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Testing the interaction of flavonoids with protein disulfide isomerase PDIA3 and the effects on protein biological properties

DIPLOMA THESIS

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1. Introduction

The protein PDIA3 or ERp57 (Endoplasmic Reticulum protein 57) is a part of the family of disulfide isomerase (PDI). The proteins belonging to this family are located predominantly in the endoplasmic reticulum (ER) but this is not their only location. These proteins are characterized by multiple domains, each structurally similiar to thioredoxin, a small protein and ubiquitous responsible for a variety of cellular redox processes. Unlike the thioredoxin, members of the protein disulfide isomerase family may have two or three active sites, characterized by two vicinal cysteins (-C-X-X-C-). The catalytic activity of ERp57 is not manifested only in reduction and formation of disulfide bonds, but also for the isomerization of the disulfide bonds that are formed during the correct folding of newly synthesized proteins (Turano et al, 2011).

As well as in the endoplasmic reticulum, which is reponsible for the proper protein folding, ERp57 was found in other cellular compartments such as the cytosol, the cell surface, in the mitochondria and nucleus.

In the recent years some studies have shown hyper-expression of ERp57 in the cells which are stressed. This stressing conditions cause protein misfolding and other degenerations, but ERp57 with its chaperonic and redox activities may help to reduce these harmful effects.

1.1. Protein disulfide isomerase family (PDI)

Protein disulfide isomerase is a member of the thioredoxin superfamily of redox proteins, mainly localized in the endoplasmic reticulum and characterized by the presence of a thioredoxin-like folding domain. PDIs constitute a family of structurally related enzymes which catalyze disulfide bonds formation, reduction or isomerization of newly synthesized proteins in the lumen of the ER. They act also as chaperones and are part of quality-control system for the correct folding of the proteins in the same subcellular compartment (Turano et al 2002).

PDI consists of four thioredoxin-like domains *(a, b, b', a')*, a linker (x) and an acidic C-terminal extension. Only *a* and *a'* contain the catalytic site with –Cys-X-X-Cys- motive. The *b'* domain provides the primary peptide or non-native protein binding site but other domains also contribute to the binding. It is seen that the *a* and *a'* domains isolated can catalyze thiol-disulfide exchange reactions, but only in combination with *b* domains isomerization reactions are possible.
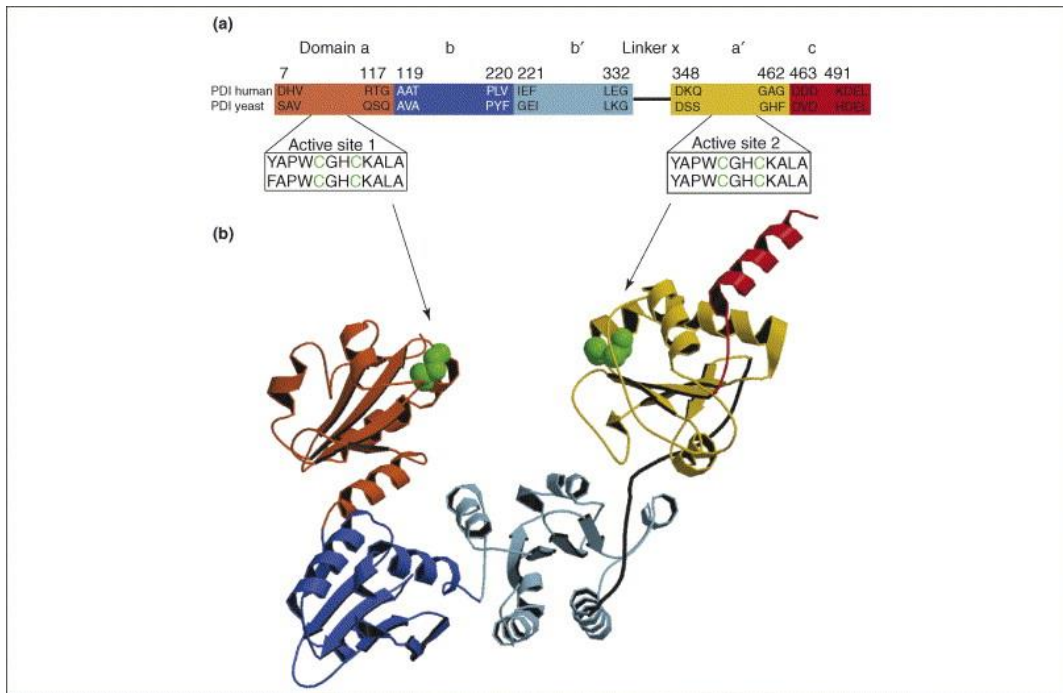
Figure 1. a) Representation of PDI domains; b) Protein structure of yeast PDI with catalytic sites in green

A great amount of proteins are part of this family in humans. The number and the arrangement of the thioredoxin-like domains with the specific sequence of the catalytic active site can be used to define different members of this family.

1.2. Endoplasmic reticulum protein 57 (ERp57)

One of the family members of the protein disulfide isomerase is PDIA3, also known as GRP57 (glucose-regulated protein 58), ERp57, ER-60, ERp58, Q2 and 1,25D3-MARRS. It is coded by the gene PDIA3 on chromosome 15, it weighs about 57 kDa and is formed by 505 amino acids of which first 24 represent the signal peptide (Turano et al 2011). The protein is mainly localozed in endoplasmic reticulum and has an established function as a chaperon and disulfide-rearrangements enzyme which helps proper folding in newly synthesized proteins (Coe and Michalak, 2010).

