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Antti Lehtinen

Expression and Purification of Plant-derived Enzymes

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Bachelor of Laboratory Services

Laboratory Sciences

Bachelor's Thesis

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Gerbera hybrida on asteraceae-heimon kasvi, jota on ehdotettu uudeksi mallilajiksi kehitysbiologian tutkimuksissa. Muita tunnettuja mallilajeja ovat esimerkiksi banaanikärpänen ja *Escherichia coli*-bakteeri. Kukkakasveista on toistaiseksi vain yksi hyvin tunnettu mallilaji, *Arabidobsis thaliana.*

Yksi mielenkiintoinen piirre on Gerberan sekundaariset metaboliatuotteet, erityisesti gerberiini ja parasorbosidi. Näiden metaboliatuotteiden tiedetään toimivan Gerberan puolustusmekanismissa taudinaiheuttajia ja kasvissyöjiä vastaan.

Tämän työn tarkoituksena oli ilmentää ja puhdistaa Gerbera hybridan entsyymejä *E. coli* -kannassa Lemo 21. Näiden entsyymien tiedetään tai niiden ehdotetaan toimivan gerberiinin ja parasorbosidin biosynteeseissä. Nämä entsyymit ovat G2PS1, G2PS2, PKS8 (gerberan polyketidit, ~45 kDa), GTKPR1, GRED1 ja GRED2 (gerberan reduktaasit, ~35 kDa). Nämä ovat toistaiseksi heikosti tunnettuja entsyymejä ja niitä ei olla tutkittu missään muualla.

Heterologinen ekspressio aloitettiin viljelemällä valmiita ekspressio-isäntäkonstrukteja. Isännät oli transformoitu aikaisemmin pHis8:lla, joka on muunneltu pET-28-vektori. Se sisälsi geenin, joka tarvitaan entsyymien ilmentämiseen multikloonauskohdassa. Ekspressoitujen proteiinien N-terminuksessa oli 8x-histidiinihäntä, jota hyödynnettiin tuotteen puhdistamiseen ja tunnistamiseen. Isäntäsolut lysaattiin ja ekspressoidut entsyymit puhdistettiin His SpinTrap -kitillä. Puhdistusvaiheiden fraktiot kerättiin ja analysoitiin Bradford-määrityksellä. Fraktiot analysoitiin lisäksi SDS-PAGE- ja dot blot -menetelmillä tuotteen koon ja histidiinihännän läsnäolon määrittämiseksi.

Havaittiin, että GRED1 ja GRED2 eivät tuottaneet yhtä vahvaa signaalia dot blot -menetelmällä, kuin muut entsyymit. Tämä saattoi johtua proteiinin väärästä laskostumisesta jo ekspressiovaiheessa, jolloin se ei eluoitunut halutussa vaiheessa. Niiden ekspressio kuitenkin tuotti runsaasti tuotetta ja GRED1:n saantoa saatiin lisättyä 51,45 % muokkaamalla ekspressio- ja puhdistusmenetelmiä. Kaikki tuotetut entsyymit varastoitiin jatkotutkimuksia varten.

Avainsanat	Proteiinieksprressio, biosynteesi, metaboliatuotteet, entsyymit

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Gerbera hybrida is a plant in the asteraceae family that has been proposed as a new model species for development biology studies. Other well-known model species include the fruit fly and *Escherichia coli,* for example. Of flowering plants there is only one well known model species so far, *Arabidobsis thaliana.*

One feature of interest is *Gerberas* secondary metabolite pathways, notably gerberin, parasorboside. Gerberin and parasorboside are known to function in the defense mechanism of *Gerbera* against pathogens and herbivores.

The aim of this work was to express and purify a set of enzymes of *Gerbera hybrida* in *Escherichia coli* strain Lemo 21. These enzymes are known or are proposed to function in the biosynthesis of gerberin and parasorboside. These enzymes are G2PS1, G2PS2, PKS8 (gerbera polyketides, ~45 kDa), GTKPR1, GRED1 and GRED2 (gerbera reductases, ~35 kDa). These enzymes are still not very well known, and they have not been used in research anywhere else.

The heterologous exspression was begun by culturing the expression host constructs that had been provided. The hosts were previously transformed with pHis8, a pET-28 vector that included the gene necessary for the expression of the enzymes in the multicloning site. The expressed proteins had an 8x histidine tag to the N-terminus which was used to purify and identify the product. The host cells were lysated and the expressed enzymes purified with His SpinTrap kit. The fractions of the purification steps were collected and analyzed with Bradford assay. The fractions were additionally analyzed with SDS-PAGE and dot blot methods to determine the product size and the presence of the 8x histidine tag respectively.

It was discovered that GRED1 and GRED2 did not produce as strong a signal in the dot blot analysis as the other enzymes. This might have been due to incorrect folding of the protein where the histidine tag was folded inside the protein structure and thus was not purified in the correct fraction and did not produce a strong signal in dot blot. The expression and purification methods were modified and the yield of GRED1 was increased by 51.45 %. All the expressed enzymes were stored for further study.

Keywords	Protein expression, biosynthesis, metabolites, enzymes

Contents

Abbreviations

G2PS1, G2PS2	*Gerbera* 2-pyrone synthases
GTKPR1	*Gerbera* tetraketide α-pyrone reductase, specific to the anther region of *Gerbera*
GRED1, GRED2	Similar to GTKPR1, but found in other regions of the plant as well
HPLC	High-performance liquid chromatography
PKS8	Polyketide synthase
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TLC	Thin-layer chromatography

1 Introduction

This work was conducted at the Faculty of Agriculture and Forestry, University of Helsinki. The “Gerbera Lab” research group focuses on Asteraceae developmental biology and secondary metabolism. The group has studied the model plant *Gerbera hybrida* for decades and has published numerous papers in the field of plant biology and biotechnology. This subject was chosen because of the author’s interest in biology and biochemistry.

