2. Material and Methods

2.1. Material

2.1.1. Plants

Plant Material and Growth Conditions

Spinach *(Spinacia oleracea* L. cv Monnopa) was grown in a growth chamber with 12 h of light at 150 μmol quanta m^{-2} s^{-1} at 25 °C and 12 h of dark at 15°C at 50% RH (relative humidity). (Production: Julius Wagner GmbH)

Cabbage *(Brassica oleracea* L. cv Grüfewi) was grown in the garden for several months and then transferred to pots. Plants were harvested, and leaves were stored frozen at
-20°C.

2.1.2. Bacterial strain:

E. coli M15[pREP4], SG13009[pREP4] Qiagen
For regulated high-level expression with pQE Vectors (Cells contain pREP4 plasmid encoding lac repressor in trans, ensuring tightly regulated expression).

2.1.3. Genes used in this study (Accession no. provided from NCBI)

Gene	Acc. no. :	Reference
LTI 29	D17714	Kiyosue T. and Yamagutchi-Shinozaki K., 1994
ERD 14	D17715	Kiyosue T. and Yamagutchi-Shinozaki K., 1994
COR 47	X59814	Thomashow, M.F., 1992
RAB 18	ATRAB18A	Lang V. and Palva, E.T., 1992

2.1.4. Protein and DNA standards

1) Wide-Range Unstained Standard for SDS-PAGE

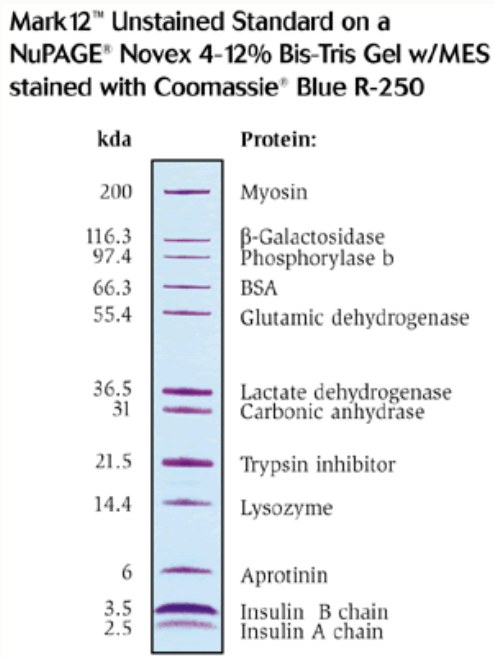

Fig. 2.1. The Mark12™ Standard is an unstained protein standard that allows the closest estimation of molecular weight of sample protein over a wide molecular weight range. 12 polypeptides resolved into sharp, tight bands in the range of 2.5 kDa to 200 kDa

2) GeneRuler™ 100bp DNA Ladder Plus (MBI Fermentas)

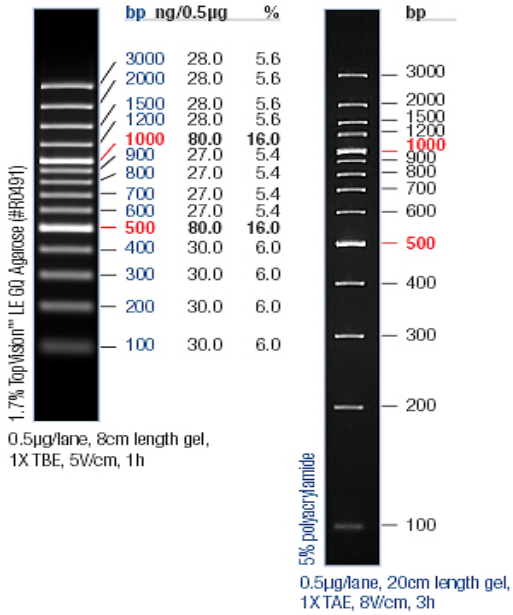

Fig. 2.2. Designed for sizing and approximate quantification of PCR products or other wide range double-stranded DNA fragments in agarose or polyacrylamide gels.

Two reference bands: 1000 bp and 500 bp.

Range

14 fragments (in bp): 3000, 2000, 1500, 1200, **1000**, 900, 800, 700, 600, **500**, 400, 300, 200, 100.

2.1.5. Expression vector

The expression vector **pJTS1** containing the cloned dehydrin genes were obtained from Jan Svensson (Sweden, Upsala University) (Svensson, 2000). The vector pJTS1 is a modified pQE-60 (Qiagen) vector. The expression vector pQE-60 was digested with *Bam*HI and *Hind*III, removing the multicloning site and the sequence encoding the C-terminal His-tag. To generate a new multicloning site, primers where synthesized with a 5' *Bam*HI and a 3' *Hind*III overhang respectively. After ligation digested pQE-60, yielding a plasmid with a multicloning sites *Nco*I, *Bam*HI, *Sph*I, *Sac*I, *Kpn*I, *Sma*I/*Xma*I, *Sal*I and *Pst*I. The dehydrin genes were cloned into pJTS1 vector (Svensson et al., 2000).

Expression vectors containing genes for analysed proteins were transferred to E. coli **M15[pREP4], SG13009[pREP4]** strain.

2.2. Methods

2.2.1. *E. coli* transformation

2.2.1.1. Preparation of chemically competent cells

Competent cell - cell which has been chemically treated to allow the diffusion of DNA into the cell.

The component cells where prepared as follows:

Day 1

Streak out the *E.coli* strain on an LB plate (no ampicillin!) to isolate colonies and incubate at 37 degrees C overnight (16-20 hours).

Day 2

Use a sterile inoculating loop to collect cells from a single colony and inoculate 10 ml sterile 1X LB Grow at 37 degrees C overnight (16-20 hours) in a shaker incubator.

Day 3

1. Add 10 ml of the overnight culture to each 400 ml LB flask. Grow the cultures to OD_{600} = 0,6-0,7 (approximately 3 hours).
2. Pellet the cells in chilled autoclaved large centrifuge bottles using the Sorvall RC-5B centrifuge and F-16/250 rotor (must be cold!) at 5000 rpm

for 10 minutes. Subsequent resuspensions may be done in the same bottle. *Cells must remain cold for the rest of the procedure:* Decant supernatant and transport tubes and store on -20 °C for further use.

3. Resuspend the cells in 1/20 original volume (17.5 ml) of ice cold 100 mM $CaCl_2$. Hold on ice for 20 minutes. Pellet as above 4000 rpm for 10 minutes.
4. Decant the supernatant and resuspend the cell pellet in 1/100 original volume (3.5 ml) of a solution that is 85% v/v 100 mM $CaCl_2$ and 15% v/v glycerol (100%). For each culture processed chill approximately 15 labeled eppendorf tubes in a dry ice-EtOH bath. Pipet 300 ul cells into each tube and place immediately into the dry ice-EtOH bath. Transfer the frozen competent cell aliquots to -80 degrees C.

