µl thylakoid suspension (final chlorophyll concentration 90 µg/ ml) was added 200 µl of glucose of different molarities.

3.12.3. Analyses of CPP by Light scattering system technique

To see whether CPP as cryoprotective protein have influence on the value of LSS signal and on kinetics of curve, in the next experiment, before adding of glucose, a 65 μg/ml (final concentration) of active CPP has been added. A different LSS signal was recorded in contrast to control (without protein).

When CPP is added to thylakoid suspension, with final concentration of 65 μg/ml LSS signal increases gradually contrary to the control.

This might be due the forming of clusters of thylakoids or the thylakoids are shrinking in the presence of CPP. On the Fig. 3.24. a change in the form of LSS signal after adding of CPP in comparison to control can be seen.

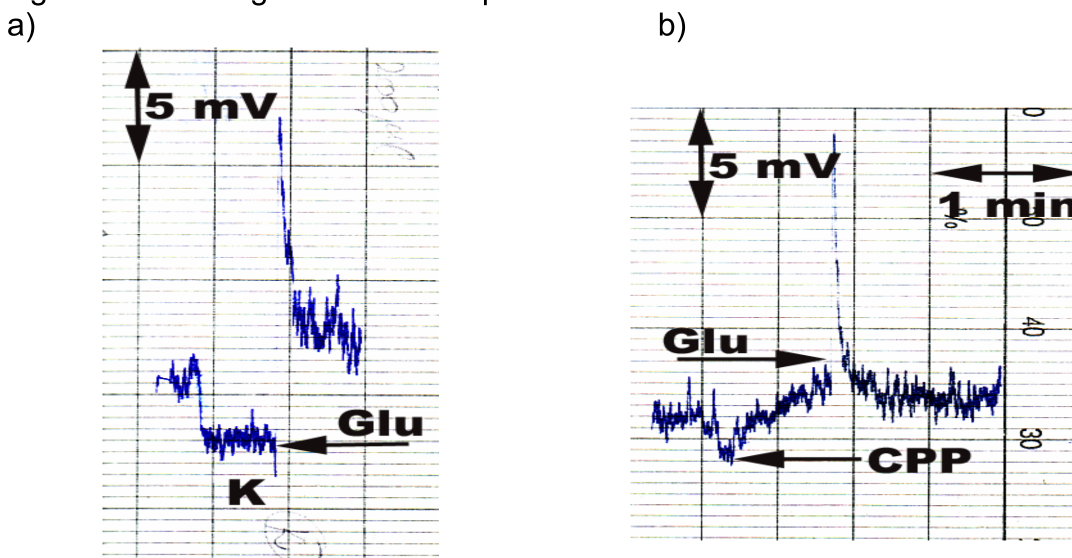
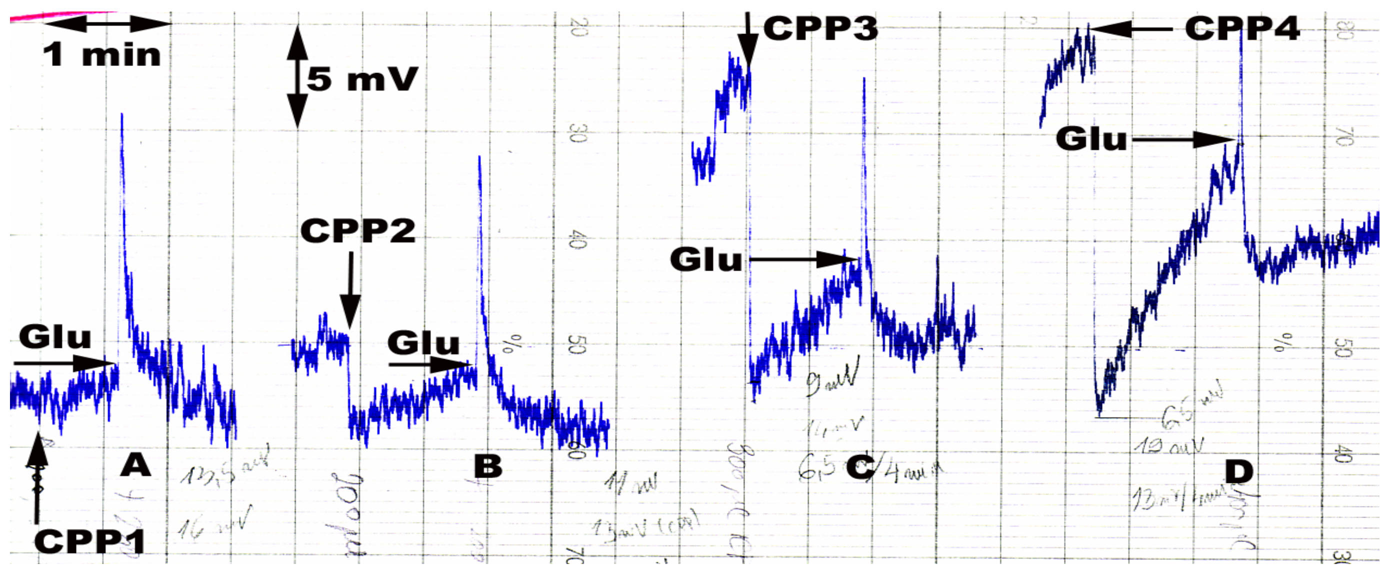


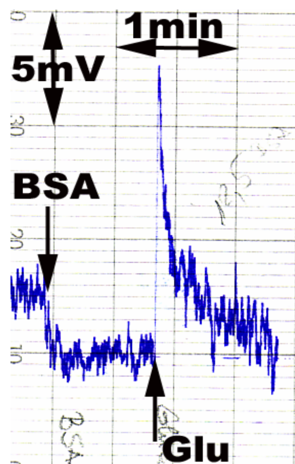
Fig. 3.24. LSS signal after adding of 200 μl 1M Glucose in suspension thylakoids-CPP.

a) LSS signal after adding 200 μl of buffer (10 mM sucrose). After 1 min of stirring in suspension was added 200 μl 1 M glucose (final conc. 100 mM)
b) LSS signal after adding 200 μl of active CPP (conc in assay. 65 μg/ml). After 1 min of stirring in suspension was added 200 μl 1 M glucose (final conc. 100 mM).Final chlorophyll concentration 105 μg/ ml. Mn^{2+} and Ca^{2+} ions are not present in the thylakoid solution.