Gerbera hybrida is a relatively new model organism in plant biology. It belongs to the large Asteraceae (alternatively Compositae) family, also known as the sunflower, or daisy family, which consist of around 25 000 species. It is well known as ornamental crop around the world and grown in the tens of millions annually. 60 million plants are grown in California alone and over 100 million in Europe each year. The economic importance of the plant is therefore significant. [1.]

G. hybrida shows a wide range of morphological variations, useful for developmental, evolutionary and genetic reseach. It also produces a wide range of the secondary metabolites, some of them only found in the Asteraceae family. These traits make it an excellent model plant of its family. [1; 2].

The aim of this work was to express and purify key enzymes in the biosynthetic pathways of *Gerbera.* These enzymes work in conjunction to produce secondary metabolites that have a significant role in the defense mechanism of *Gerbera* against fungal and bacterial infections. Knowledge of this pathway may have significant applications in the future, in farming for example. Detailed knowledge of the metabolic pathways mentioned provides tools to modify them in other plant species also. Transforming a plant with a gene that provides a resistance to infections or from herbivore attack would reduce or remove completely the need for herbicides in the future.

2 Asteraceae and *Gerbera hybrida*

Asteracea is a large family that includes many commonly known edible, herbal and ornamental plant genuses such as sunflower, lettuce, artichoke, chrysanthemum, aster and of course the genus *Gerbera*. Asteraceae is thought to originate from the area around modern Patagonia, Argentina when the continents of South America and Africa were still connected. Since then members of the family have spread to all parts of the globe except Antarctica.

A common feature among all Asteraceae species is the inflorescence that resembles a single flower but is rather a dense structure of multiple smaller, specialized flowers. This feature is believed to be a key factor for the success of Asteracae Moreover Asteraceae species show wide range of colors and shapes attracting pollinators and humans alike.

The genus *Gerbera*, sometimes called the African daisy, includes around 30 species that grow in southern Africa, Madagascar and the tropical regions of Asia. Economically and scientifically the most important is a hybrid cultivar of two South African species, *Gerbera jamesonii* and *Gerbera viridifolia* called *Gerbera hybrida* (figure 1). [2; 3.]

Figure 1. *G. jamesonii* (left) *[4]* and *G. viridifolia (right) [5]*

G. hybrida has three specialized flower types in its inflorescence: colorful “ray” flowers in the outermost form the most visible part or the plant with their elongated petals (figure 2). The middle part consists of central “disc” flowers that have very small petals. Between the ray and disc are so-called “trans” flowers that are similar to ray flowers but have shorter petals. [6.] Ray and trans-flowers are female, while disc flowers are both female and male [2].

Figure 2. Comparison of the inflorescense of sunflower (*Helianthus annuus)* and *G. hybrida*. Note how the sunflower lacks trans-flowers. [6]

3 Polyketide Biosynthetic Pathways in Gerbera

3.1 Secondaty Metabolites

Secondary metabolites are as their name suggest, secondary in importance for the basic biomechanical processes of a plant. Secondary metabolites are a diverse group of low-molecular weight compounds that are important in mechanisms such as defense against pathogens and herbivores, floral scent, pigments for attracting pollinators or preventing water loss during drought. Therefore, they are not directly important for the growth and life of the plant but are necessary when interacting with external factors. Without them an organism would not thrive in nature. [7.]

Prasorboside and gerberin are two very similar compounds involved in the defense against plant pathogens. In addition, parasorboside is regarded as a precursor to para-sorbic acid which in turn is related to sorbic acid, a common food preservative. It is pre-sent also in other plants, such as the berries of *Sorbus aucuparia,* where the compound got its name. [7; 8.] The proposed biosynthetic pathway is presented in figure 3. Note that this is a proposition based on current knowledge and it may change in the future.