Solutions:

100 mM $MgCl_2$:

1:10 dilution of lab stock; use sterile ingredients or filter sterilize

100 mM $CaCl_2$:

1:10 dilution of lab stock; use sterile ingredients or filter sterilize

85% 100 mM $CaCl_2$, 15% glycerol:

42.5 ml 100 mM $CaCl_2$
7.5 ml 100% glycerol
50 ml total volume;

Mix well and use sterile ingredients or filter sterilize

2.2.1.2. Transformation of frozen competent cells (Protocol):

1. Defrost frozen component cells slowly on ice (15- 30 min).
2. Add 200 µl of competent cells to the 20 µl of DNA solution in tubes, swirling gently.
3. Incubate on ice for 30 minutes.
4. Heat shock cells for 90 sec at 42 °C. (time varies with different *E.coli* strains).
5. Return tubes to ice and Incubate for 2 minutes.
6. Bring up in 1 ml LB and shake gently for 1 hour at 37°C.

2.2.2. Colony screening by PCR

Bacterial clones can be screened for the correct ligation products. Selected colonies are picked with a sterile toothpick from an agarose plate and dabbed into the master mix or sterile water.

Notes

This is the fastest way to screen bacterial colonies.PCR is used to amplify specific regions of a DNA strand. This can be a single gene, just a part of a gene, or a non-coded sequence. PCR typically amplifies only short DNA fragments, usually up to 10 kilo base pairs (kb).

Protocol:

Set up 26 PCR tubes.

Touch a fresh toothpick (or yellow tip) onto a colony, dip it into a PCR tube, then streak it onto a fresh replicate agar plate using a numbered template (that is, all 26 colonies on a single agar plate). Repeat this for the 22 (or whatever) colonies, for tube 26 (positive control) use a colony that will yield a product with your primers.
Incubate the replicate agar plate at 37C.

These streaked colonies will be visible within 6-8 hours so you can set up overnight miniprep cultures on the same day.

Set up a 26 x PCR pre-mix as follows:

Tab. 2.2. Pre-mix solution used for 26 PCR probes

Pre-mix solution:

1 x		26 x
2,5µl	10 x PCR buffer	65µl
3 µl	$MgCl_2$ (3 mM)	78µl
0,12µl	d NTP	3,12µl
0.2µl	Taq Pol	5,2µl

In pre-mix solution for 26 tubes add 409 µl of H_2O

In each tube add 21,5 µl of pre-mix solution.

In each tube add primers and DNA template:

Tab. 2.3. Amount of primers and template DNA used for 26 PCR probes

1 x pre-mix		26 x pre-mix
0.5µl	primer 1 (100mM)	6µl
0.5µl	primer 2 (100mM)	6µl
3µl	DNATemplate	78µl

The following colony PCR program has been designed to be performed in reaction tubes.

Tab. 2.4. Program used for analyzing of transformed bacteria in colony screening PCR.

Cycles	Temperature	Duration
1	94 C	60 seconds
35	94 C	30 seconds
	* C	30 seconds
	72 C	1 minute

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

This PCR should take less than 2 hours.

2.2.3 Design of primers

The primers were designed in the manner to comprehend the restriction sites. Also we took an account of following:

1. We used primers that produce stable base pairing with the template DNA under conditions appropriate for cycle sequencing.

2. Length of primers should be between 18 and 35 nt. Length of used primers in this study was between 21 and 37 nt.

3. G-C content of 40-60% is desirable.

4. The Tm (annealing temperature) should be between 55 C and 75 C. The old "4 degrees for each G-C, 2 degrees for each A-T" rule works poorly, especially for oligos shorter that 20 or longer than 25 nt.

5. The Tm can be calculated by computer program, in our case correct annealing temperatures were found experimentally.

6. We discard candidate primers that show undesirable self-hybridization.

7. We verified the site-specificity of the primers and performed a sequence homology search (e.g. dot-plot homology comparison) through all known template sequences and check the alternative priming sites. Any primers that display 'significant' tendency to bind to such sites were discarded.
We can provide only rough guidelines on that what is 'significant'. It that sense we excluded the alternative sites present with (1) more than 90% homology to the primary site or (2) more than 7 consecutive homologous nucleotides at the 3' end or (3) abundance greater than 5-fold higher than the intended priming site.

According to those norms we designed primers for colony PCR (Tab. Material and methodis). PCR products were observed on the agarose gel with expected sizes (Figure 4.1.)

Primers:

Tab 2.1. Lists of oligonukleotides (primers) used in this study

Name of seq.	Sequence:
LTI29	
F LTI29	**5' GAAAAGA ATGGCAGAAGAGTACAAGAACACC 3'**
R LTI29	**3' TTAATCAGACACTTTTTCTTTCTTCTCT 5'**
ERD14	
F ERD14	**5'CCGCTCGAGAAAAGA TGGCTGAGGAAATCAAGAATG 3'**
R ERD14	**3' GCTCTAGA TTATTCTTTATCTTTCTTCTCC 5'**
COR47	
F COR47	**5' GAAAAGA ATGGCTGAGGAGTACAAGAACAACG 3'**
R COR47	**3' TTAATCATCAGACTCTTTTTCTTTCTTCACTTCC 5'**
RAB18	
F RAB18	**5'CCGCTCGAGAAAAGA TGGCGTCTTACCAGAACCGTCCAGG 3'**
R RAB18	**3' GCTCTAGA TTAACGGCCACCACCGGGAAGCTTTTCC 5'**

PQE F1	**5' CCCGAAAAGTGCCACCTG 3'**
PQE F2	**5' CGGATAACAATTTCACACAG 3'**
PQE R	**3' GGTCATTACTGGAGTCTTG 5'**

2.2.4. Electrophoresis of DNA in agarose gels

DNA electrophoresis is an analytical technique used to separate DNA fragments by size. An electric field forces the fragments to migrate through a gel. DNA molecules normally migrate from negative to positive potential due to the net negative charge of the phosphate backbone of the DNA chain.

1. Agarose concentrations: 0.8% Agarose (w/v) was used for high molecular weight DNA fragments, and 1 - 1.2% for smaller DNA fragments. 0.5% gels are very flimsy and need to be handled with caution.

2. Buffers & Electrophoresis conditions: TBE buffers are for high voltage electrophoresis greater than 60 volts. Resolution is higher for DNA fragments with lower molecular weight when using TBE.

Electrophoretic conditions: For quick electrophoretic separations, we used 1X TBE at 50-100 volts for a few hours (baby gels 80cV 60 min). For overnight runs we used 40 constant volts for the large 12-20 square cm gels. For critical measurements, run the gel overnight at the lower voltage. High voltages lead to recreased resolution.