Increasing the concentration of previously added CPP protein in thylakoid suspension, the amplitude of recorded LSS signal, before adding of sugar, was also increased (Fig.3.25.).



As negative control BSA was used with the same concentration as CPP protein.



e)

Fig. 3.25. LSS signal after adding of Glucose in suspension thylakoids–CPP.

A) LSS signal after adding of 100 µl CPP1 (final conc. 25 µg/ml). After 1 min of stirring in suspension was added 200 µl 1 M glucose (final conc. 100 mM)
B) LSS signal after adding of 200 µl CPP2 (final conc. 50 µg/ml). After 1 min of stirring in suspension was added 200 µl 1 M glucose (final conc. 100 mM)
C) LSS signal after adding of 300 µl CPP3 (final conc. 75 µg/ml). After 1 min of stirring in suspension was added 200 µl 1 M glucose (final conc. 100 mM)
D) LSS signal after adding of 400 µl CPP4 (final conc. 100 µg/ml). After 1 min of stirring in suspension was added 200 µl 1 M Glucose (final conc. 100 mM)
E) LSS signal after adding of 300 µl BSA (final conc. 80 µg/ml). After 1 min of stirring in suspension was added 200 µl 1M Glucose (final conc.100 mM). Initial chlorophyll conc.115 µg/ ml

In these experiments where glucose as osmotic active substance was used, the acclivity in the slope of LSS signals after adding the CPP, is shown to be dependent on the concentration of previously added CPP protein. This type of dependency is shown on the Fig. 3.26.

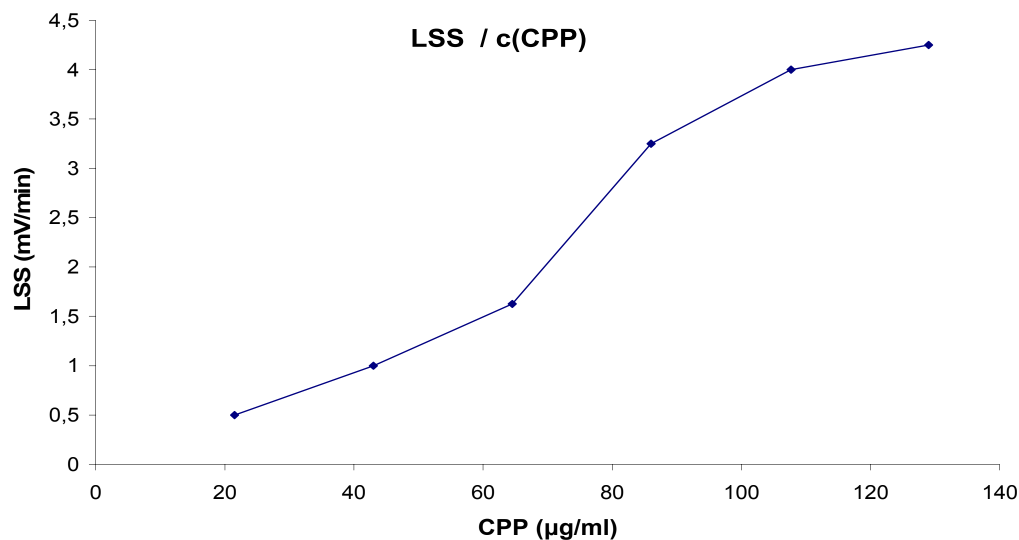


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Fig. 3.26. Dependence of slope of LSS signal on the concentration of CPP protein. Slope of LSS signal is dependent on the concentration of previously added CPP protein. Glucose (final conc.100 mM). Initial chlorophyll conc.115 µg/ ml

It is also seen that after adding of glucose, the LSS peak is dependent on CPP concentration. The higher CPP concentration is the smaller LSS peak becoms. This type of dependency is shown on the Fig.3.27.

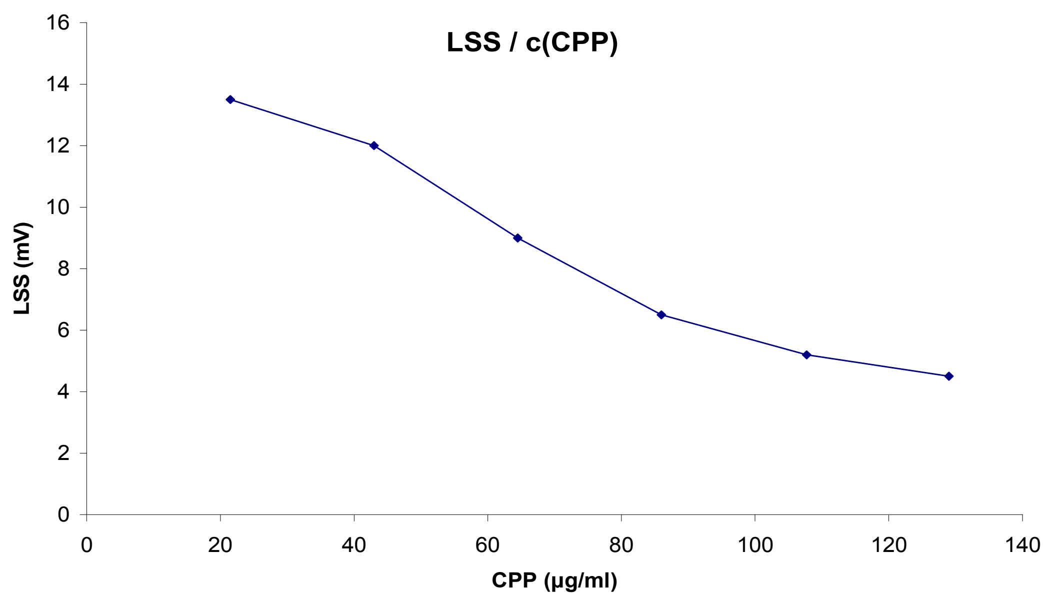


Fig. 3.27. Dependence of measured LSS peak on CPP concentration.