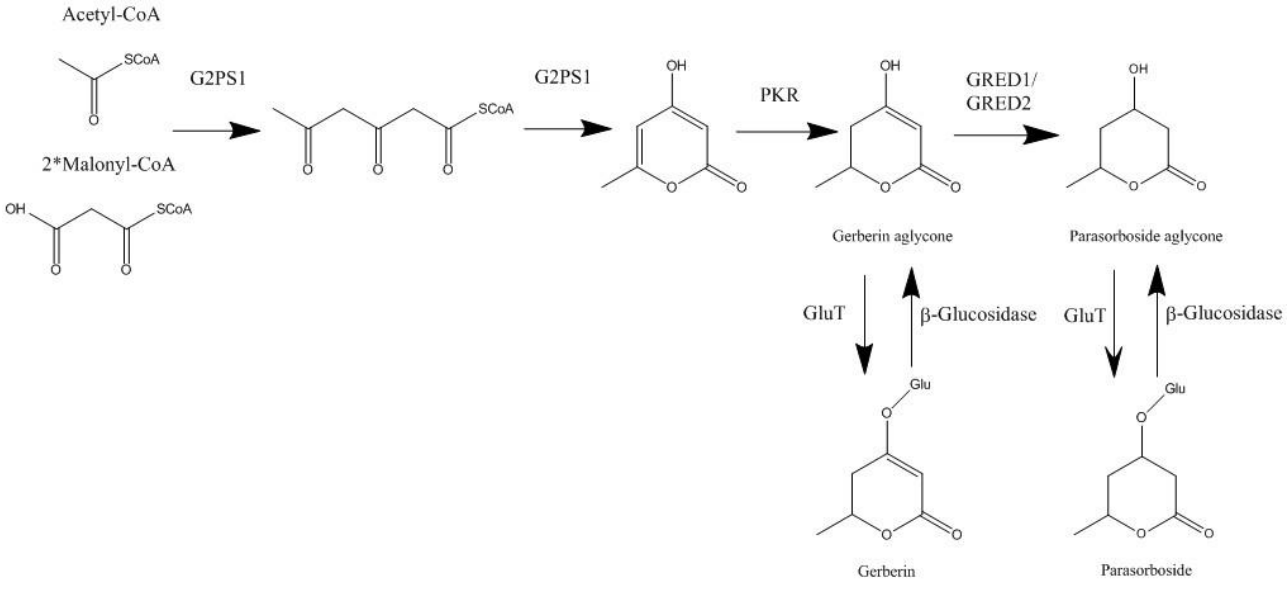

Figure 3. Proposed biosynthetic pathways of gerberin and parasorboside. The first stages are catalyzed by 2-pyrone synthase G2PS1 and the aglycones are thought to be catalyzed by reductase enzymes (PKR, GRED1 and GRED2). Glucosyltransferases (GluT) attach a glucose to the aglycones to form gerberin and parasorboside. Upon infection, β-glucosidase removes the glucose to convert them back to bioactive aglycone forms. [9]

Gerberin and parasorboside are synthesized in a storage form and are not bioactive until they are needed. It is anticipated that once an attack by pathogens occurs, the glucose is separated from the compounds by β-glucosidase and they become bioactive as seen in figure 3. [7; 9.] These aglycone forms of gerberin and parasorboside are classified as phytoalexins, meaning that they are not detectable unless the plant is infected by pathogens. Phytoanticipins on the other hand are antipathogenic agents that are synthesized throughout the growth and development of the plant. [7.]

Sporopollenin forms the outer shell of pollen called exine. It is a biopolymer structure that surrounds the inner layers. Sporopollenin protects pollen from pathogens, UV radiation, and dehydration and interacts with female reproductive cells during pollination. Its pathway has been identified in *Arabidobsis thaliana* and *Gerbera* is thought to have similar pathway and enzymes (figure 4). [10; 11.]

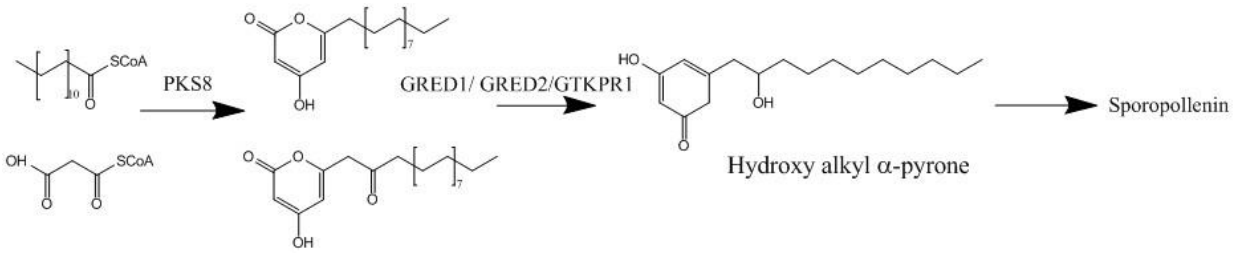

Figure 4. Proposed biosynthetic pathway of Hydroxy alkyl α-pyrone, a sporopollenin precursor. In *A. thaliana* the functioning enzymes are APKSB, ATKPR1 and ATKPR2 [11].

3.2 Polyketide and reductase enzymes

It has been discovered in a previous study that without the ability to express G2PS1 (*Gerbera* 2-pyrone synthase) enzyme, *Gerbera* is unable to produce gerberin and parasorboside and therefore cannot resist fungal infection effectively [7].

Polyketide synthases are a large group of enzymes present in plants, fungi, bacteria and some animals. Their purpose is to synthesize secondary metabolites from precursor molecules. Many secondary metabolites synthesized by polyketides are toxic to micro-organisms. Polyketide synthases are used in pharmacological industry to produce antibiotics, for example. Polyketides form the first part in gerberin and parasorboside biosynthesis in *Gerbera*.

Reductase enzymes catalyse biochemical reduction reactions by lowering the energy needed for electron gain for target molecules. In *Gerbera* the reductases GRED1 and GRED2 catalyse the final steps in gerberin and parasorboside biosynthesis. GRED1 and GRED2 are two novel enzymes similar to tetraketide α-pyrone reductase 2 (TKPR2), which is specific to the anther region of *Gerbera.* TKPR2 is itself similar to GTKPR1. GRED1 and GRED2 are thought to function in the sporopollenin biosynthetic pathway as well as the biosynthetic pathways of gerberin and parasorboside. They are expressed in many tissues of *Gerbera,* not just the anther region and their genes are co-expressed with that of G2PS1.

Overall aim of the expression and purification of these enzymes was to conduct an *in vitro* enzymatic assay. In the assay G2PS1 will be provided with malonyl-CoA and acetyl-CoA to provide substrate for GRED1 and GRED2. The end products will be analysed with GC-MS. Furthermore, the enzymes will be used in pollen grain viability tests to study the development of pollen grains.