3. Gel preparation: Measure agarose and dilute buffer to appropriate concentration. Boil in an ehrlenmeyer ml flask. Cool to 60-65°C before pouring onto a clean, leveled, glass plate or plastic tray surrounded by masking tape. Insert the comb parallel to the plate's edge, with the bottom of the teeth about 2mm above the plate. The thinner the comb is, the higher the resolution. The gel will solidify in the cold room in about 20-25 minutes, or about 45 minutes at room temperature. The gel is opaque when solid. Remove the comb carefully or you may rip out the bottom of the wells. Transfer the gel to the electrophoresis chamber. If the gel won't be run for 1-2 hours, submerse it in running buffer.

4. Sample preparation: Heat DNA samples to 65°C, 10-15 minutes before loading to disassociate overhanging ends of molecules which may have re-annealed. Load 0.4 - 0.6 µg of DNA per lane for analytical gels (depending on the number of expected bands) and up to 20 µg of genomic DNA per lane.
Load molecular weight markers. Gently overlay with buffer, or alternatively, load samples through the buffer. Do not puncture the bottom of the wells.

5. Staining the Gel: Stain gels with 1 µg/ml ethidium bromide in running buffer for 20-45 minutes depending on the gel thickness after electrophoresis. This is preferred to running gels with stain in them since DNA may migrate aberrantly with ethidium bromide. Large agarose gels stain in about 45 minutes; baby gels take about 15 minutes.

Wear gloves when working with ethidium bromide, as it is carcinogenic and mutagenic. Dispose of the ethidium bromide solution in the decontamination carboy.

10X TBE (1M Tris, 1M Boric Acid, 20mM EDTA, pH 8.3)

For 2 l:
242.2 g Tris
123.66 g boric acid
14.89 g EDTA

Adjust pH to 8.3. Autoclave. (*Biotechniques* 10:182, 1991 claims that filtering up to a 20x TBE solution through 0.2 - 0.45µ cellulose acetate or cellulose nitrate filters prevents formation of precipitants during long-term storage. The solution may be reautocalved to dissolve precipitates that form.)

Ethidium Bromide Stock Solution (10mM Tris-HCl, 1 mM EDTA, 1 mg/ml ethidium bromide)

For 50 ml:
0.5 ml 1M Tris-HCl, pH 8.0
0.1 ml 0.5M EDTA
1 mg ethidium bromide

Add ethidium bromide to Tris-HCl, EDTA and about half of the water, and stir overnight to dissolve. Store in a brown bottle at room temperature.

2.2.5. DNA Concentration Determination

DNA concentration was measured by spectrophotometer on A260 for Plasmid minipreps and DNA PCR products.

1. Dilute 1 µl of midiprep DNA into 10 µl TE.
2. Add 1 µl of diluted DNA to 99 µl ddH2O in microcuvette.
3. If A260 is too low, measure the other 9 µl of diluted DNA and adjust calculations.
4. Measure A260 after dilution of DNA sample.

A260 X 50 X dilution factor = µg DNA/ml in undiluted sample. 1 A260 = 50 µg DNA/ml.

2.2.6. Protein expression

A typical expression experiment consists of the following step:

1. **Picking of a single colony** from a freshly streaked plate of the expression host containing the recombinant vector. When the heterologous protein is toxic for the cells, higher expression levels are obtained by using the so-called "plating method".

2. **Growing of a starter culture**. Inoculate with the picked colony up to 50 ml of rich medium (**LB**) containing the appropriate antibiotic (Ampicilin and Kanamicin). When a larger starter culture is required, inoculate 4 ml of rich media with the single colony; grow for 4-8 hours at 37°C; and use this to inoculate the starter culture.

Do not let cultures grow at 37°C overnight! It is better to grow overnight cultures at 30°C or lower. Alternatively, the culture can be incubated at 37°C until the OD_{600} is approx. 1. Then store the culture at 4°C overnight. The following morning, collect the cells by centrigfugation, resuspend them in fresh medium and use this to inoculate the main culture.

The use of ampicillin requires special care. The selectable marker, β-lactamase, is secreted into the medium where it hydrolysis all of the ampicillin. This point is already reached when the culture is barely turbid. From here on, cells that lack the plasmid will not be killed and could overgrow the culture.

1. grow overnight cultures at 30°C or lower.

2. spin overnight cultures and resuspend the pellet in fresh medium to remove β-lactamase.

Inoculation of the main culture and incubation until OD_{600} reaches 0.4-1. The optimal OD value depends on the culture method and the medium. For flask cultures using LB-medium an **OD_{600}** of **0.6** is recommended. To increase the growth rate, we carry out the cultures at 37°C until the OD for induction is reached. Then the cultures are cooled to the induction temperature in ice-water.

Remark: For good aeration, don't use more medium than **20%** of the total flask volume.

3. **Induction of protein expression**. Protein expression is induced by the addition of the proper inducer or by changing the growth conditions. From this point on the cells will use most of their resources for the production of the target protein and will not grow much further.

For the used promoter induction conditions are listed below.

Promoter	induction	typical condition	range
T 7-lac operator	**addition of IPTG**	**0.2 mM**	**0.05 -2.0 mM**

After induction the cultures are incubated from 3 hours to overnight depending on the induction temperature. Guide lines are given below.

Incubation temperature	incubation time
15°C	overnight
20°C	overnight
25°C	overnight
30°C	5-6 h
37°C	3-4 h

4. **Harvesting** of the cell pellet by centrifugation (20 min at 6000 g).

Cell pellets are stored at -20°C.

2.2.7. Protein isolation from *E.coli*

1. **Completely resuspend the pellet** from last step in 4 ml of cold Tris-HCl-Puffer, pH 8,0 (50 mM Tris-HCl, 2 mM EDTA) to yield a concentration factor of 5X (20 ml culture to 4 ml buffer volume).

Note: Some proteins may exhibit higher solubility when the cells are lysed in a buffer containing salt. If desired, NaCl up to 0.5 M may be added to this buffer. Other proteins, such as those associated with membranes, may partition into the soluble fraction if a zwitterionic detergent (e.g., 10 mM CHAPS), is added to the lysis buffer.

2. **Completely lyse the cells** by following method:

Lysozyme treatment plus sonication.
Add lysozyme to a final concentration of 100 µg/ml from a freshly prepared 10 mg/ml stock in water. Incubate at 30°C for 15 min.
Mix by swirling and sonicate on ice using the power level set between 4–5.
Sonicate 4 times for 45 seconds.

Note: Optimal conditions for a given sonicator may be quickly determined by performing a time course analysis. Remove samples at various times during the sonication,

centrifuge at 12,000 x g for 5 min, and then determine the protein concentration in the supernatant by a standard assay, e.g., Bradford, BCA, etc. When the protein concentration in the supernatant reaches a plateau, proceed to the next step.

3. **Incubate at 75°C for 10 min.**

4. Take a 1.5 ml sample of the lysate and centrifuge at 14,000 g for 10 min to **separate the soluble and insoluble fractions**. Transfer 100 µl of the soluble supernatant to a new tube.