Adding of glucose LSS peak is dependent of CPP concentration. The higher is CPP concentration the smaller is LSS peak. Glucose (final conc.100 mM). Initial chlorophyll conc.115 μg/ ml

One of the conclusions based on Boyle Van't Hoff analyse´s results is that the dehydrins, in the high sucrose concentrations increase membrane permeability. Following this trail it was of an interest to examine whether dehydrins can have the similar effect on the membrane permeability in LSS analysis. By this method it was possible to detect fast changes in the thylakoids membrane permeability in presence of dehydrins.

Previously it was established that presence of sucrose in the thylakoid suspension causes shrinking of thylakoids (Fig. 3.21.a). This is a consequence of establishing of osmotic equilibrium inside and outside of thylakoid vesicle.

Sucrose can not penetrate the membrane and the thylakoids become osmotically dehydrated (water goes out). In the presence of proteins which are able to change (i.e. increase) the thylakoid membrane permeability, sucrose can penetrate trough the membrane, what causes increasing of thylakoid vesicle volume. This change can be recorded by LSS method.

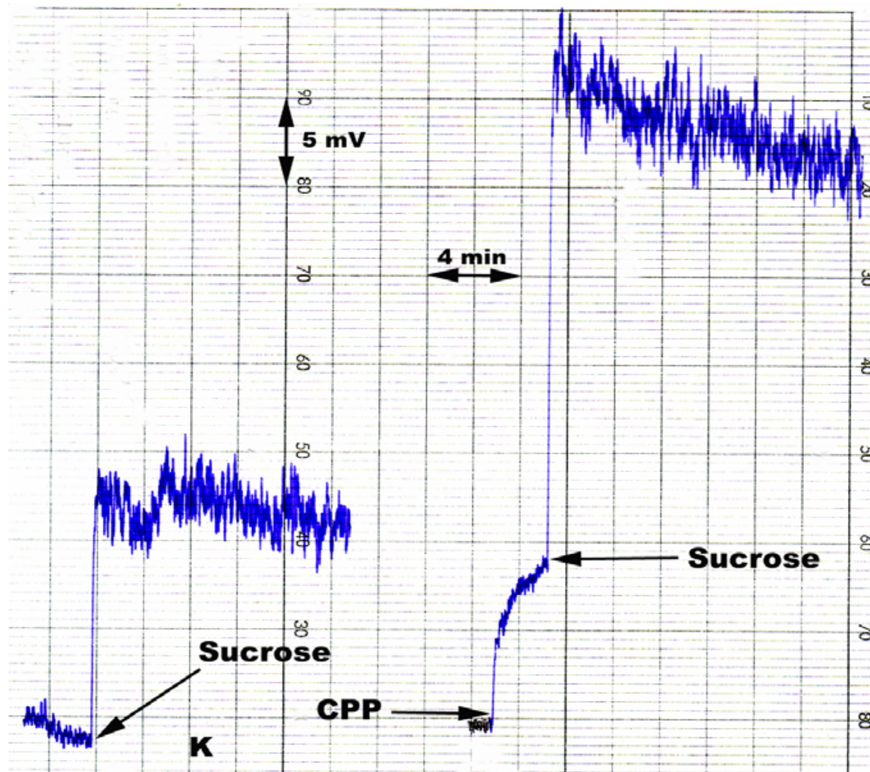


Fig. 3.28. Influence of CPP to the thylakoid membrane permeability in LSS technique.

After adding of CPP (final concentration in assay 90 μg/ ml) in thylakod suspension LSS signal was increased. After 2 min of stirring sucrose (final conc. 100 mM) was added and amplitude of LSS signal was recorded. After apparent shrinking of thylakoids in presence of CPP LSS amplitude decreases during the time commensurate to swelling of thylakoids (increased membrane permeability). Final chlorophyll conc. 115 μg/ ml

After adding of sucrose in the thylakoid suspension in the presence of CPP a different LSS signal was detected in the contrast to the control. LSS signal in due cause of time slightly decreases (thylakoids swelling) which indicates the increasing of membrane permeability in the presence of CPP. In the presence of CPP it was also observed that LSS signal increases more comparatively to the control. (Fig. 3.28).

To see whether thylakoids can be “sensitive” to additional adding of the sucrose, the equally amount of this sugar was added three times in thylakoid suspension. Different LSS signals were recorded in the presence of CPP in the contrast to control.