4 Protein Expression

4.1 Expression Host

Lemo 21 is an *E. coli* strain that is widely used in protein expression. Lemo 21 is a versatile T7 polymerase-based strain that has a resistance to chloramphenicol. This strain can be used to create protein expression constructs by transforming them with a vector that contains a T7 promoter, such as pET-28. [13.]

PHis8 is a slightly modified pET-28 vector. It contains T7 promoter and therefore is compatible with Lemo 21 that expresses the T7 polymerase. It also has a kanamycin resistance for selective growth and contains a multicloning that is used to integrate the protein encoding sequence for expression. Expressed protein receive an 8x histidine tag in the N-terminus that is applied for identification throughout the process. [13.] The expression, under control of the *lac* operon can be induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) to the growth culture. IPTG mimics the substrate of *lacI* repressor, which is bound to the *lac* operon. The repressor, however, cannot digest the

substrate and remains inactive. The RNA polymerase is then able to start gene transcription. [14.]

4.2 Purification method

His SpinTrap column is optimized for histidine-tagged protein purification and contains nickel ions (Ni^{2+}) in a polyethylene frit. In principle, it is an immobilized metal ion affinity chromatography (IMAC) column in which the histidine-tagged target proteins are bound by the column matrix. All other biomolecules without any affinity towards the matrix flow through the column after which the target protein may be eluted with elution buffer. The elution buffer has a stronger affinity towards the matrix and knocks the target proteins into the elution buffer. [15.]

4.3 Dot Blotting

Dot blotting is a detection method for proteins in which a primary and secondary antibody is bound to a protein sample. It is a simplified version of Dot blotting, where the proteins are not separated with electrophoresis. The protein themselves are blotted on a nitrocellulose membrane that is blocked to ensure that the antibodies only bind to the intended targets and not the membrane. The antibodies mark the target protein, so it can be detected from a solution containing other proteins and biomolecules. These antibodies are usually specific to the target protein or a group of proteins.

The primary antibody binds to the target site in the protein and the secondary antibody binds only to the primary antibody. After binding, a detection reagent is used to visually identify the secondary antibody. This reaction can be for example formation of colour or luminescence. Excess antibodies are washed from the membrane. [16.]

5 Methods

All the reagents and solutions used in this work are listed in the appendix sections 1 and 2.

5.1 Expression Culture

A set of polyketide synthases and reductase enzymes had been proposed to function in the biosynthetic pathways of gerberin, parasorboside and sporopollein. These enzymes are:

Polyketide synthases: G2PS1, G2PS2, PKS8

Reductase enzymes: GTKPR1, GRED1, GRED2

The Lemo 21 constructs were revived from -80 °C deep freeze, cultured on LB (+50 µg/ml kanamycin + 25 µg/ml chloramphenicol) plates and grown overnight. A single colony from each plate culture was inoculated in 5 ml LB-broth (+kan +cam) in 15 ml plastic tubes and cultured for 5 h in 37 °C, 200 rpm to provide a starter culture.

OD_{600} of the starter cultures were measured and the cultures were diluted sufficiently to reach an OD_{600} of 0.1 in two 25 ml cultures (50 ml in total per culture). These expression cultures were grown for 1 h to reach an OD_{600} of 0.2-0.6 after which they were combined to 50 ml volume in 250 ml flasks for protein induction.

The expression cultures were induced with 20 µl 1 M IPTG (40 mM) to start protein expression. This was continued overnight in 16 °C (figure 6). The temperature was set to focus on protein production and inhibit cell division (propagation). Before adding IPTG, 2 ml of the cultures were stored as control samples.

After induction the culture tubes were centrifuged with 4000 rpm for 20 min to harvest the pellets. The pellets were resuspended in 1 ml LB and transferred to 1.5 ml centrifuge tubes, two for each pellet. The tubes were centrifuged again (5000 G, 10 min), the supernatant was removed, and the pellets were weighted and stored in -20 °C.

Control culture was a Lemo 21 strain with pHis8 vector without any insert in the multicloning site.

5.2 Protein Purification

Purification was conducted according to the user manual provided with Ge Healthcare His SpinTrap kit (GE Healthcare product code 28932171) [15].

For extraction of total proteins, the pellets were lysated in 4x volume (4x volume in ml to the weight of the pellet in mg) of extraction buffer (100 µg/ml lysozyme, 2.6 units Dnase (Thermo Scientific, 2600 unit/ml stock), 1x protease inhibitor. 10x inhibitor stock solution was made by dissolving a tablet in 1 ml B-PER reagent. B-PER reagent was used as solvent), incubated for 15 min and centrifuged at 15 000 G for 5 min. The column was prepared with binding buffer (10 mM imidazole, 1x phosphate buffer (from GE Healthcare Buffer Kit, product code 11003400) in H_2O) by adding 600 µl of binding buffer and centrifuging the column for 30 s at 100 G. After this the lysate supernatant solution was pipetted on the column and centrifuged for 30 s at 100 G. Before adding the lysate, 50 µl of the solution was stored. This was called the total soluble protein fraction (TOTAL). Next the column was washed using 600 µl of binding buffer and finally the proteins were eluted twice using 200 µl of elution buffer (500 mM imidazole, 1x phosphate buffer in H_2O) and centrifuging for 30 s at 100 G.