5. **Add 100 µl of 2X SB Sample Buffer** (2X SB = 100 mM DTT, 2% SDS, 80 mM Tris-HCl, pH 6.8, 0.006% bromophenol blue, 15% glycerol) to the 100 µl soluble fraction sample.

Store at –20°C until SDS-PAGE analysis. Supernatant and solubilized cell debris were analyzed for the presence of inclusion bodies.

Solutions:

Tris-HCl-Puffer, pH 8,0
50 mM Tris-HCl, 2 mM EDTA

2X SB Sample Buffer
100 mM DTT, 2% SDS, 80 mM Tris-HCl, pH 6.8, 0.006% bromophenol blue, 15% glycerol

2.2.8. Ammonium sulphate precipitation $(NH_4)_2SO_4$

Analyzed proteins were precipitated in 80% (561 g/l) ammonium sulphate solution:

1. Place the sample in a beaker or flask that can contain twice the sample's volume. Place the container on a stir plate and add a stir bar.

2. While stirring, add the saturated ammonium sulfate solution drop-wise to the protein solution until precipitates start to form.

3. Allow sample to precipitate for 30 minutes at room temperature or 6 hours to overnight at 4°C.

4. Centrifuge sample at 4°C, 12 000 rpm 10 minutes.

5. Discard supernatant and dissolve the precipitate in a buffer. Use a volume of buffer from 0.1 to 1.0 times the original sample volume.

2.2.9. Gel filtration

After dissolving of ammonium sulphate precipitate in a suitable buffer it was necessary to remove salt from the sample:

PD-10 desalting Columns

Amersham Biosciences PD-10 Desalting columns are prepacked, disposable columns containing Sephadex™ G-25 Medium for group separation of high (Mr > 5000) from low molecular weight substances (Mr < 1000) by desalting and buffer exchange.

Procedure:
1. Cut off bottom cap, remove top cap and pour off excess liquid.

2. If available mount the LabMate Buffer Reservoir on top of the PD-10 column and place the columns in the PD-10 Desalting Workmate.

3. Equilibrate the column with approximately 25 ml elution buffer. Discard the flow-through (you can use the plastic tray to collect the flow-through)

4. Add sample of a total volume of 2.5 ml. If the sample is less than 2.5 ml, then add buffer until the total volume of 2.5 ml is achieved. Discard the flow-through.

5. Elute with 3.5 ml buffer and collect the flow-through. A typical chromatogram is showed in Figure

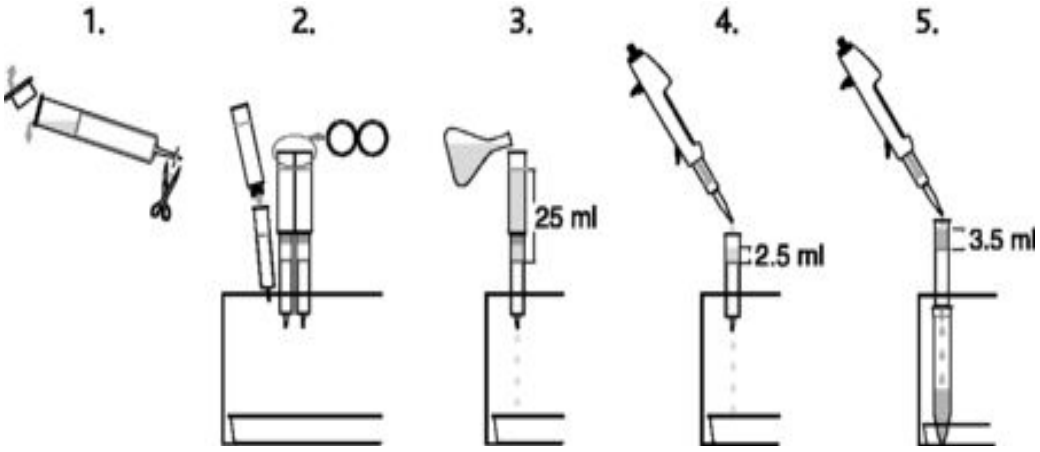

Fig. 2.3. Schematic of the method used with PD-10 desalting Columns.
(1) Preparation of the column; (2) attachment of the LabMate Buffer Reservoir; (3) column equilibration; (4) sample application; (5) elution and collection of sample.

PD-10 desalting column characteristics:

Matrix Sephadex G-25 Medium
Particle size range 85–260 µm
Bed volume: 8.3 ml
Bed height: 5 cm
Rec. sample volume 2.5 ml
Exclusion limit Mr 5 000
Chemical stability All commonly used buffers
Working pH range 2–13
Storage temperature +4 to +30°C
Supplied in Distilled water containing 0.15%
Kathon™ CG/ICP Biocide

2.2.10. Isolation and purification of Cabbage Cryoprotectin

Cabbage leaves (1 kg) were homogenized in a blender (Ika, Staufen, Germany) in 600 ml of an ice-cold solution comprising 50 mM Tris, 2% (w / v) Polyclar AT (insoluble PVP), and 300 p mercaptobenzothiazole (pH adjusted to 7.8 with acetic acid).
The homogenate was filtered through 50-µm nylon mesh and then centrifuged for 30 min at 23,000xg.
The supernatant solution was incubated in a boiling-water bath for 8 min and immediately transferred to an ice-water bath. From the cooled solution the precipitated proteins were removed by a 15-min centrifugation.
Further precipitation was done by the addition of solid ammonium sulfate to 60% saturation. The solution was stirred for 1 h at 4°C and, after centrifugation as above; the pellets were resuspended in Probe buffer.

Solutions:

Homogenizations buffer:
50 mM Tris
2% (w / v) Polyclar AT (insoluble PVP)
300 p~ mercaptobenzothiazole (pH adjusted to 7.8 with acetic acid)