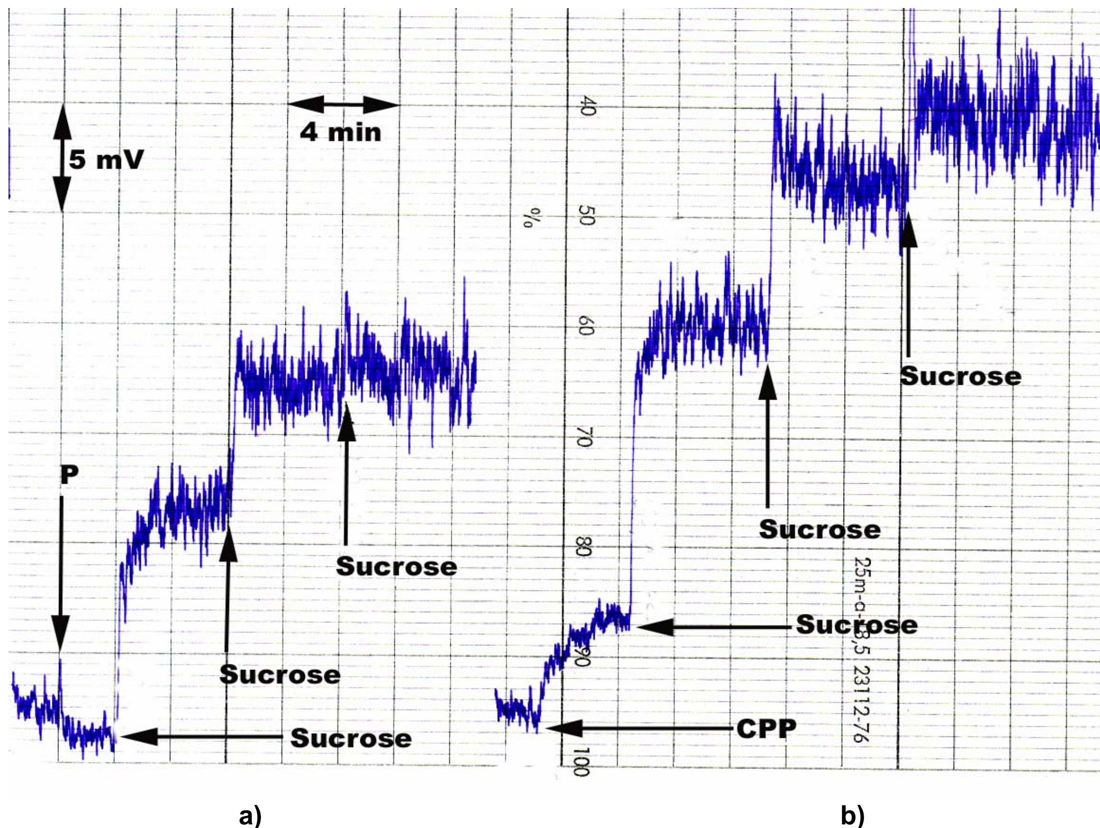


Fig. 3.29. LSS signal after several adding of sucrose in thylakod suspension.

a) After a constant baseline was established in thylakoid suspension was added buffer (10 mM sucrose). After 2 min of stirring 200 μl of 1M sucrose (final conc. 100 mM) was added. Fist adding of sucrose was followed with second and third addition of sucrose (200 μl of 1M sucrose). Initial chlorophyll conc. 115 μg/ ml

b) After a constant baseline was established in thylakoid suspension was added CPP (final conc. 65 μg/ ml). After 2 min of stirring 200 μl of 1M sucrose (final conc. 100 mM) was added. Fist adding of sucrose was followed with second and third addition of sucrose.

From the Fig. 3.29 a) it can be seen that after the second adding of an equal concentration of sucrose (200μl (1M)), the thylakoids are “reacting” – LSS signal increases. It is also visible that increasing of LSS signal is lesser then in the case of the first adding of sucrose. After third adding of sucrose the change in LSS signal is barely noticed.

Contrary to the results obtained for control Fig.3.29 b) in the presence of CPP the increasing of LSS signal is higher after each individual adding of sucrose. By third adding of sucrose in the presence of added CPP the increasing of LSS signal is detected. This was not a case in the examination of control.

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

Hincha and Schmitt (2001) have shown that Mn^{2+} and Ca^{2+} ions have an influence to CPP cryoprotective activity. It was of an interest to determine the effect of these ions on the LSS signal.

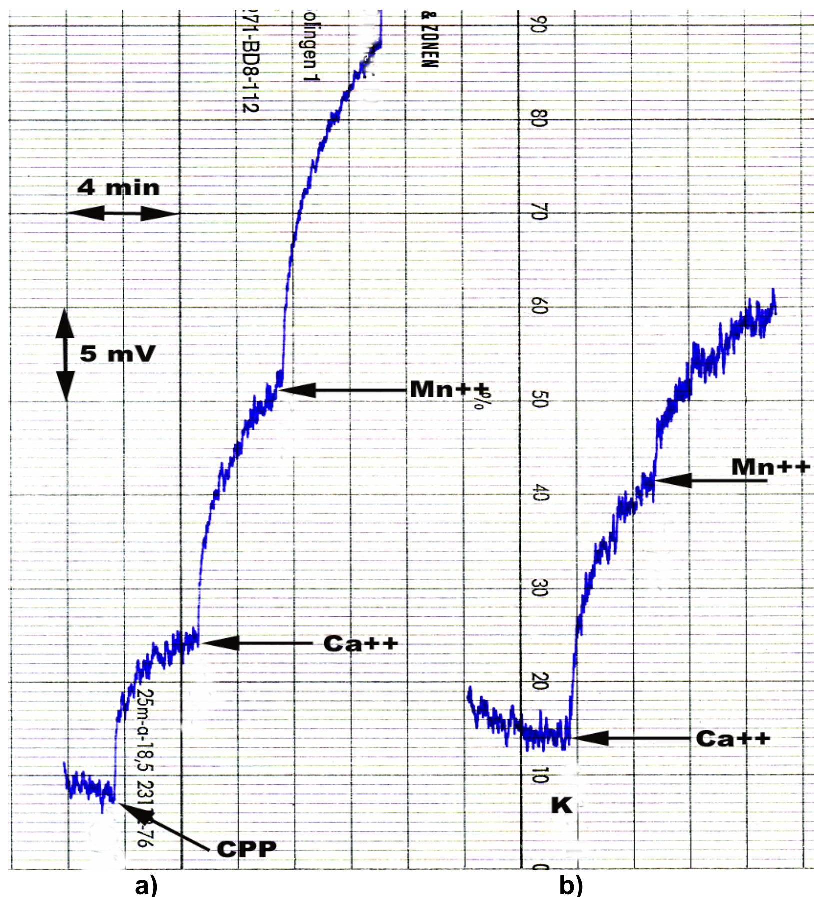
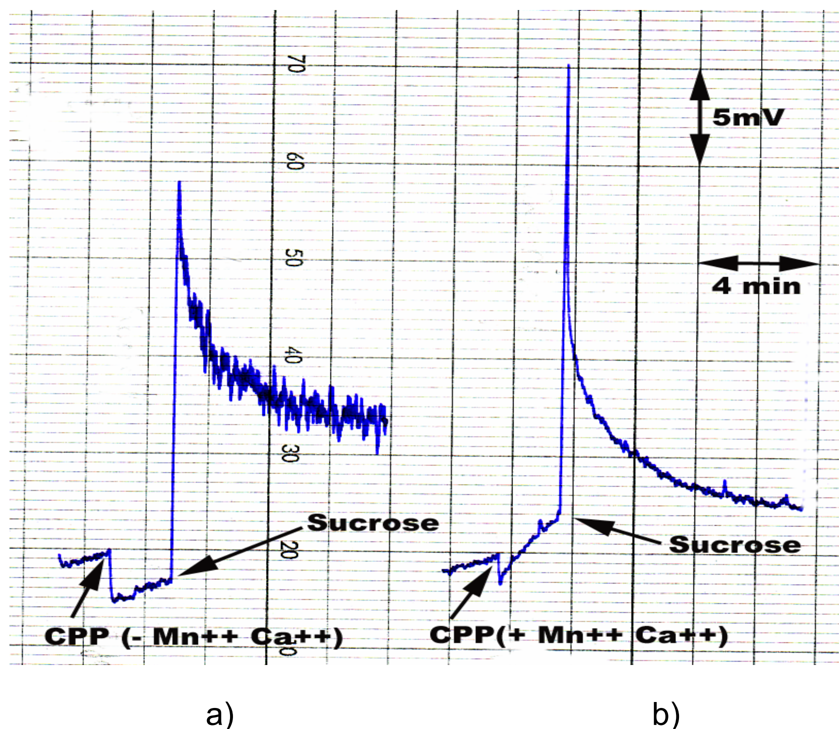