ERp57 consists of four domains named *a, b, b', a',* each one characterized by a thioredoxin-like active site with alternating alpha-helices and beta-strands. The first and the fourth domain, that is *a* and *a',* are catalytic domains containing the thioredoxin-like active site sequence -Cys-Gly-His-Cys-, whereas domains *b* and *b'* are not only necessary for the full activity of ERp57, but also provide the binding sites for calreticulin and calnexin (Turano et al, 2011). Three-dimensional structure of ERp57 shows a U-shape structure with catalytic sites of

the two active thioredoxin domains facing each other. Interior surface is hydrophobic and contributes to ability to bind unfolded proteins and catalyze the formation of correct disulfides (Cygler et al, 2006).
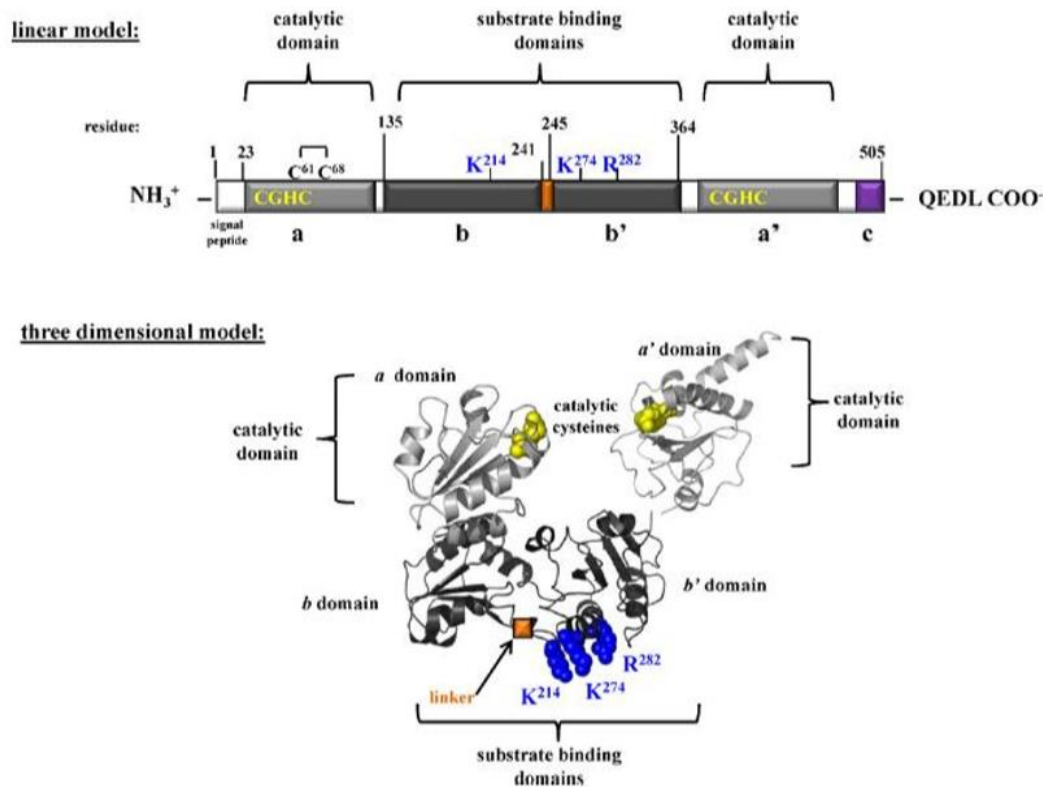
Figure 2. Linear and three dimensional model of ERp57 structure

Apart from localization in endoplasmic reticulum, ERp57 is also present in the cytosol, in the nucleus, in mitochondria and on the cell surface.

1.2.1. ERp57 in the endoplasmic reticulum

The endoplasmic reticulum (ER) provides an environment that allows the oxidative folding and post-translational modifications of protein that enter the secretory pathway (Jessop et al, 2007). The first function of ERp57 highlighted within ER has been to participate in the proper folding of newly synthesized glycoproteins and to control that were secreted into the cell membrane or positioned correctly. This requires the interaction of ERp57 with calnexin (CNX) and calreticulin (CRT), which are responsible for recognizing and binding newly synthesized glycoproteins (High, 2000). These endoplasmic chaperones determine if the proteins are to be released from the endoplasmic reticulum to be expressed, or alternatively, if they are to be sent

to the proteosome for degradation (Bedard et al, 2005). Glycoproteins do not directly interact with ERp57 but are first bound to the lectins which in turn mediate the binding to ERp57 (Cohen-Doyle, 2002).

ERp57 is also involved in the assembly of the major histocompatibility (MHC) class I complex in human cells. MHC class I complex has the function to bind short antigenic peptides derived from cytosol and to present them on the cell surface of cytotoxic T-lymphocytes, permitting the detection and elimination of pathogen-infected cells. Mature MHC class I is composed of two subunits: a trans-membrane heavy chain (HC) and a small soluble protein, β_2-microglobulin. The function of ERp57 is to allow the formation of the correct disulfide bonds in the heavy chain permitting its association with β_2-microglobulin. Later, ERp57 participates in forming of the peptide-loading complex (PLC) responsible for adding peptide fragments on the MHC class I complex (Cohen-Doyle, 1996).

1.2.2. ERp57 in the cytosol

The presence of the ERp57 protein in the cytosol is important for the interaction of this protein with the retinoic acid receptor and STAT3. STAT3 belogs to the STAT (Signal Transducer and Activator of Transcription) protein family. In response with cytokines and growth factors, STAT family members are phosphorylated by receptor-associated kinases and then form homo- or hetero-dimers that translocate to the cell nucleus, where they act as transcription activators (Darnell, 1997). All cytoplasmic proteins can be imported in nucleus if they possess a specific aminoacid sequence recognized by the nuclear import system. Since STAT3 is devoid of such nuclear localization signal, it is thought that STAT3 uses interaction with ERp57 to enter the nucleus (Altieri et al, 2002).

1.2.3. ERp57 in the nucleus

Initially the idea that the protein could have a nuclear localization was not considered because it was thought there was a contamination from the endoplasmic reticulum, where the protein is normally present. However, further studies confirmed the presence of ERp57 in the nucleus. It was also shown that several proteins can interact with ERp57 within the nuclear compartment, such as APE/Ref-1, Ku80, Ku70 and nuclear matrix proteins (Altieri et al, 2006). Further proof of ERp57 nuclear localization was the discovery that ERp57 can be associated with DNA, showing preference for AT-rich sequence and S/MAR (scaffold/matrix associated regions)-like regions (Coppari et al, 2002).

ERp57's DNA-binding properties are strongly dependent on the redox state of the protein. The a' domain is resposible for the binding properties and it has been proved that the oxidized form of the domain is the competent one (Grillo et al, 2002).