Probe buffer:
10 mM Sucrose
1 mM MnCl2
1 mM CaCl

Ammonium sulfate

2.2.11. Freezing test (Hincha and Schmitt, 1992a)

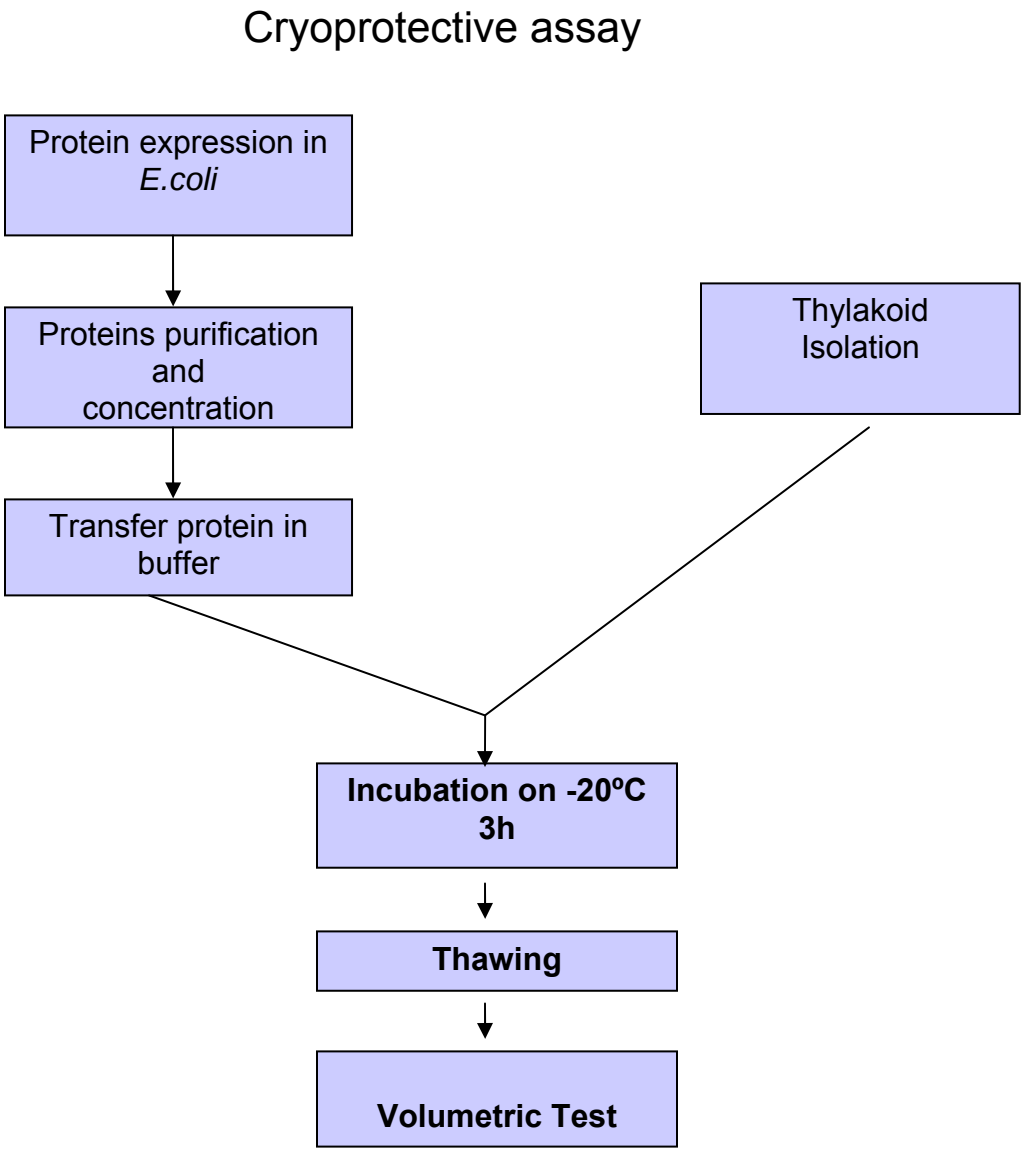

Fig. 2.4. Schematic retrospective of cryoprotective assey

Thylakoids are sensitive to elevated temperatures, therefore all solutions and glassware should be cooled to 4°C before use. Isolated membranes should always be kept on ice, preferably in dim light. All centrifugation steps take place at 4°C.

1. **Thylakoid isolation**:

Homogenize 50g of leaves with 100 ml of homogenization buffer in a blender for approx 10 s. Add 125 µl 1M Na-ascorbate and 340 µl 1M cysteine to the buffer immediately before use, as these substances are unstable in solution. Filter the homogenate through nylon mesh to remove coarse particles. Centrifuge filtrate for 5 min at 2000g. Discard supernatant and resuspend pellets in aprox 50 ml of washing solution. This is most easily performed using a Pasteur pipet. Centrifuge for 5 min at 7000g and discard supernatant. Repeat this washing procedure twice. Resuspend the pellets from the last centrifugation in a minimum volume of washing solution. Mix 10 µl of the thylakoid suspension with 990 µl of 80% (v/v) acetone and centrifuge for 2 min in a benchtop centrifuge. Measure absorbance of the supernatant at 663 and 645 nm with 80% (v/v) acetone as the reference.

Chlorophyll content is calculated as:

(8.02 * A663 + 20.2 * A645) * 0.1 = mg Chlorophyll / ml

Dilute the thylakoid suspension with washing buffer to a concentration of at least 1 mg chlorophyll/ml

2. Mix 100 µl of the thylakoid suspension with an equal volume of cryoprotectant solution (protein in suitable buffer) in eppendorf tubes. Place in a freezer at -20°C. No special precautions are necessary for the freezing step. Samples are most conveniently thawed for 2 min. in a water bath at room temperature and should be transferred in an ice bath immediately when the ice in the tubes has melted.

Solutions:

Thylakoidisolation buffer:

240 mM Sucrose
160 mM NaCl
1 mM $MgCl_2$ (Hexahydrate)
1 mM $MnCl_2$ (Dihydrate)
2 mM EDTA
1 mM KH_2PO_4
50 mM Tris-HCl, pH 7,8

Before using add 1,25 mM Ascorbat (Na-salt)
3,3 mM Cystein

Washing buffer: 10 mM NaCl

Protein buffer :
10 mM Sucrose
1 mM $MnCl_2$ (Dihydrate)
1 mM $CaCl_2$ (Dihydrate)

2.2.13. Thylakoid volume measurements for freezing test

For the volumetric assay, samples were diluted with an equal volume of 10 mM $MgCl_2$. Aliquots of the thylakoid suspension were filled into glass capillaries and the packed volume was determined by hematocrit centrifugation (Hincha and Schmitt, 1992a). Four measurements were taken from each sample and averaged. Control samples without added protein were held at 0°C (100% protection) or at −20°C (0% protection).

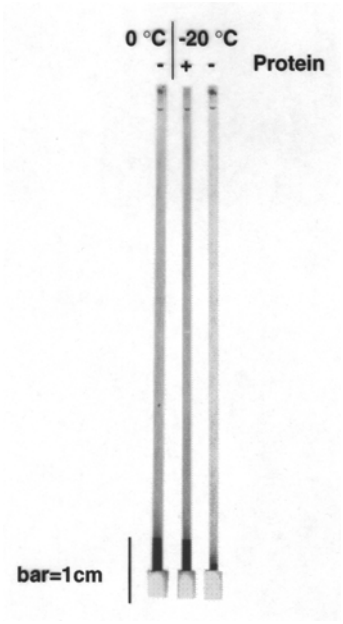

Fig. 2.5. Volumetric test – Hematocrit capilars were used to measure cryoprotective activity. Controls on 0°C and -20 °C shows whole volume of thylakoids at these temperatures after centrifugation**.**

The cryoprotective activity (in percentage) was calculated by formula:

TKV(PP -20°C) - TKV(-20°C) / TKV(0°C) - TKV(-20°C) = X/100

TKV(PP -20°C) - thylakoid volume in presence of analyzed protein at -20°C
TKV(-20°C) - thylakoid volume without protein at -20°C
TKV(0°C) - thylakoid volume without protein at 0°C

Solutions: Test buffer: 10 mM $MgCl_2$ (Hexahydrate)

2.2.13. Thylakoid volume measurements for Boyle van't Hoff plot analyzes

Protocol:

1. Mix aliquots of thylakoids in cryopreservation solution with sucrose solutions ranging in concentration from 20 to 500 mM sucrose finally. Make all sucrose solutions in 5 mM $MgCl_2$
2. Determine the osmolality of the resulting solutions with an osmometer. These measurements are made easier if you use the cryopreservation solution diluted 1:1 with the washing solution instead of thylakoids. The final results will be the same.
3. Load hematocrit capillaries with the diluted thylakoid suspensions and seal the capillaries at one and. Centrifuge for 15 min in a hematocrit centrifuge and measure pellet heights with a magnifying glass and a 0.1 mm scale.