Fig. 3.30. Mn and Ca ions have a influence on CPP activity in LSS technique.

a) After adding of CPP to thylakoid suspension $CaCl_2$ was added (final conc. 1 mM). After adding of ions LSS signal was extremely increased. This effect is even stronger after adding of $MnCl_2$ ions in the same final concentration.
b) In thylakoids suspension were added Ca^{2+} and Mn^{2+} ions ($CaCl_{2;}$ $MnCl_{2,}$ final conc. 1mM) Final chlorophyll conc. 105 μg/ ml. CPP (final conc. 65 μg/ ml)

By adding of $MnCl_2$ and $CaCl_2$ (1mM final concentration) in the thylakoid suspension LSS signal increases faster. This effect is stronger in the presence of CPP (Fig. 3.30 a).
This points out to the conclusion that Mn^{2+} and Ca^{2+} ions increase apparent membrane permeability and that they have an influence on the cryoprotective activity.

Thylakoids are reaching their equilibrium much faster when $MnCl_2$ and $CaCl_2$ are added in the thylakoid suspension (Fig.3.31.) The membrane is more permeable for sucrose molecules in presence of Mn^{2+} and Ca^{2+} ions.This effect is much stronger in the presence of CPP (Fig.3.31). In control experiment sucrose without added CPP behaved as expected.



a) b)

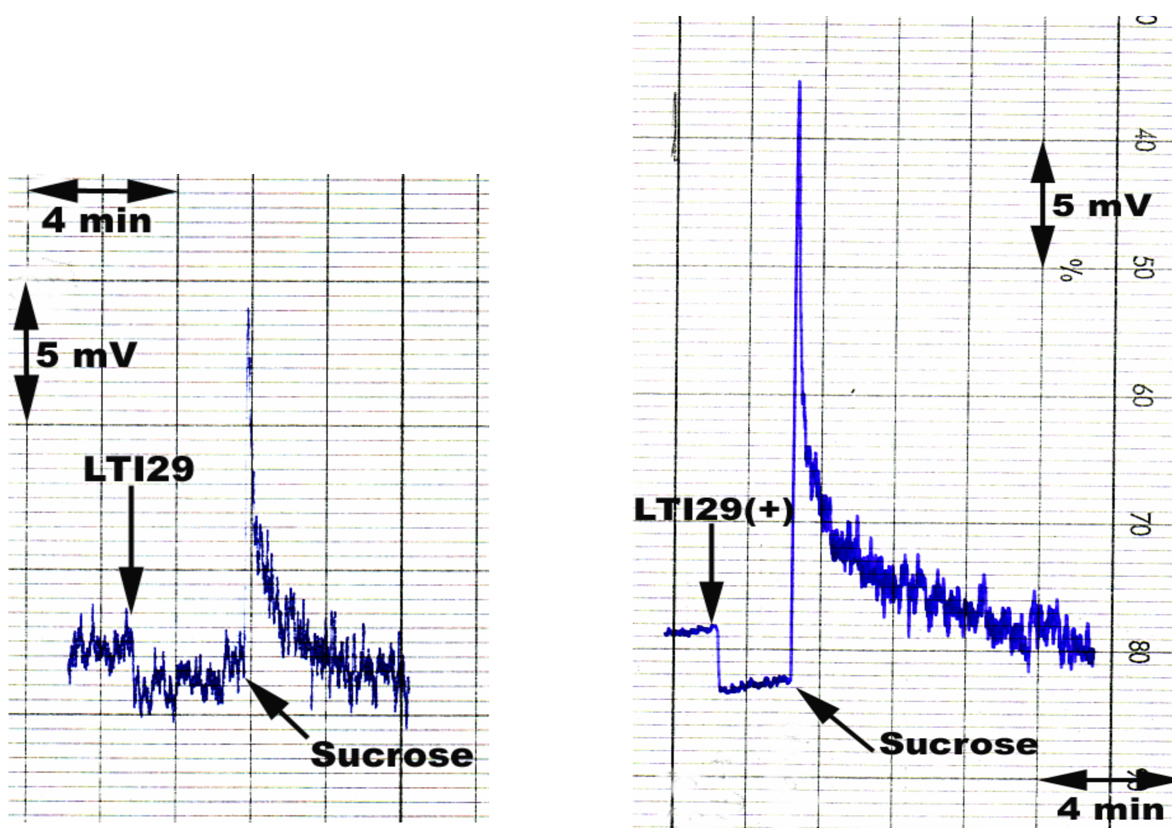
Fig. 3.31. Time course of the light scattering signal in sucrose permeation experiment.