The following fractions were collected:

- Total soluble protein, collected before purification, 50 µl (TOTAL)
- Sample application flow through, volume depended on the volume of the lysate, 200-400 µl (FLOW)
- Washing flow through, 600 µl (WASH)
- Elutions, 200 µl each (E1, E2)

Of the pHis8 control sample, only the total soluble protein was collected. After purification, protein concentration of the collected samples was measured with Bradford assay.

In a 96-well plate 2 µl of the sample was diluted with 198 µl of water and mixed 20 times with 50 µl of dye reagent. Elution buffer from the purification stage was used as a blank sample. Samples that initially gave results above 0.6 were diluted sufficiently and measured again until the results were within range.

5.3 Identification

The expressed and purified protein were identified by their size and the presence of the histidine tag.

5.3.1 SDS-PAGE

SDS-PAGE analysis was performed to determine whether the produced enzymes were of correct size.

A total of 10 µg of protein was pipetted in the SDS-gel wells. For each sample, the volume needed was calculated according to the concentration given by Bradford assay. The samples were denatured by adding 1/3 volume of SB denaturing reagent (100 mM Tris-Cl, pH 6.9, 20 % glycerol, 4 % SDS, 10 % β-Mercapto EtOH, 0.2 mg/ml Bromophenol

blue) to the volume of sample. Denaturation was done by heating the sample for 5 min in 96 °C. The sample was pipetted into the gel wells and the gel was run for 35 min with 200 V after which it was stained with PageBlue™ (Thermo Scientific, cat #24620) and washed with water.

5.3.2 Bioluminecent Dot Blotting

The method used in this work was based on a bioluminescence reaction that could be recorded on a film placed against a nitrocellulose membrane (Amersham Hybond-ECL, GE Healthcare, cat #RPN303D) in a closed film cassette. Depending on the quantity of his-tag sites in the sample (i.e. amount of target proteins) the more intense the luminescence was. This corresponded to the size of a dot in the exposed film.

Volumes of the sample solution corresponding to 5 µg of total protein was pipetted on the membrane. All fractions from the purification stage were included (total, flow, wash and elutions) for each enzyme. In addition, 2 µl of the pellets from the extraction step and 5 µl of the uninducted samples were included.

After applying samples on the membrane, it was stained with Ponceau S by taking the following steps. All steps were done in room temperature and while shaking.

Staining steps:

1. The membrane was placed inside a plastic box with a lid

2. Staining with 50 ml Ponceau S staining solution (0.1 % Ponceau S, 2.5 % glacial acetic acid in H_2O) for 1 h. Afterwards the staining solution was poured back to the bottle.

3. The membrane was rinsed with water until the background colour was gone

4. Most of the excess water was removed with a paper towel

Note that the staining was not a necessary step for dot blotting, and it was only used to get a visual interpretation of proteins on the membrane.

Next the membrane was blotted according to Amersham ECL Prime Dot Blotting Detection Reagent instruction manual [17]:

1. The membrane was washed with 50 ml tTBS buffer (20 mM Tris-Cl, 150 mM NaCl, 0.1% Tween® 20 (Sigma-Aldrich cat #P1379)) for 5 min

2. tTBS was poured off and 25 ml of blocking buffer (5 % non-fat dry milk powder (Valio cat #84062)) was added, and the membrane was shaken for 1 h

3. Washing with 100 ml tTBS buffer for 10 min

4. The membrane was rolled inside a 50 ml tube and the primary antibody solution (4 µl His-tag polyclonal antibody (Cell Signalling Technologies cat #2365S) in 20 ml blocking buffer) was added. The tube was placed on a roller in +4 °C overnight.

5. The membrane was rinsed and washed with tTBS three times for 10 min each.

6. The secondary antibody (5 µl Anti-Rabbit IgG HRP-linked antibody (Cell Signalling Technologies cat #7074P2) in 40 ml blocking buffer) was added on the membrane for 1 h

7. Washing four times with 100 ml tTBS for 10 min each.

8. The membrane was dried with a paper towel and 1 ml Amersham ECL Dot Blotting Detection Reagent™ (50:50 mix of ECL A and ECL B solutions (GE Healthcare cat #RPN2232)) was added and rolled on the membrane with a roller pin.

9. The membrane was dried and placed inside a film cassette and the film was placed on top of it and the cassette was closed

10. Three exposures were made: 3 s, 2 min and 90 min.

The film was removed from the cassette and submerged into a developing solution, washed with water and fixed with a fixing solution. Placing the film inside the box and treating it with developing and fixing solutions needed to be done in total darkness, because the film was too sensitive to have a red light on.

6 Results

6.1 Bradford Assay

Bradford assay showed that the first elution had more protein than the second, as expected (Table 1). From the absorption value the protein concentration (C) was calculated, taking in to account the volume of sample in the wells and the dilution factors. For SDS-PAGE the volume that corresponded with 10 µg of protein was calculated from these results, taking to consideration the addition of 1/3 volume SB-solution. Bradford assays showed that the *E. coli* heterologous expression system works and produced a lot of protein, and some of the produced proteins could be purified by the his-tag purification system

Table 1. Bradford assay tables. The total protein fraction volume is the amount of extraction buffer used (4x the weight of the pellet). This volume was pipetted on the purification column. Flowthrough volume is the volume after 50 µl of sample was removed. Note that 8*A_{595} is a factor in the method instructions. The 8x multiplication is derived from the calibration slope of the laboratory instructions.