2.2.14. Protein analyzing

2.2.14.1. Protein determination using the Bradford method

This method is based on the idea that an acid solution of Coomassie Brilliant Blue G can be changed from 465nm to 595nm by protein binding. The resulting complex can be measured at 595nm in a spectrophotometer, so that the protein content of a solution after preparation can be used to create a calibration curve.

Coomassie Brilliant Blue (CBB) is a tri-anilo-methane. CBB-G250 is greenish with an anionic absorption maximum of 575-585nm and is used in the determination of proteins. Without aryl-methyl groups, CBB receives the additive R250 (reddish, with a maximum of 555-570nm) and is rarely used.

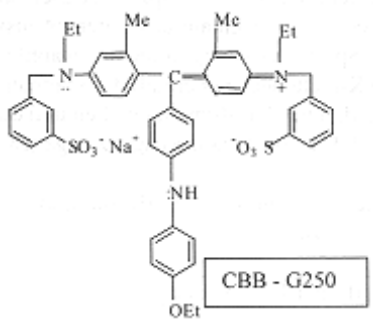

Fig. 2.6. Structural formula of CBB-G250.

Coomassie Briliant Blue G-250 appears in three different states absorb at varying wavelenghts. By biding the dye with a protein it changes from a cationic to an anionic state and its absorption level is 595nm. This absorption change is proportional to the protein concentration over a wide range, and it was first utilized in concentration analysis by Bradford.

CBB-G250 binds primarily to basic amino acids (arginine most of all) and aromatic at a rate of approx. 1mg CBB per 1mg prtein. This accounts for the difference in the level of absorption of varying proteins. For this reason it is highly recommended measuring the absorption level on a calibration curve using BSA.

Preparation of a standard curve

A BSA stock solution of 10mg/ml was made. This stock solution was used to set up different concentrations BSA standard (see Tab.2.5.).To make calibration curve we measured **OD_{595}** for different BSA concentrations.

Tab. 2.5. Different concentrations of BSA standards

BSA Solution	µg BSA/ml	Stock Solution (µl)	µl H_2O_{dist}	V_f (µl)
S	0	0	1000	1000
1	50	5	995	1000
2	100	10	990	1000
3	200	20	980	1000
4	300	30	970	1000
5	400	40	960	1000
6	500	50	950	1000

BSA standard are prepared at a concentration of 0-500µg/ml.
To make calibration curve the Bradford assay was set up:

750 µl H2O
200 µl Roti-Quant
50 µl BSA standard
(see Tab. 2.5)

1. Mix by inverting repeatedly

2. Measure the OD_{595} of the standard solutions (S) and samples (1-6) after 5 to 30 min against zero value.

3. Plot and compare OD_{595} of standard solution (S) to amount of protein used. The amount of protein in sample can be read on calibration curve.

Tab. 2.6. OD_{595} values for different BSA(µg/ml) concentrations.

BSA Solution	µg BSA/ml in stock solution	µg BSA/ml in the Bradford assay	OD_{595}
S	0	0	0
1	50	2.5	0.103
2	100	5	0.210
3	200	10	0.417
4	300	15	0.650
5	400	20	0.808
6	500	25	0.932

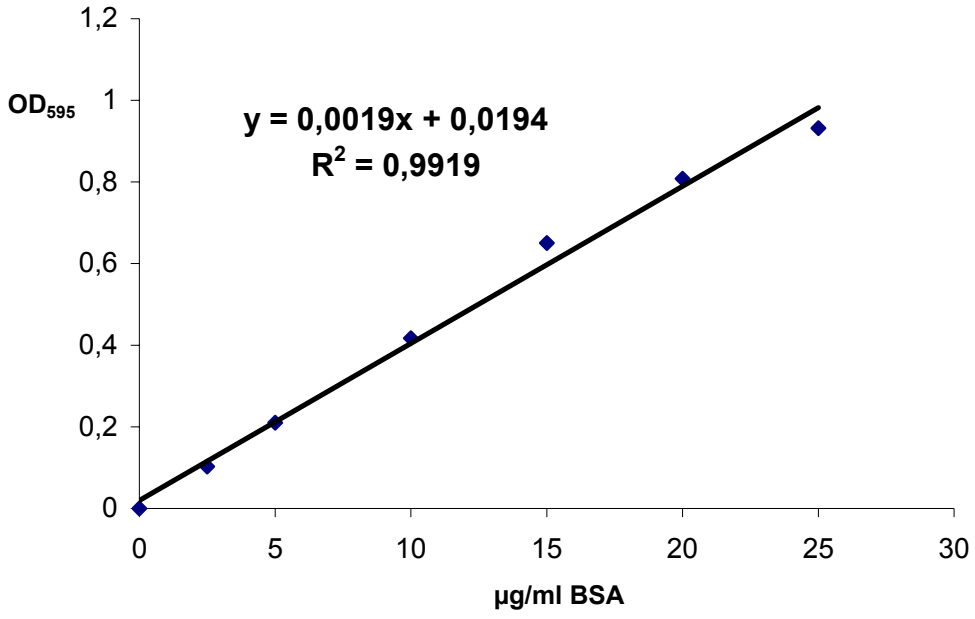

Fig. 2.7. Standard curve made by measuring concentration of BSA on OD $_{595}$

2.2.14.2. SDS-PAGE

SDS-polyacrylamide gel electrophoresis is a very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The most commonly used system is also called the Laemmli method after U.K. Laemmli, (Laemmli et al., 1970) who was the first to publish a paper employing SDS-PAGE in a scientific study.

SDS (also called lauryl sulfate) is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field.