ERp57 can regulate gene expression contributing to the correct folding of multiprotein complexes that are directly involved in DNA binding or maintaining the appropriate redox state of transcription factors (Altieri et al, 2002).

1.2.4. ERp57 on cell membrane

Numerous studies have shown presence of the ERp57 protein on the cell surface, alone or in complex with membrane proteins (Hirano et al, 1995). One of the possible roles for the oxidoreductase ERp57 activity on cell membrane is to participate in gamete fusion, where ERp57 is located on the acrosome of the sperm (Ellerman et al, 2006). ERp57 was reported as the cell surface receptor for the vitamin D metabolite 1,25-dihydroxyvitamin D3 [1,25(OH)$_2$D3] and thus was also called 1,25D3-MARRS (Membrane Associated, Rapid Response Steroid binding) receptor (Khanal and Nemere, 2007).

1.3. Flavonoids

Flavonoids are broad class of molecules with low molecular weight characterized by flavone core. This group of naturally occurring abundantly present compounds are distributed in leaves, cortex, seeds and flowers of plants where they have various functions: protection from ultraviolet radiation, pathogens and herbivores (Harborne and Wiliams, 2000). They are present mainly in fruits, vegetables, wine, tea and cocoa. Flavonoids were discovered by Nobel Prize laureate Albert Szent Gyorgyi in the year 1930.

Flavonoids are the most important plant pigments for flower coloration, producing yellow or red/blue pigmentation in petals designed to attract pollinator animals. In higher plants, they are involved in UV filtration and symbiotic nitogen fixation. They may act as a chemical messenger or physiological regulator and they can also act as cell cycle inhibitors (Barile et al, 2008).

Many of their health benefits is attributed to their anti-oxidant and chelating ability. They are capable of scavenging hydroxyl radicals, superoxide anions and lipid peroxy radicals. They can also participate in the regeneration of other antioxidants, such as vitamin E. They are used in prevention and treatment of cardiovascular diseases due to their ability to inhibit lipid peroxidation. It has been reported that they also show anti-bacterial, anti-inflamatory, anti-

allergic, anti-mutagenic, anti-viral, anti-neoplastic, anti-thrombotic and vasodilatory effects (Bast et al, 2001; Jovanovic et al, 1998; Allan and Miller, 1996).
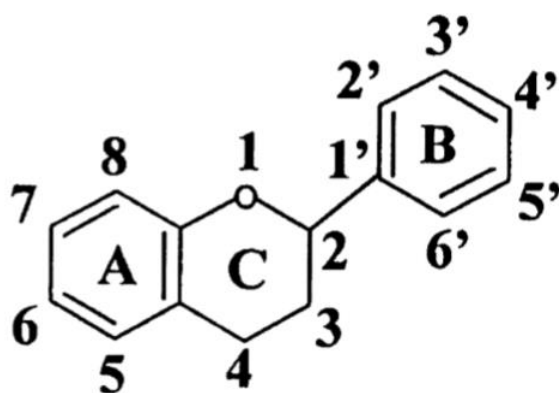
Figure 3. General structure of flavonoids

The basic structure of flavonoids consists of two benzene rings, A and B, linked through a heterocyclic pyrane ring, C. They differ in their structure from each other at the ring C. Dietary flavonoids differ in the arrangements of hydroxyl, methoxy, glycosidic group and in the conjugation between A- and B- rings. Hydroxyl groups are added, methylated, sulfonated or glucuronised during metabolism (Kumar and Pandey, 2013; Plaza et al, 2014; Heim et al, 2002). The in vivo anti-oxidant activity of flavonoids and their metabolites mainly depends on the arrangement of functional groups on the skeleton of the base structure. According to the oxidation form of the central ring (C) and the different substitutions, flavonoids are classified in various classes: flavone, isoflavone, flavonol, flavanone, anthocyanin and flavans (catechins, biflavans).

1.3.1. Eupatorin

Eupatorin or 5,3'-dihydroxy-6,7,4'-trimethoxy-flavone is extracted from the leaves of *Orthosiphon stamineus Benth* (Lamiaceae). This plant has been used in Indonesia and Malaysia for hundreds of years for healing effect kidney stones, atherosclerosis, rheumatism, gout and diabetes.
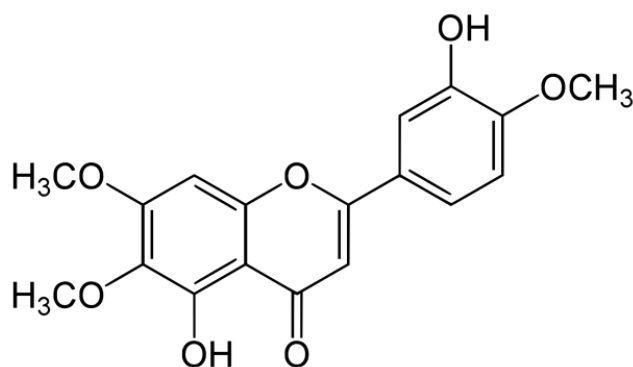
Figure 4. Molecular structure of eupatorin

It is a free radical scavenger, possesses an anti-oxidant, anti-inflammatory and anti-tumor effect (Yam et al, 2010). Among other abilities, eupatorin can block the cell cycle arresting cells in G2/M phase, can induce apoptosis, destroy the mitotic spindle and inhibit tumor angiogenesis. All these features make this molecule an excellent candidate for future studies as a possible antitumor therapeutic strategy. In addition, it shows significant inhibiting activity on various kinases such as EGFRs (epidermal growth factor receptor), PDGFR (platelet derived growth factor receptor), VEGFR (vascular endothelial growth factor receptor) and CDKs (cyclin-dependent kinases). All these anti-proliferative activities of eupatorin were tested on different tumor cell lines, such as cells of adenocarcinoma of the cervix (HeLa), breast cancer cells (MDA-MB468) and finally those of gastric adenocarcinoma (B16F10). It was observed that almost all beneficial effects were reached at very low concentrations, in micromolar range, and doses that are cytotoxic to cancer cells do not kill healthy cells instead. Thus it has been hypothesized the fact that eupatorin is able to differentiate between cancer cells and normal cells (Dolečková et al, 2012; Salmela et al, 2012).