enzyme	fraction	volume of fraction (µl)	dilution factor	A 595	8*A595	C (µg/µl)	protein (µg)
G2PS1	otal soluble protei	360	4	0.369	2.952	11.808	4250.9
1 µl /well	flowthrough	310	4	0.294	2.352	9.408	2916.5
	wash	600	1	0.488	3.904	3.904	2342.4
	elution 1	200	1	0.452	3.616	3.616	723.2
	elution 2	200	1	0.181	1.448	1.448	289.6
G2PS2	otal soluble protei	288	4	0.283	2.264	9.056	2608.1
1 µl /well	flowthrough	238	4	0.133	1.064	4.256	1012.9
	wash	600	1	0.327	2.616	2.616	1569.6
	elution 2	200	1	0.112	0.896	0.896	179.2
	elution 1	200	1	0.012	0.096	0.096	19.2
PKS8	otal soluble protei	336	4	0.42	3.36	13.440	4515.8
1 µl /well	flowthrough	286	4	0.403	3.224	12.896	3688.3
	wash	600	1	0.407	3.256	13.024	7814.4
	elution 1	200	1	0.141	1.128	1.128	225.6
	elution 2	200	1	0.056	0.448	0.448	89.6
GTKPR1	otal soluble protei	323	8	0.559	4.472	17.888	5777.8
2 µl /well	flowthrough	273	8	0.245	1.96	7.840	2140.3
	wash	600	1	0.622	4.976	2.488	1492.8
	elution 1	200	1	0.416	3.328	1.664	332.8
	elution 2	200	1	0.08	0.64	0.320	64.0
GRED1	otal soluble protei	258	8	0.548	4.384	17.536	4524.3
2 µl /well	flowthrough	208	8	0.146	1.168	4.672	971.8
	wash	600	1	0.584	4.672	2.336	1401.6
	elution 1	200	1	0.354	2.832	1.416	283.2
	elution 2	200	1	0.196	1.568	0.784	156.8
GRED2	otal soluble protei	160	8	0.277	2.216	8.864	1418.2
2 µl /well	flowthrough	110	8	0.498	3.984	15.936	1753.0
	wash	600	1	0.52	4.16	2.080	1248.0
	elution 1	200	1	0.307	2.456	1.228	245.6
	elution 2	200	1	0.131	1.048	0.524	104.8
Control	otal soluble protei	232	1	0.336	2.688	10.752	2494.5

6.2 SDS-PAGE

SDS-PAGE analysis showed that GRED1 and GRED2 had bands that were of different size than predicted. The predicted sizes of GRED1 and GRED2 are ~35 kDa but the strongest bands in the elution fraction were approximately 40-45 kDa according to SDS-

PAGE. 35 kDa bands can be seen in the flow through (F) and wash (W) fractions. There was also a considerable band around 70 kDa (figure 5).

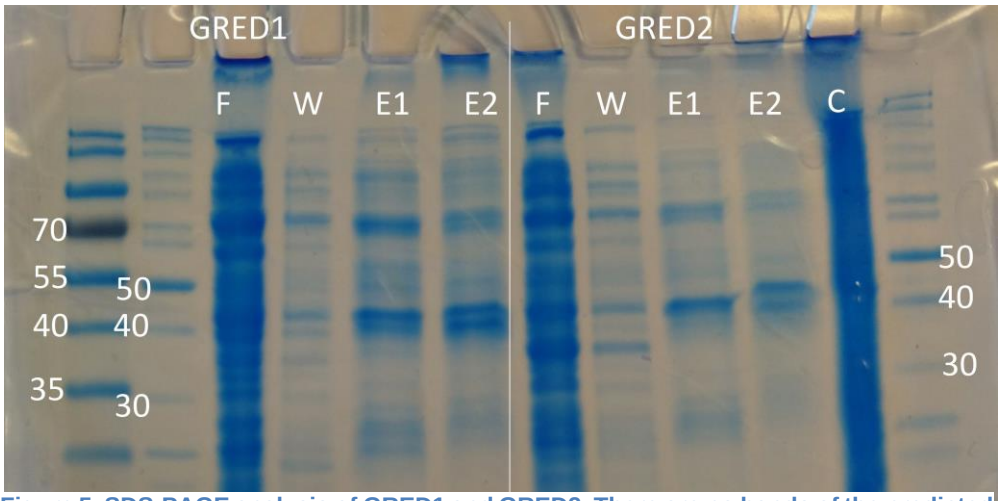

Figure 5. SDS-PAGE analysis of GRED1 and GRED2. There are no bands of the predicted size in the elution fractions. The samples are: flowtrhough (F), wash (W), elutions (E1, E2). 10 µg of protein was pipetted on each well and 1/3 of that volume of SB denaturating reagent was added.

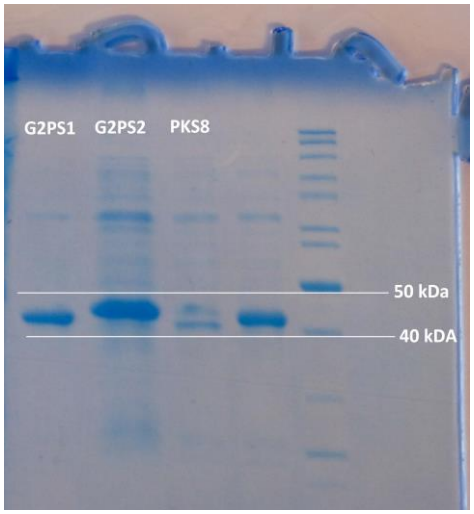

Figure 6. SDS-PAGE analysis of G2PS1, G2PS2 and PKS8. GTKPR1 not shown. Only E1 fractions shown.