Tab.2.7. Buffers used for SDS-PAGE

	Cathode buffer	**Anode buffer**	**Gel buffer**
Tris	100 mM	200 mM	3 M
SDS	0,1 %	-	-
Tricin	100 mM	-	-
pH	8,25	8,9	8,45
pH- adjusting	no	with HCl	with HCl

Gel preparation:

Gel was prepared with size of 7x8x0.1 cm. It consists a resolving gel of 16.5% acrylamide and stacking gel of 4% acrylamide. The folloving table showed how these gels were prepared:

Tab.2.7. Preparing of resolving and stacking gels

	Resolving gel (16,5 %)	**Stacking gel (4 %)**
Acrylamid/Bisacrylamide*	6,6 ml	0,83 ml
Gel buffer*	4 ml	1,56 ml
Bidest	72 µl	3,85 ml
Glycerin	1,33 ml	-
APS*	37 µl	62 µl

Temed*	4 µl	6 µl
Total volume	12 ml	6,25 ml

Solutions (*):
Acrylamid-Bisakrylamide: N,N´-Methylenbisacrylamid 37,5:1
Gel buffer: 3 M Tris, pH 8,45
APS: Ammoniumpersulfat, 10% (w/v) solution in deionised water
Temed: N, N, N´, N´-Tetramethylethylendiamin

The polymerisation of the 16.5% resolving gel was started by adding of APS. The solution was poured into gap between the glass plates. Using a Pasteur pipette the gel was overlaid with isobutanol. After polymerization isobutanol was removed by filter paper and the top of the gel was washed several times with deionised water to remove any unpolymerized acrylamide. The stacking gel prepared as describe above was poured directly on the surface of the polymerized resolving gel. Immediately a clean Teflon comb was inserted into the stacking gel solution. The gel was placed in a vertical position at room temperature.

2.2.14.3. Sample preparation

One volume of protein sample was mixed with one volume of the sample buffer containing DTT with a concentration of 100 mM. The sample was boiled in a water bath for 2 min at 100°C.

Tab. 2.8. Sample buffer

3x Sample buffer	
Tris-HCl pH 6,8	0,135 M (titration with HCl)
Glycerin	30 %
Bromphenolblau	0,03 %
DTT (Dithiothreitol)	0,15 M
SDS (Natriumdodecylsulfat)	3 %

2.2.14.4. Coomassie blue staining

Coomassie Brilliant Blue R250, binds nonspecifically to virtually all proteins. Coomassie Blue staining is less sensitive than silver staining, but effective nonetheless - in addition to being easy to perform.
The gel is soaked in dye for 30 – 40 minutes and then distained for thirty minutes or more. This treatment allows the visualization of bands indicating the protein content of the gel. The visualization on the gel usually contains a set of

molecular weight marker so that protein MW can be determined in an unknown solution.

Staining solution:

2.5 g Coomassie Brilliant Blue R
455 ml ethanol
455 ml deionized / distilled water
90 ml glacial acedic acid.

Destaining solution:

455 ml methanol
455 ml deionized / distilled water
90 ml glacial acetic acid (but it can also be destained using only distilled water and heating)

Coomassie dye is an integral component of the Bradford method for determining protein concentration solution.
This is based on the fact that Coomassie Brilliant Blue G-250 in acid solution has an absorbance shift from 495nm to 595 nm when it is bound to protein. The absorbance data can then be used in Beer's low to determine protein concentration and ultimately the actual amount of protein in a given solution. The Coomassie dye bonds to proteins via physisorption.

A common formula for mixing Bradford reagent:

0.01% Coomassie Brilliant Blue G-250
4.7% ethanol
8.5% phosphoric acid in distilled water.

2.2.15. Light scattering method:

Light scattering technique was used to measure the volumetric changes associated with adding of sugar (sucrose or glucose) into thylakoid suspension. When highly concentrated sucrose is added to a thylakoid solution, the vesicles initially lose water and shrink. In presence of proteins which can change permeability of thylakoid membrane, thylakoid vesicle begins to swell following diffusion of sugar and its associated water into vesicles, achieving the osmotic equilibrium. These volume changes can be monitored using changes in the light scattering properties of the suspension.

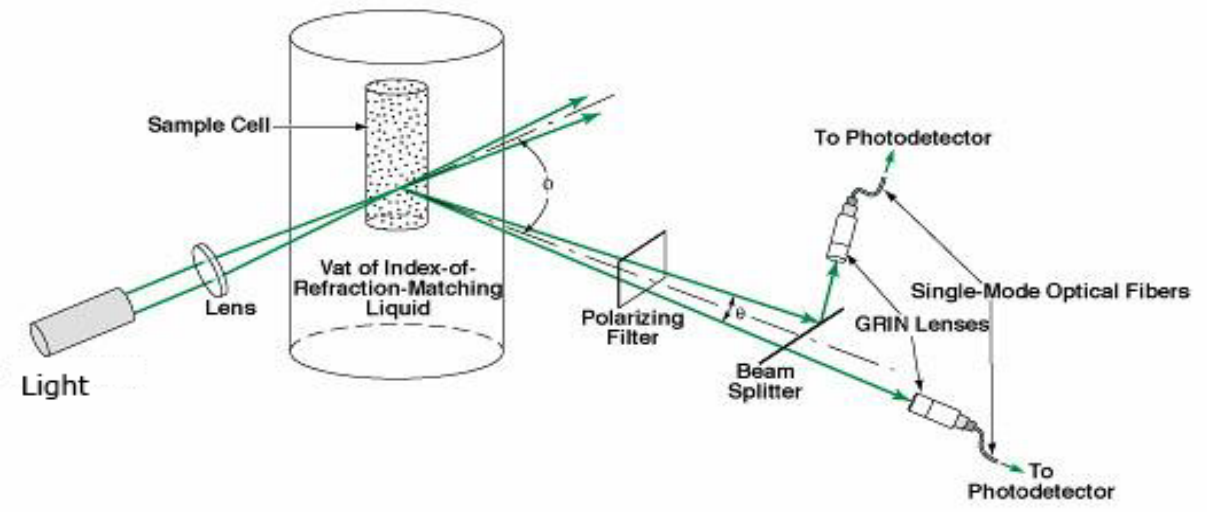

Fig. 2.8. Scattered Light enters two adjacent optical fibers attached to photodetectors. Cross-correlations of the outputs of the photodetectors are obtained for several different values.

Changes in scattering intensities of thylakoid suspension were measured at the angle of 90° to the incident beam in a spectrofluorimeter (SFM 25, Kanton Instruments). The excitation and emission monochromators were set at **535nm**. The voltage analogue $\Delta\Psi$ of the optical changes of the samples (**2 ml, 0.8-1.2 mg chlorophyll/ml**) was recorded from a 1 cm cuvette at room temperature (approx. 21°C) under continuous stirring.
200 µl of highly concentrated sucrose (1M) solutions were added in 1.8 ml thylakoid suspension and the change in scattering intensity was recorded.