1.3.2. Apigenin and apigenin-7-glucoside

Apigenin (4',5,7-trihydroxyflavone) is a common dietary flavonoid abundantly present in fruits and vegetables such as oranges and grapefruit, but also in parsley and onions. However, major source of apigenin is dry extract of *Matricaria chamomilla* (Asteraceae) flowers which is consumed in form of a tea. It also exsits as a dimer and is extracted from the buds and flowers of *Hypericum perforatum* (Hypericaceae). In nature it is present as apigenin-7-O-glucoside (apigetrin-found in dandelion coffee) and in various acetylated derivatives.
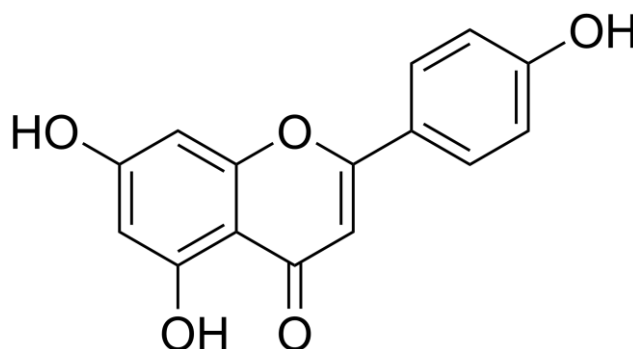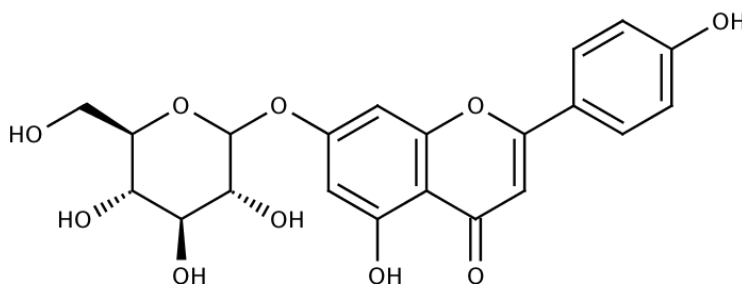
Figure 5. Molecular structure of apigenin

Figure 6. Molecular structure of apigenin-7-glucoside

Many of the biological effects of apigenin are associated with its anti-oxidant power. It also has anti-mutagenic, anti-viral and purgative effect. It seems to give good results in recent studies on breast cancer, as well as cervix, colon, lungs and prostate cancers. Another biological

effect induced by apigenin is the reduction of plasma levels of low density lipoprotein (LDL), inhibiton of platelet aggregation and the reduction of cell proliferation (Shukla and Gupta, 2010). Apigenin has a good inflammatory activity with many different targets, including the inhibition of the NF-κB pathway as one of the most known mechanisms (Kang et al, 2011). However, further research is required before apigenin could be brought to the clinical trials, but it has the potential for further investigation and development as a cancer chemopreventive and/or therapeutic agent.

1.3.3. Kaempferol

Kaempferol (3,5,7-trihydorxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a yellow compound and phytoestrogen found in many edible plants (tea, broccoli, cabbage, kale, beans, endive, leek, tomato, strawberries and grapes) and in plants or botanical products commonly used in traditional medicine (e.g. Ginkgo biloba, Tilia spp, Equisetum spp, Moringa oleifera, Sophora japonica and propolis).
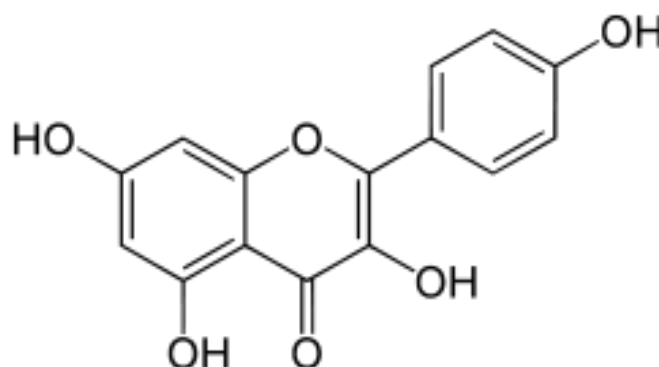
Figure 7. Molecular structure of kaempferol

Its anti-oxidant/anti-inflammatory effects have been demonstrated in various disease models, including those for encephalomyelitis, diabetes, asthma and carcinogenesis. Kaempferol act as a scavenger of free radicals and superoxide radicals as well as preserve the activity of various anti-oxidant enzymes such as catalase, glutathione peroxidase, and glutathione-S-transferase (Rajendran et al, 2014). More detailed studies should be performed to confirm anti-cancer effects of kaempferol for clinical use (Seung-Hee and Kyung-Chul, 2013).

1.3.4. Naringenin

Naringenin (5,7-dihyroxy-2-(4-hydroxyphenyl)chroman-4-one) is flavan found in grapefruits, oranges and tomatoes (Vallverdu-Queralt et al, 2012).
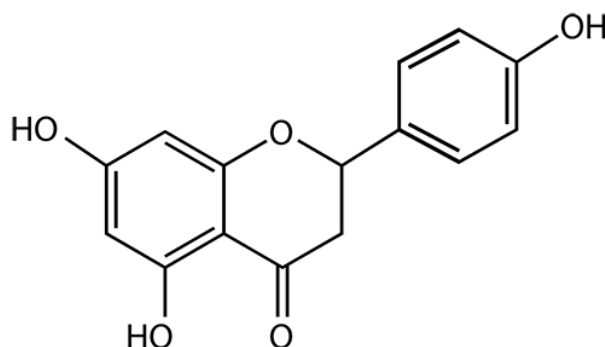
Figure 8. Molecular structure of naringenin

Naringenin showed lipid lowering properties and it can inhibit VLDL secretion from cultured hepatocytes in a manner resembling insulin. For these reasons it represents a promising therapeutic approach for metabolic syndrome (Mulvihill et al, 2009). It has an inhibitory effect on the human cytochrome P450 isoform CYP1A2 (Fuhr, 1993) so may have effect on pharmacokinetics of several drugs.