G2PS1, G2PS2, PKS8 and GTKPR1 had bands in the elution fractions were of the predicted sizes (figure 6). They also seemed to purify more completely and did not show unspecific bands in the gel.

6.3 Dot Blot

The Westen blot results showed that G2PS1, G2PS2, PKS8 and GTKPR1 had histidine-tagged proteins in the elution fraction. Additionally, GTKPR1 had his-tagged proteins spread in all fractions. GRED1 and GRED2 did not have any considerable amounts of target proteins in the elution fraction, but instead a strong signal was detected in the pellet and in the total protein sample (figure 7).

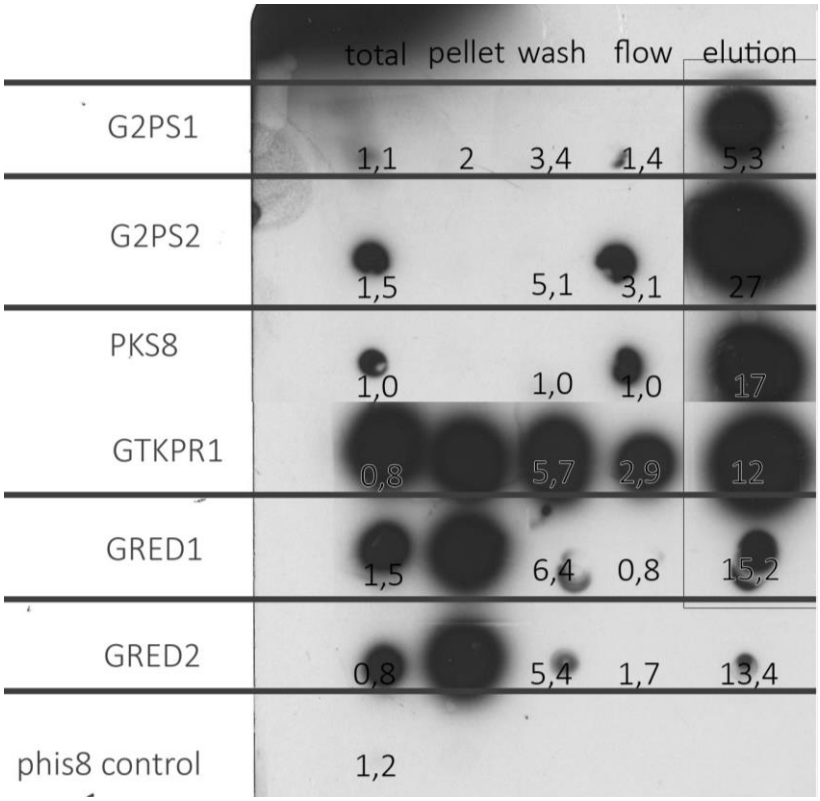

Figure 7. Dot blot results. The numbers show the amount of sample (µl) pipetted on the membrane that corresponded to 10 µg of protein. Image is from a film exposed for 3 hrs.

6.4 Modifications to Protocol

A modification to the protocol was made in order to increase the amount of protein in the elution fraction. The modification was to remove imidazole from the binding buffer and re-applying the flow through three times to the purification column. The protocol was identical to previous procedures in all other aspects. This modification was performed with the enzyme GRED 1.

Table 2. Bradford assay results of purification fractiong of GRED1. The amount of protein increased from the previous purification of GRED1 from 283,2 to 361,6 µg in the first elution for example.

enzyme	fraction	dilution factor	A 595	8*A595	C	V1 (10 µg/C)	V2 (4/3*V1)
GRED1	total soluble protein	8	0.548	4.384	17.536	0.570	0.760
	flowthrough	8	0.146	1.168	4.672	2.140	2.854
	wash	1	0.584	4.672	2.336	4.281	5.708
	elution 1	1	0.354	2.832	1.416	7.062	9.416
	elution 2	1	0.196	1.568	0.784	12.755	17.007

Bradford assay results showed that both the concentration and total amount of enzyme increased (table 2) in both elution fractions. The increase of protein increased by 51.54 % from the first purification when calculating with the combined amounts of both elutions:

$$\frac{(362.6\ \mu g+304.8\ \mu g)-(283.2\ \mu g+156.8\ \mu g)}{(283.2\ \mu g+156.8\ \mu g)} * 100 = 51.45\ \% \quad (1)$$

7 Discussion

Working with proteins always requires good planning and scheduling and results may vary even with the most careful work.

All the enzymes were expressed with the heterologous expression method as the his-tag signal was detected in all of the total protein samples. It seems that GRED1 and GRED2 did not express as hoped. According to SDS-PAGE and dot blot the target proteins remained in the pellet fraction and were possibly of different size than predicted. One possibility is that the proteins dimerizied and produced a band on 70 kDa given that GRED1 and GRED2 were of 35 kDa size and a dimer would therefore be around 70 kDa. It was discussed that Lemo 21 might not be the optimal expression host for recombinant reductases and Rosetta-strain was proposed as a replacement for future experiments. The rest of the proteins were successfully purified and were identified in the expected manner. GTKPR1 had histidine tagged protein in all fractions but still the elution fraction produced a strong signal in dot blotting.