a) LSS after adding of 200µl of 1M Sucrose. (In suspension previously was added 200µl of CPP (conc.in assay 70 µg/ml (without Ca^{2+} and Mn^{2+} ions).

b) LSS after adding of 200μl of 1M Sucrose. (In suspension previously was added 200μl of CPP (200μl concentration in assay 70 μg/ml) with Ca^{2+} and Mn^{2+} ions. Final chlorophyll conc. 85 μg/ ml

3.12.5. Analyzing of dehydrins by LSS technique

The presence of LTI29 dehydrin in LSS system triggers effects which are different in comparison to the effects caused by CPP protein. Firstly, LTI29 dehydrin do not increase LSS signal alone before adding of sucrose. Secondly, the amplitude of the LSS signal is different in comparison to the amplitude of the LSS signal triggered by CPP protein.
The conclusions are that these two proteins interact differently with thylakoid membrane and that they activate different mechanisms of cryoprotective activity (Fig 3.32.). Also, in absence of Mn^{2+} and Ca^{2+} ions the equilibrium was faster reached in relation to CPP.



a) b)

Fig. 3.32. Time course of the light scattering signal in sucrose permeation experiment.

a) LSS after adding of 200μl of 1M Sucrose. (In suspension previously was added 200μl of LTI29 (conc. in assay 60 μg/ml (without Ca^{2+} and Mn^{2+} ions). Final chlorophyll conc. 115 μg/ ml

b) LSS after adding of 200μl of 1M Sucrose. (In suspension previously was added 200μl of LTI29 (conc. in assay 60 μg/ml) with Ca^{2+} and Mn^{2+} ions. Final chlorophyll conc. 85 μg/ ml

The conclusion coming out of this result would be that, in comparison to CPP, LTI29 causes higher membrane permeability. The kinetic of LTI29 LSS curve is similar both in the case when Mn^{2+} and Ca^{2+} ions are added to the system, as well as in the case when they are not a part of the system. It is possible that Mn^{2+} and Ca^{2+} ions do not have important role in the functioning of LTI29 and other dehydrins.

Changing of the concentration of added LTI29 the amplitude of LSS signal after adding of sucrose also changes. The higher concentration of added LTI29 is, the LSS amplitude becomes smaller Fig. 3.33

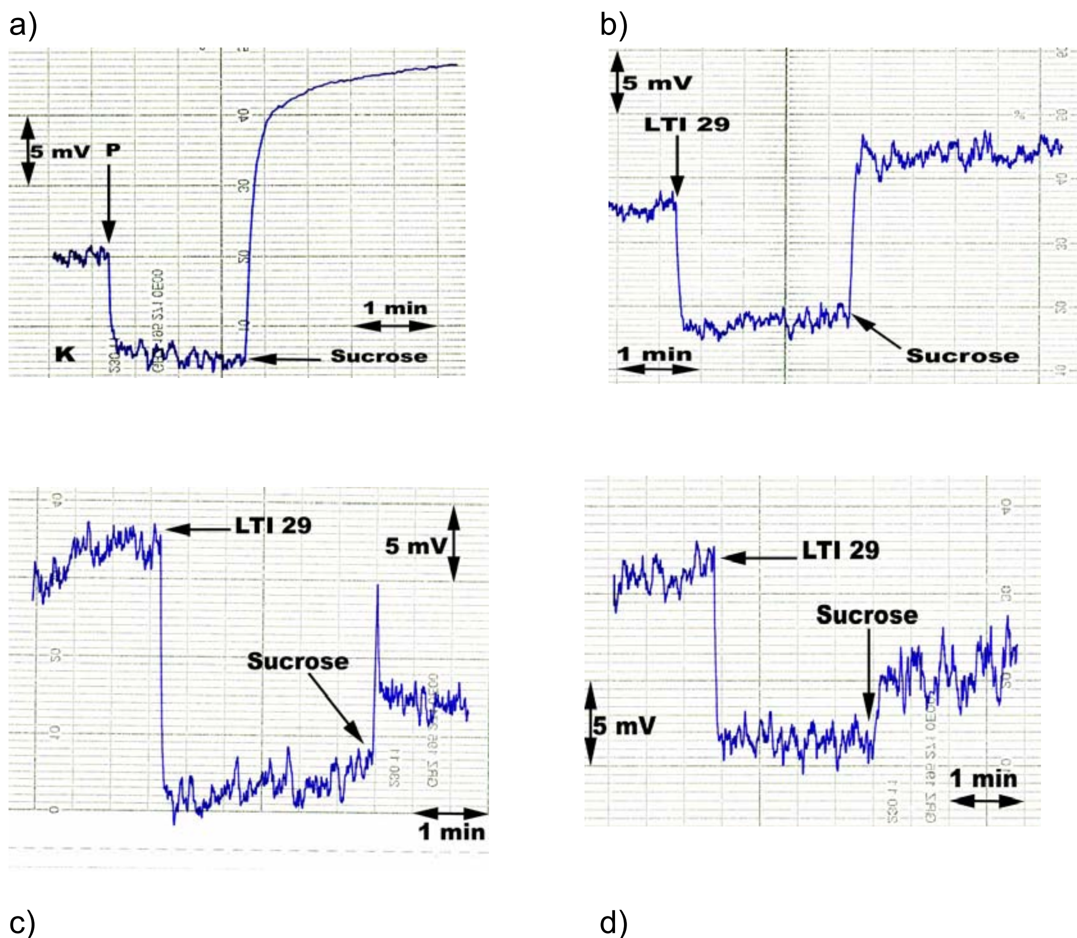


Fig. 3.33. Time course of the light scattering signal in the sucrose permeation experiment in the presence of LTI29.