1.3.5. 3-O-Methylquercetin

The 3-O-methylquercetin, also called quercetin-3-O-methyl-ether, is one of quercetin derivatives that is frequently found in plants used in medicine. There are a few data in the literature on the the presence this molecule in nature and its biological activity.
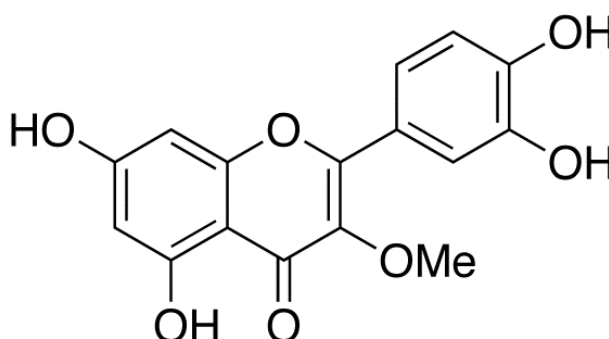
Figure 9. Molecular structure of 3-O-methylquercetin

A study performed on mice FL83B hepatocytes in culture showed that this molecule reduces the formation of ROS induced by copper and protect the cells from cell death caused by the presence of copper. This effect is due to an increase in biosynthesis of GSH and superoxide dismutase (SOD) after the ROS formation and the activation of the PI3K/Akt and MAP/Erk pathways (Tseng et al, 2012).

1.3.6. Cyanidin

Cyanidin belongs to a group of anthocyanins and is water-soluble pigment. The natural electron deficiency of anthocyanins makes these compounds particulary reactive, rendering them also very sensitive to pH and temperature changes. Cyanidin is present in berries (blackberry, blueberry, cherry, cranberry, elderberry and others) but also in other fruits including apples, pears, peaches and plums.
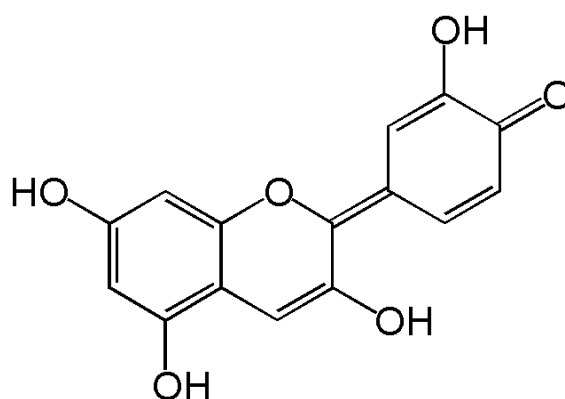
Figure 10. Molecular structure of cyanidin

Literature data confirm its antioxidant activity and biological properties with a potential beneficial role in human health.

1.3.7. Genistein

Genistein (4',5,7-trihydroxyisoflavone) is a phytoestrogen with a wide variety of pharmacological effects in animal cells, including tyrosine kinase inhibition. It is found in lupin, fava beans, soybeans, kudzu and psoralea. Dietary genistein ingestion has been linked to a wide range of potential beneficial effects that include chemoprevention of breast and prostate cancers, cardiovascular disease and post-menopausal ailments (Kaufman et al, 1997; Dixon RA and Ferreira D, 2002).
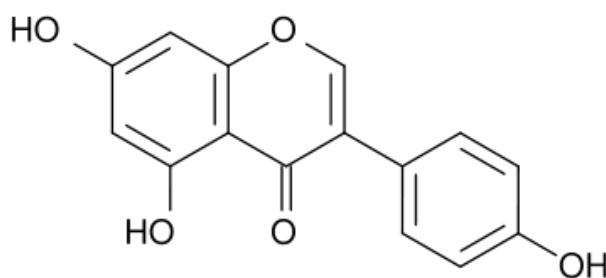
Figure 11. Molecular structure of genistein

However, it may stimulate existing breast tumor growth and antagonize the effects of tamoxifen (de Lemos, 2001).

1.3.8. Luteolin-7-glucoside

Luteolin-7-glucoside, also known as glucoluteolin and cynaroside is a flavone found in Campanula persicifolia and C. rotundifolia, Teucrium gnaphalodes and in artichokes (Teslov and Teslov, 1972; Nüβlein and Kreis, 2005).
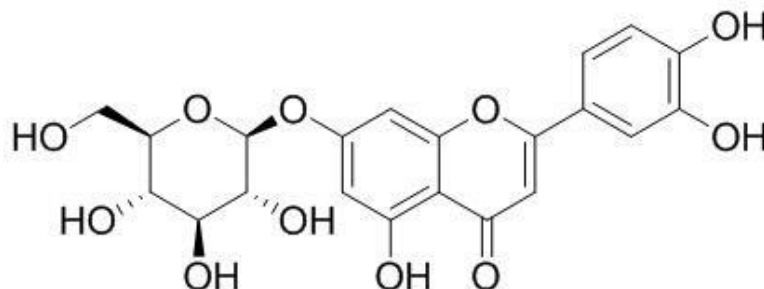
Figure 12. Molecular structure of luteolin-7-glucoside

Cynaroside is a flavonoid compound that exhibits anti-oxidative capabilities. A study showed pretreatment of H9c2 cardiomyoblasts with cynaroside significantly reduced the apoptotic rate enhancing the endogenous anti-oxidative activity of superoxide dismutase, glutathione peroxidase, and catalase, thereby inhibiting intracellular reactive oxygen species (ROS) generation (Sun X et al, 2011).

2. Aim of the project

Protein PDIA3/ERp57 is an important protein with multiple functions and is distributed in several cellular compartments. ERp57 is involved in various diseases and has a potential to be a pharmacological target. This is further supported by the obsevartion that ERp57 can interact with flavonoids, known for their antioxidant capacity.

Following a previous research performed in this laboratory, where the binding of ERp57 to catechins, a class of flavonoid, as well as some derivatives of quercetin was investigated, the aim of this project is to reach a deeper understanding of the interaction between ERp57 and other classes of flavonoids (particulary kaempferol, eupatorin, cyanidin, naringenin, apigenin, genistein, 3-O-methylquercetin, apigenin-7-glucoside and luteolin-7-glucoside).