Reductase enzymes are known to be difficult to express and purify. It was speculated that the reductases may not fold correctly during the expression or they denaturized quickly after. The strong signal in the pellet fraction in dot blot also suggests that the

reductases did not solve into the lysate buffer completely. The his-tag might be folded inside the protein structure, which makes the purification and identification of the protein impossible. It is also possible that the enzymes lose activity during the extraction and purification stages because of an incorrect folding. In addition, finding the isoelectronic point (PI) of the reductases and checking the pH value of the lysate buffer is advisable. This would make it possible to adjust the pH of the buffer so that the enzymes would solve. The expression and purification protocols were modified in order to increase yield and the final concentration of the enzyme. An enzymatic assay was also attempted with GTKPR1, ATKPR1 and ATKPR2 (*A. thaliana* reductases) but the enzymes showed no activity.

Even though the yield of GRED1 increased considerably, the signal produced remained relatively weak compared to other enzymes. Steps should be taken in order to express the enzymes and retain the natural form. In the future, a different protocol for the expression and purification of the reductases is needed in order to properly study the biosynthetic pathways of gerberin and parasorboside. Also, modifications to existing protocols should be explored further and repeated to obtain reliable data on their effect on the yield.

The study of this pathway and related genes could result in significant applications in farming economically important plants, for example producing mould-resistant strawberries.

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Appendix 1: Reagents

Protein expression

Reagent	Manufacturer	C	CAS #	Lot #	notes
LB-broth	internal	-	-	-	autoclaved
LB-agar	internal	-	-	-	autoclaved
Kanamycin	Sigma-Aldrich	solid	25389-94-0	SLBR6873V	store at - 20 °C
Chloramphenicol	Duchefa Biochemie	solid	56-75-7	009104.03	store at - 20 °C
IPTG (Isopropyl-beta-D-thiogalactopyranoside)	Sigma-Aldrich	solid	367-93-1		store at +4 ° C 

Protein Extraction and purification

Reagent	Manufacturer	C	CAS #	Lot #	notes
Bacterial Protein Extraction Reagent (B-PER™)	Thermo Scientific	-	-	TK274478	-
Lysozyme	Sigma-Aldrich	solid	9001-63-2	57H7045	store at - 20 °C
Dnase	Thermo Scientific	2616 U/ml	9003-98-9	TI270908	store at - 20 °C
Imidazole	GE Healthcate	2 M	288-32-4	10222985	store at +4 ° C 
Phosphate buffer	GE Healthcate	8x stock	-	10222984	store at +4 °C
Protein Assay Dye Reagent Concentrate	Bio-Rad	-	-	-	store at +4 °C 
Protease inhibitor tablets	Roche	solid	-	38591400	store at +4 °C dissolve 1 tablet in 1 ml H2O for 10x stock

SDS-PAGE

Reagent	Manufacturer	C	CAS #	Lot #	notes
Tris Base	Fisher BioReagents	solid	77-86-1	178204	
Glycine	Fisher Chemical	solid	56-40-6	1869865	-
SDS	Fluka	solid	151-21-3	409773/1	
SB solution	internal	4x stock	-	-	store at -20 °C 
PageBlue™ Protein staining solution	Thermo Scientific	-	67-63-0	725515	

Western blot					
Reagent	**Manufacturer**	**C**	**CAS #**	**Lot #**	**notes**
TWEEN®20	Sigma-Aldrich	-	9005-64-5	SLBV0519	highly viscous, cut the pipette tip
Acetic acid	Sigma-Aldrich	≥ 99,8 %	64-19-7	SZBD1910V	
Ponceau S	Hoechst	solid	6226-79-5	49097	can be reused
TRIS-HCl	internal	1 M	1185-53-1	-	autoclaved, pH 8 
NaCl	internal	5 M	7647-14-5	-	autoclaved
Skimmed milk powder	Valio	solid	-	7,096E+10	-
His-Tag Polyclonal antibody	Cell Signaling Techno	-	-	3	store at - 20 °C
Anti-rabbit IgG, HRP-linked antibody	Cell Signaling Techno	-	-	28	store at - 20 °C
Amersham™ ECL ™ Solutions A and B	Ge Healthcare	-	-	16919972	-

Appendix 2: Buffers and solutions

Extraction and purification	
Extraction buffer, 1 ml	
B-PER	888 µl
lysozyme	10 µl
Dnase	2 µl
10x inhibitor stock	100 µl
total	**1000 µl**
Binding buffer, 1 ml	
8x Phosphate	125 µl
2 M imidazole	5 µl
H_2O	870 µl
total	**1000 µl**
Elution buffer, 1ml	
8x phosphate	125 µl
2 M imidazole	250 µl
H_2O	625 µl
total	**1000 µl**

SDS	
Running buffer, 10x stock, 1 l	
Tris Base	30,3 g
Clycine	144,1 g
SDS	10 g
H_2O	**1000 ml**
SB solution, 4x stock, 25 ml	
Tris-HCl	2,5 mM
clycerol	5,8 ml
SDS	1 g
β-Mercapto EtOH	2,5 ml
Bromophenol blue	5 mg
H_2O	**fill to 25 ml**

Western blot	
tTBS buffer, 1 l	
Tris-HCl	20 ml
NaCl	30 ml
TWEEN® 20	1 ml
H_2O	fill to 1000 ml
5 % Blocking buffer, 50 ml	
Milk powder	1 g
TBS buffer	50 ml
note:	Mix with magnetic stirrer for 30 min
Poinceau S staining solution, 200 ml	
Poinceau S	0,2 g
Acetic acid (glacial)	5 ml
H_2O	fill to 200 ml
Primary antibody	
His-Tag polyclonal a.b.	4 µl
5 % Blocking buffer	20 ml
Secondary antibody	
Anti-rabbit IgG HRP-linked a.b.	5 µl
5 % Blocking buffer	40 ml