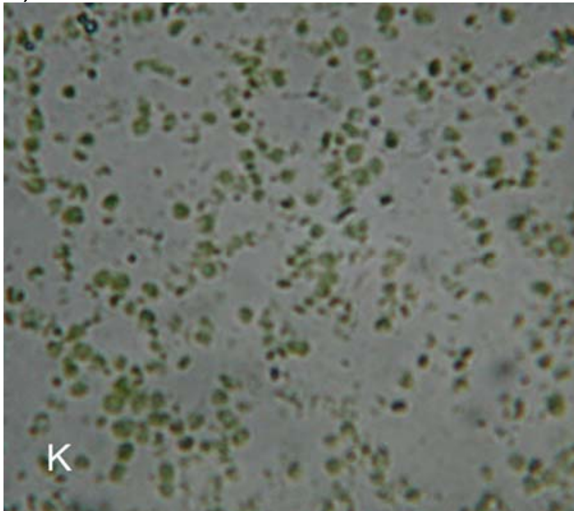
LSS after adding of 100µl of 1M sucrose.
a) In suspension previously was added 200µl of Probe buff. (without Ca^{2+} and Mn^{2+} ions)
b) In suspension previously was added 50 µl of LTI29 (concentration in assay 20 µg/ml) (without Ca^{2+} and Mn^{2+} ions)

c) In suspension previously was added 150µl of LTI29 (conc. in assay 60 µg/ml (without Ca^{2+} and Mn^{2+} ions)
d) In suspension previously was added 250µl of LTI29 (concentration in assay 100 µg/ml) (without Ca^{2+} and Mn^{2+} ions). Final chlorophyll conc. 90 µg/ ml

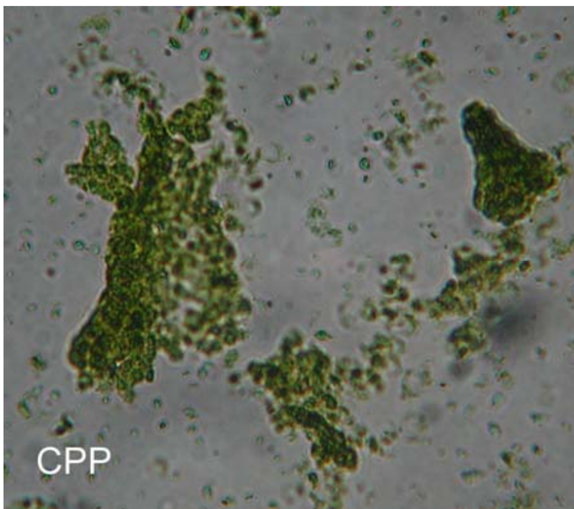
3.13. Analyses done by light microscope

The previous assumption is that aggregations of thylakoids can be the reason for increasing LSS signal in the presence of CPP protein. The light microscope observation is done in order to find out if this hypothesis is correct. The figures show, in the contrast to control, that thylakoids aggregate in the presence of CPP protein. Aggregations are also noticed in the presence of LTI29 dehydrin, but not in the same extent as in the presence of CPP protein. The same concentration of CPP and LTI29 were added to the thylakoid suspension.

a)



b)



c)

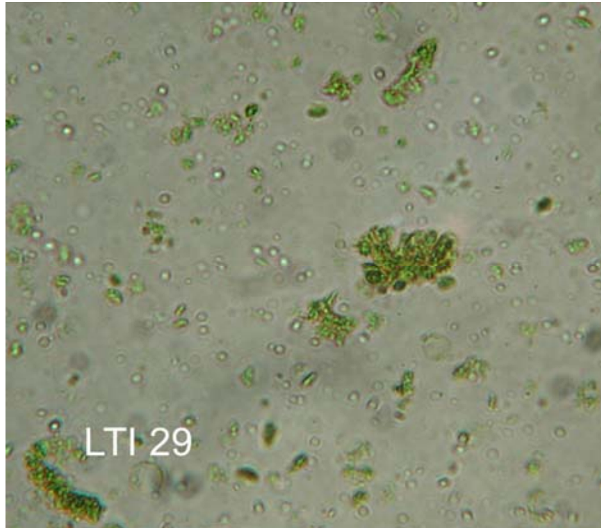


Fig. 3.34. Light microscope analyses of thylakoid suspension in presence of LTI 29 and CPP.

a) Light microscope analyses of thylakoid suspension without proteins CPP and LTI29
b) Light microscope analyses of thylakoid suspension. In 900 µl of thylakoid suspension was added 100 µl of CPP (final conc 65 µg/ml)
c) Light microscope analyses of thylakoid suspension. In 900 µl of thylakoid suspension was added 100 µl of LTI29 (final conc. 65 µg/ml) Final chlorophyll concentration 70 µg/ ml

3.14. Sedimentation of thylakoids in presence of cryoprotective proteins and sucrose

The aggregations of thylakoids could cause sedimentation in due course of time. That was the reason to measure OD_{535} of thylakoid suspension at the certain time points. Several plastic cuvettes were filled with thylakoid suspension containing specified concentration of CPP protein, LTI29 and sucrose.