The goal is to understand how different substitutions on the flavonoid basic structure can modify the interaction with ERp57 and have also effects on protein activity, stability and DNA-binding capability. Fluorescence quenching is used to measure flavonoid binding to ERp57, while electrophoretic mobility shift assay (EMSA) is used to evaluate flavonoid effect on ERp57-DNA interaction. Influence of mentioned flavonoids on protein activity is performed using a substrate modified with a fluorescent dye, dieosin glutathione disulfide, and the effect on protein stability was tested by differential scanning fluorimetry (DSF).

3. **Materials and methods**

Instruments and materials:

- PCR instrument GenAmp PCR System 2400 (Perkin Elmer, USA)

- Laboratory cetrifuge (Hettich, Germany)

- Anion exchange column Macro-Prep Q (BioRad, USA)

- Affi-Prep Heparin column (BioRad, USA)

- Spectrofluorometer FluoroMax (HORIBA Scientific, Japan)

- Automatic single-channel pipettes, adjustable volume (Eppendorf, Germany)

- Test tubes Falcon (BD Biosciences, USA)

- Analytical balance (Mettler, USA)

- Chemidoc MP^{TM} Imaging System (BioRad, USA)

Standards, reagents and other chemicals:

- plasmid pET21 (Clonetech Laboratories, USA)

- LB Broth powder (Sigma-Aldrich, St Louis, USA)

- Yeast extract (Sigma-Aldrich, St Louis, USA)

- IPTG isopropyl-β-thiogalactopyranoside (Sigma-Aldrich, St Louis, USA)

- ammonium sulphate (Sigma-Aldrich, St Louis, USA)

- DTT dithiothreitol (Sigma-Aldrich, St Louis, USA)

- PBS buffer (Sigma-Aldrich, St Louis, USA)

- Coomassie Brilliant Blue color (BioRad, USA)

- Tris 1 M, pH 8.0 (Sigma-Aldrich, St Louis, USA)

3.1. Expression and purification of ERp57, its *abb'* fragment and *a'* domain

3.1.1. ERp57

Bacterial BL21 cells were used for protein expression because they have a high capacity to synthesize proteins if stimulated with IPTG (isopropyl-β-thiogalactopyranoside). BL21 were first transformed with a pET21 plasmid containing coding sequence of the protein of interest and then grown on LB agar supplemented with ampicilin. Later a single colony was picked-up and grown as following:

- Preinoculum (in a small flask previuosly autoclaved) with 100ml of YT medium, 100µL of ampicilin (100 mg/mL) and one colony picked from the agar plate with a sterile stick

- Cells were grown at 37ºC with shaking for 24 hours

- Inoculum: 2 flasks each containing 50mL preinoculum added to 450mL YT, 450µL ampicilin (100 mg/mL)

- Incubation of inoculum (2h) at 30ºC until A_{600} reaches 0,6 OD (optical density)

- Adding 400µL of IPTG 1mM in each flask, expression of the protein is being induced

- Cells were left to grow for other 5 hours at 30ºC and centrifugated at 5000rpm at 4ºC for 15 min

- Cells harvested by centrifugation were resuspended in NEN buffer (20mM Tris-HCl pH 8.0, 100mM NaCl, 0.5mM EDTA) containing 0.25% TritonX-100, 5mM DTT and 1mM PMSF (phenyl-methyl-sulfonyl fluorid), using 5 mL for every 125 mL of culture

- Cell suspension was subjected to lysis by sonication using Ultrasonic Device Hielscher UP100H for 20 seconds with a pause of 20sec in between, 6 times repeated (the process is done on ice due to production of warmth that can cause damage to the sample) and cleared by centrifugation at 12000rpm for 15min at 4ºC

- Supernatant was collected and left on ice while pellet was resuspended with half of the resuspension buffer used above and the sonication procedure was repeated as previously described

- Supernatant collected after second sonification was united with the first one and NEN buffer added up to 100mL

- At this point begins precipitation with ammonium sulfate in two steps:

  1. precipitation of bacterial proteins that are insoluble in 30% saturation solution of ammonium sulfate by adding 176 g/L. After incubation under stirring conditions at 4ºC for 2h and then centrifugation at 12000rpm for 20min at 4ºC, pellet was discarded and supernatant subjected to next step

  2. precipitation of proteins that are insoluble in 75% saturation solution of ammonium sulfate by adding additional 340 g/L respect to the initial volume. Mixing under stirring conditions and centrifugation steps are repeated but at this point pellet is collected (supernatant is thrown away)

- The next step of purification is dialysis in which buffer Tris HCl 20 mM pH 8.0, NaCl 40 mM (40 mL of Tris 1 M pH 8.0, 4.68 g NaCl and water to fill up to 2L) was used

- After 24 hours of dialysis, buffer was changed to Tris HCl 20 mM pH 8.0, NaCl 20 mM (40 mL of Tris 1 M ph 8.0 and 2.34 g NaCl and water to fill up to 2L) and dialysis step is repeated

- After dialysis and centrifugation at 10000 rpm, 10 min, supernatant was loaded on an anion-exchange column Macro-Prep Q (Biorad) which was then washed in Tris HCl 20 mM pH 8.0, NaCl 20 mM buffer.Elution was done with Tris HCl 20mM pH 8.0, NaCl 150 mM (flow rate 1 mL/min)

- Purification quality was tested by SDS electrophoresis

- Eluted material was dialysed against Tris HCl 20 mM pH 8.0, NaCl 20 mM overnight

- Dialyzed proteinswere loaded on Affi-Prep Heparin column (which was washed with the same buffer used for dialysis) and eluted using a NaCl gradient from 20 mM to 1 M in Tris HCl 20 mM pH 8.0 buffer (gradient elution in 60 min, flow rate 1 mL/min)

- Collected fractions were analyzed using SDS gel electrophoresis and those containing ERp57 were collected and dialyzed against Tris HCl 20 mM pH 8.0, NaCl 20 mM overnight

- Ion-exchange chromatography was repeated for the third time (anion-exchange column Macro-Prep Q washed in Tris HCl 20 mM pH 8.0, NaCl 20 mM). This

time the elution was done with a NaCl gradient from 20 mM to 250 mM in Tris HCl 20 mM pH 8.0 (elution 35 min, flow 1 mL/min)

- At the end, purification quality was tested by SDS gel electrophoresis and quantity of protein by spectrophotometer measurement

3.1.2. Abb' fragment

To express the protein portion of ERp57 corresponding to abb' domains, the same procedure described for the whole protein was followed, with some minor differences. The first sammonium sulphate precipitation step was performed with a50%saturation ammonium sulphate solution instead of 30%. Dialysis stepafter ammonium sulphate precipitation was performed using the same buffer both times (Tris HCl 20 mM pH 8.0, NaCl 20 mM). The fractination on the first anion-exchange column and on the Heparin column were performed as described for the whole protein, but the second fractination step on the anion-exchange column was not required since the collected protein was pure enough.