2.2.16. Binding study of dehydrins with thylakoids

Thylakoids were incubated at 0°C for 10 min and than subjected to three washing steps by centrifugation (10 min at 16 000 x g). After centrifugation thylakoids were resuspended in the protein free buffer containing 10 mM sucrose, 5 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. After every washing step an aliquot of thylakoid membrane suspension was used for a cryoprotective activity assay to determine whether the membranes were still protected from freeze-thaw damage.
Aliquots of the resulting supernatant after washing steps were then analyzed for the presence of dehydrin proteins by western blots.

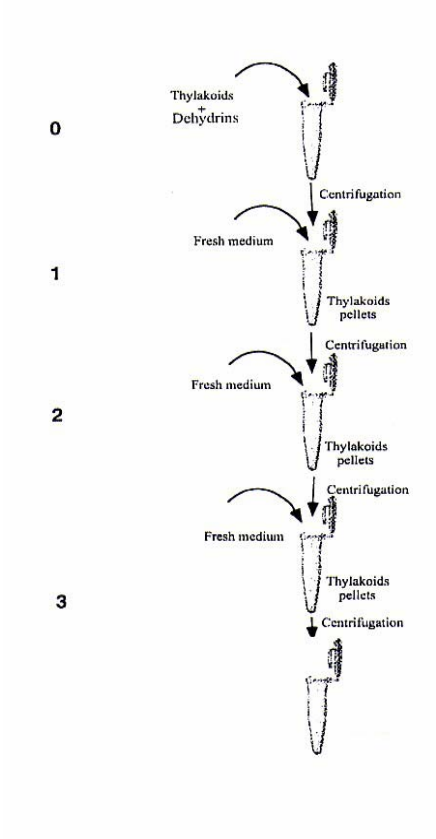

Fig. 2.9. Schematic representation of the binding experiment with thylakoid membranes.

Thylakoids from nonhardened spinach leaves were incubated with dehydrins and than subjected to three rounds of sedimentation and resuspension in protein-free solution.

2.2.17. Western blotting

The immunoblotting (Western Blotting) technique provides information about the presence, molecular weight, and/or quantity of an antigen by combining protein separation via gel electrophoresis with specific recognition of antigens by antibodies.
Immunoblotting is useful when the antigen of interest is insoluble or readily degraded and cannot be easily immunoprecipitated. Since most gel electrophoresis procedures result in denaturation of the antigen, only polyclonal and monoclonal antibodies that recognize the denatured form of an antigen can be utilized in immunoblotting.

2.2.17.1. Procedure

1. **Separate the proteins** using SDS-polyacrylamide gel electrophoresis.

2. **Place a nitrocellulose membrane on the gel** and, using electrophoresis, drive the protein (polypeptide) bands onto the nitrocellulose membrane. You want the negative charge to be on the side of the gel and the positive charge to be on the side of the nitrocellulose membrane to drive the negatively charged

proteins over to the positively charged nitrocellulose membrane. This gives you a nitrocellulose membrane that is imprinted with the same protein bands as the gel.

One thing to be aware of is that proteins bind better to nitrocellulose at a low pH. You may need to go through some trial-and-error to find the optimal pH. You also need to be sure there are no air bubbles between the nitrocellulose and the gel or your proteins will not transfer.

3. **Incubate the nitrocellulose membrane with a primary antibody.** The primary antibody, which is the specific antibody mentioned above, sticks to your protein and forms an antibody-protein complex with the protein of interest.

4. **Incubate the nitrocellulose membrane with a secundary antibody.** This antibody should be an antibody-enzyme conjugate. The secondary antibody should be an antibody against the primary antibody. This means the secondary antibody will "stick" to the primary antibody, just like the primary antibody "stuck" to the protein. The conjugated enzyme is there to allow you to visualize all of this. It's kind of like a molecular flare stuck on the antibodies so you can visualize what s going on.

5. **To actually see your enzyme in action**, you'll need to incubate it in a reaction mix that is specific for your enzyme. If everything worked properly, you will see bands wherever there is a protein-primary antibody-secondary antibody-enzyme complex, or, in other words, wherever your protein is.

2.2.17.2. Western Blot Protocol

1. Load 20 to 25 microgram of whole cell lysate per lane in an SDS-PAGE mini gel.
2. Run at 20 mA per gel untill the dye front is close to the bottom.
3. Transfer the proteins to a nitrocellulose membrane (S&S NCTM) at 250 mA in transfer buffer for 1-4 h, depending on the size of the target protein.
4. Incubate the blot with blocking buffer (5% non-fat dry milk in TBS) overnight at 4^{o}C or 2 h at room temperature (RT).
5. Incubate the blot with primary antibody (diluted 1:250 to 1:1000 in blocking buffer) for 1 h in blocking buffer at RT.
6. Wash the blot 3 x 10 min in washing buffer (TBS containing 0.1% Tween 20) with shaking.
7. Incubate blot with anti-rabbit IgG-HRP conjugate (Sigma) (diluted 1:10,000 - 1:2,000 in blocking buffer) for 1 h in blocking buffer at RT.
8. Wash 3 x 10 min in washing buffer with shaking.

9. Drain washing buffer, add ECL solution (Amersham) and develop for 1 min.
10. Expose to X-ray film for 1 to 30 min.

Solutions:

TBS:	125 mM NaCl
	25 mM Tris pH 8.0
SDS/Running Buffer:	25 mM Tris
	192 mM Glycine
	1% SDS
Transfer Buffer:	20 mM Tris pH 8.0
	150 mM Glycine
	20% methanol

2.3. Chemicals

Used chemicals were provided from these firms:
Merck, Serva, Sigma, Fluka, AppliChem oder Roth bezogen.
Enzyme from Stratagene, Pharmacia, Roche Molecular Biochemicals, Promega or Novagene.

2.4.Equipment

Agarose-Elektrophorese- Unit box , handmade from FU-Berlin
Blockthermostat Kleinfeld Labortechnik
Elektroblotting Unit LKB Bromma
Brutschr.nke Memmert
Flora-F Plants lamp Osram
Freeze Dreyer Edwards High Vacuum
GeneQuantII Pharmacia Biotech
Monitor Berthold
Hybridisations Unit Bachofer
Lab-Shaker Infors AG
Lab-Therm Infors AG
Laminarbox GELAIRE

pH-Meter Sentron
Protein-Elektrophorese-Units Handmade from FU-Berlin
Spectrophotometer Pharmacia Biotec
Kontron
Sonifier Branson Sonic Power Company
Stromversorgungsger.te Biotec Fischer
Thermocycler Biometra
Transilluminator UVP, Inc.
Ultraschallbad Bandelin
Video Graphic Printer Sony
Vakuum-Centrifuge Uniequip
Vortex-Genie 2 TM Bender & Holbein
Trolly Satorius
Wasserb.der K.ttermann
Centrifuges Eppendorf (Table centrifuge)
Heraeus (Biofuge 28RS)
Hettich (H.matokrit 20)
Kontron (Centrikon H401)
Sorvall (RC-5B)
Sorvall UTD (Ultrazentrifuge)