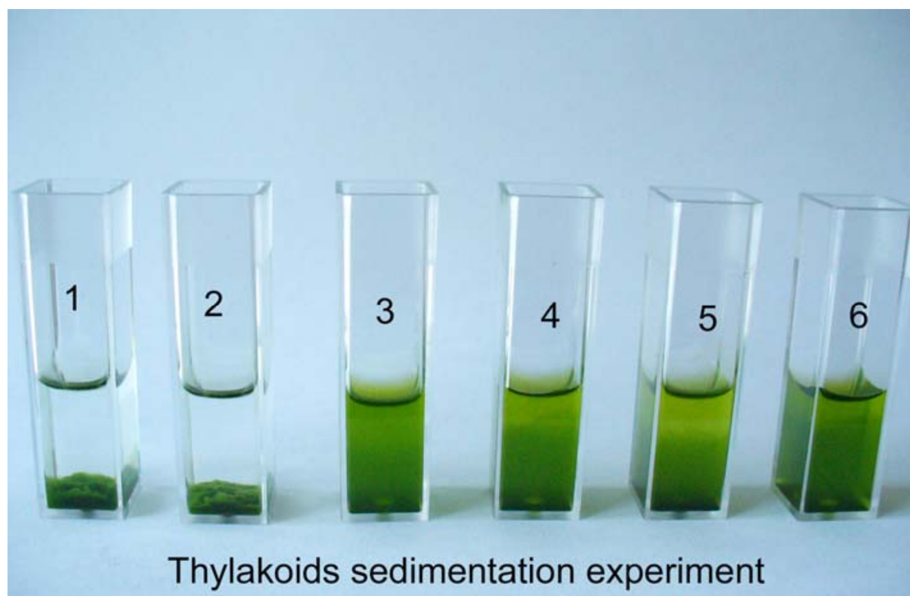


Fig. 3.35. Thylakoid sedimentation experiment .
Several plastic cuvettes were filled with thylakoid suspension in 10 mM Sucrose (without Ca^{2+} and Mn^{2+} ions) containing specified concentration of CPP protein, LTI29 and sucrose. Picture was made after 3h.

1) In thylakoid suspension was added 200 µl of 1M sucrose (final conc. 100mM)
2) In thylakoid suspension was added 200 µl of 1M sucrose (final conc. 100mM) and 200 µl of CPP (concentration in assay 60 µg/ml)
3) In thylakoid suspension was added 200 µl of 1M sucrose (final conc. 100mM) and 200 µl of LTI29 (concentration in assay 60 µg/ml)
4) Thylakoids as control without added sucrose or proteins.
5) In thylakoid suspension was added 200 µl of CPP (concentration in assay 60 µg/ml)
6) In thylakoid suspension was added 200 µl of LTI29 (concentration in assay 60 µg/ml)

Final chlorophyll concentration 100 µg/ ml

From the Fig. 3.35. it can be seen that thylakoids sediment faster in the presence of CPP protein. The sedimentation process is almost blocked in the presence of LTI29 dehydrin, with or without sucrose. This leads to the conclusion about the different mechanisms of activity of these two proteins.

Different sedimentation capacity can be observed on the next graphic.

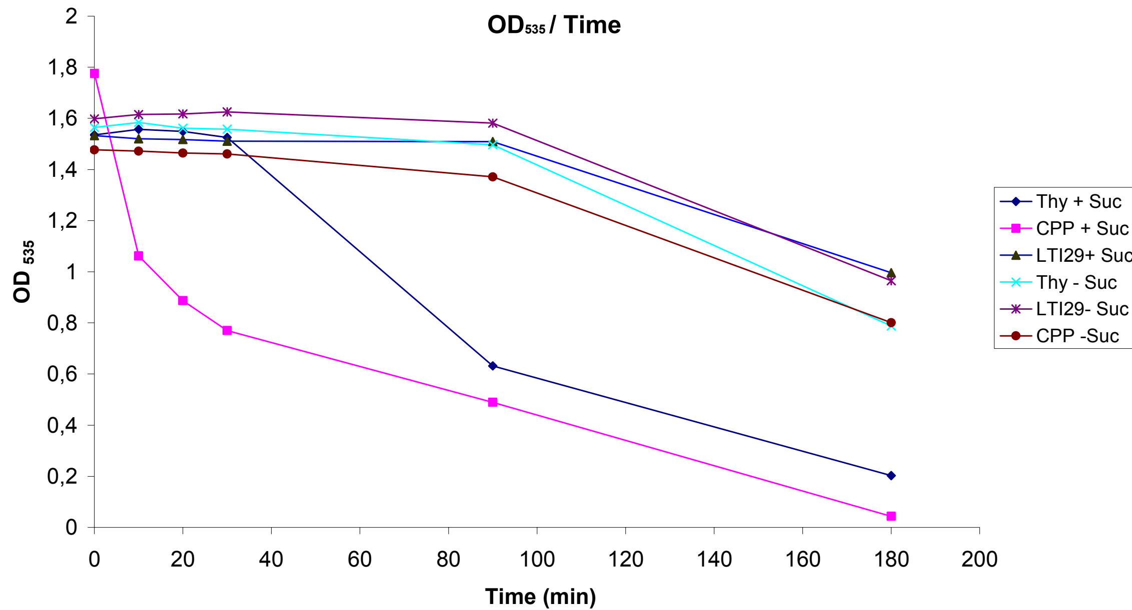


Fig. 3.36. Dependence of OD $_{535}$ on time (min).
Thylakoids have different ability to sediment in presence of LTI29, CPP and Sucrose.
Thylakoids sediment faster in the presence of added CPP protein. The sedimentation process is almost blocked in the presence of LTI29 dehydrin, with or without sucrose.
Thylakoid suspension in 10 mM Sucrose (without Ca^{2+} and Mn^{2+} ions) containing specified concentration of CPP protein (concentration in assay 60 µg/ml), LTI29 (concentration in assay 60 µg/ml) and sucrose (final conc. 100mM)
Final chlorophyll concentration 100 µg/ ml