3.1.3. a' domain

Bacterial BL21 cells were transformed with a pET29 plasmid containing the coding sequence of the protein of interest and then grown on LB agar supplemented with kanamycin.

Similar to abb' fragment, the same procedure described for the whole protein was followed, with some differences. After the two steps of ammonium sulphate precipitation (same as for the ERp57; 30 and 75%), a first dialysis step in Tris HCl 20 mM pH 8.0, NaCl 20 mM (40 mL of 1 M Tris pH 8.0 and 2,34 g NaCl with water to fill up to 2 L) was carried out. The day after the dialysis buffer was changed to Tris HCl 20 mM pH 8.0, NaCl 40 mM (40 mL of 1M Tris pH 8.0 and 4,68 g NaCl with water to fill up to 2 L) and left on dialysis for another two days. The dialyzed solution was centrifuged at 12000 rpm at 4°C for 10 min and the supernatant was loaded on an anion-exchange Macro-Prep Q (Biorad) column which was washed in Tris HCl 20 mM pH 8.0, NaCl 40 mM and elution was performed with the same buffer. The a' domain does not bind to the column, so it is found in flow through.Eluate after the elution with 1 M NaCl was also collected. The protein in flow through was further purified using an Heparin Affi-Prep column. After loading, the column was washed with Tris HCl 20 mM pH 8.0, NaCl 20 mM and eluted using a linear gradient from 20 mM to 1 M NaCl in 20 mM Tris HCl, pH 8.0. The obtained fractions were analyzed by SDS electrophoresis to evaluate the quality of the purification and those containing the *a'* domain were collected and protein concentration was evaluated by spectrophotometer measurement.

3.2.   Electrophoresis techniques

Electrophoresis is relatively simple, rapid and highly sensitive tool to study the properties of proteins. The separation of proteins by electrophoresis is based on the fact that charged molecules will migrate through a matrix upon application of an electric field provided by immersed electrodes. Generally the sample runs in a support matrix such as agarose or polyacrylamide gel.

3.2.1. SDS-PAGE

Poly-acrylamide gel is widely used to separate proteins. SDS-PAGE electrophoresis uses a discontinuous polyacrylamide gel as a support medium and the detergent sodium dodecyl sulfate (SDS) to denature the proteins allowing protein separation strictly by their size. This procedure denaturates proteins and consequently cannot be used to analyze native proteins (Hames, 2002). The negative charge of bound SDS molecules overwhelmes the intrisic charge of a protein and thereby gives all proteins a uniform negative charge density. SDS-denaturated proteins therefore migrate as polyanions through the polyacrylamid gel toward the positive electrode (the anode) according to their size. At the end of the electrophoretic separation, smaller proteins will be found near the bottom of the gel and larger proteins near the top (Goodman, 2008).

Protein samples were solubilized in Loading buffer 1x final concentration (Loading buffer 4x: SDS 4%, DTT 100 mM, Tris HCl 125 mM pH 6.8 and bromophenol blue as indicator of migration). Glycerol serves to burden sample and facilitates loading into the well, SDS denaturates proteins and DTT reduces disulfide bonds.

Protein Quadcolor (4.6-300 kDa) is used as molecular weight marker.

Polyacrylamide gel consists of two parts:

- Stacking gel that represents the upper part of the gel and its function is to concentrate the protein samples which are loaded it wells. It consists of stacking buffer, acrylamide, SDS, APS (ammonium persulfate, used as an initiator for gel formation), water and TEMED (N, N, N', N'-tetramethylethylendiamine, that stabilizes free radicals and improves polymerization).
- Running gel constitutes the lower part and its function is to separate proteins of the different samples based on their molecular weight. It consists of the same ingredients as stacking gel but in different quantity. According to desired porosity concentration

of acrylamide varies: higher concentration results smaller pores, consequently capacity to separate proteins is bigger.

Table 1. Composition of 10% polyacryamide gel

	Running	**Stacking**
Acrylamide	4 mL	0.5 mL
Lower/Upper buffer	3 mL	1.25 mL
Water	4.9 mL	3.25 mL
SDS	120 μL	50 μL
APS	100 μL	50 μL
Temed	10 μL	5μL

After running electrophoresis at 200 V, 50 min, gel is washed with bidistilled water and colored with Coomassie Brilliant Blue overnight. The next day gel is washed again and once decolored is dried in gel-dryer.

3.3. Spectrofluorimetry

3.3.1. Phenomenon of fluorescence

Luminescence is the emission of light from any substance, and occurs from electronically excited states. Luminescence is formally divided into two categories, fluorescence and phosphorescence, depending on the nature of the excited state (Lakowicz, 2006).

Fluorescence is an analytically important emission phenomenon in which atoms or molecules are excited by absorption of a beam of electromagnetic radiation. The excited species then relax to the ground state, giving up their excess energy as photon. The wavelength of absorbed radiation must be at lower values (higher energy) then the emitted (fluoresced) wavelength. The difference between these two wavelengths is known as Stokes shift and in general the best results are obtained from compounds involving large shifts (Walker and Wilson, 2005).
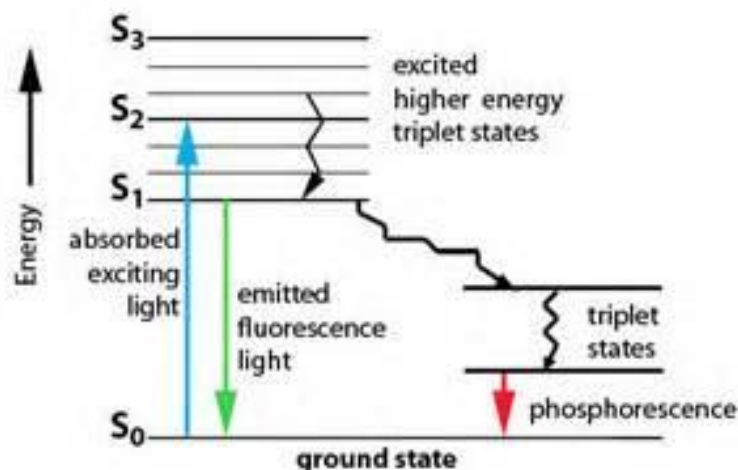
Figure 13. Jablonski diagram

State S_0 is called the ground state of the fluorophore (fluorescent molecule) and S_1 is its first (electronically) excited state. A molecule in state S_1 can relax by various competing pathways. It can undergo to 'non-radiative relaxation' in which the excitation energy is dissipated by heat (vibrations) of the solvent. Excited organic molecules can also relax via conversion to a triplet state, which may subsequently relax via phosphorescence or by a secondary non-radiative relaxation step. Relaxation of an S_1 state can also occur through interaction with a second molecule through fluorescence quenching.

Most of the intrinsic fluorescence emissions of a folded protein are due to excitation of tryptophan residues (three residues in ERp57). Tryptophan is one of the best fluorophores, with a wavelength of maximum absorption of 280 nm and emission ranging from 300 nm to 350 nm depending on the polarity of the local environment. Furthermore, tryptophan fluorescence is strongly influenced by proximity of other residues (i.e., nearby protonated groups such as Asp or Glu can cause quenching of Trp fluorescence).

3.3.2. Quenching

Quenching is a process that decreases the fluorescence intensity of a given substance. Most commonly, quenching of fluorophores occurs by one of two mechanisms: contact quenching and FRET quenching. In contact quenching, the fluorophore and quencher are in sufficiently close proximity to allow for direct electronic interaction of the excited state of the fluorophore with the quencher molecule. When the distance between the fluorophore and quencher is increased, generally to the range of 20-100 Å, the alternative mechanism of FRET (Förster resonance energy transfer) quenching is observed.

Presentation of quenching data is usually in the form of the Stern-Volmer plot:

$$\frac{F_\circ}{F} = 1 + \mathrm{Ksv}\,[Q]$$

F_0-fluorescence intensity in the absence of quencher

F-fluorescence intensity in the presence of quencher

K_{sv}-Stern-Volmer quenching constant

[Q]-concentration of quenching agent

The quenching effect of flavonoids was calculated using the following equation:

$$Q = 1 - \frac{F}{F_\circ} \times 100\%$$

Q-percentage of quenching

F_0-fluorescence intensity in the absence of quencher

F-fluorescence intensity in the presence of quencher

3.3.2.1. Fluorescence quenching of ERp57 by flavonoids

Spectroscopic measurements were recorded using a luminescence spectrometer Fluoromax in 1cm quartz cuvette. Quantitative analyses of the potential interaction between individual flavonoids and ERp57 were performed by fluorimetric titration. Briefly, solution of ERp57 (0.5μM) was titrated in quartz cuvette with successive additions of flavonoid (1mM solution), equal to 2, 4, 6, 8, 10 and 20μM in the cuvette. Flourescence emission spectra were recorded from 300 to 400nm with excitation at 290nm under continuous stirring. All experiments were carried out at 25˚C. Flourescence intensity was read at protein emission maximum of 338nm. Flourescence spectra of flavonoids diluted in buffer (0-20 μM) were recorded as blanks under the same experimental conditons and substracted from the corresponding flavonoid-ERp57 system to correct the flourescence background. Readings were performed three times for each concentration and three times for the whole titration analysis.

Flavonoids used in experiments are pure commercially available Sigma Aldrichsubstances: kaempferol (60010), eupatorin (E4660), cyanidin (79457), naringenin (52186), 3-O-Methylquercetin (90081), apigenin (A3145), genistein (G6649), luteolin-7-glucoside (49968), apigenin-7-glucoside (44692). They were dissolved in DMSO in oreder to yield 40mM stock solution. Stock solutions were preserved on -20˚C.

Solutions used in measurements are:

- Buffer PBS (10 mM, pH 7.4) adjusted by DTT (0.1 mM) and EDTA (0.2mM); prepared just before the use

- An aliquot of ERp57 (72μM) dissolved in PBS in order to yield solution with ERp57 at a final concentration of 0.5μM

- Flavonoids (kaempferol, eupatorin, cyanidin, naringenin, 3-O-Methylquercetin, apigenin, genistein, apigenin-7-glucoside, luteolin-7-glucoside; 40mM stock solution) were diluted by 50%PBS/50% ethanol to obtain 1mM solutions used for titration

Prior dilution the aliquot of protein stock solution was incubated with 0.1M DTT for 30 min to allow complete reduction of disulfide bonds (reduced protein).

3.4. Activity assay

The activity assays were made with the whole ERp57 protein. The reductase activity of ERp57 was monitored by using fluorogenic substrate compound,dieosin glutathione disulfide (Di-E-GSSG).This substrate was obtained by a reaction between oxidized glutathione and eosine-isothiocyanate, followed by chromatographic separation on Sephadex column to resolve free eosin from modified glutathion.Di-E-GSSG has low fluorescence which increasessignificantly with reduction of its disulfide bonds by ERp57 reductase activity (the fluorescence of Di-E-GSSG is lower than the fluorescence of two molecules of E-GSH, reduced glutathione linked to eosin). To analyze variation in the reductase activity of ERp57 in the presence of flavonoids the variation of Di-E-GSSG fluorescence was followed compared to protein alone. The emission of Di-E-GSSG was detected with Fluoromax fluorometer in 10 mm disposable cuvettes.

Solutions used in measurements are:

- Buffer: PBS 10 mM pH 7.4, EDTA 0.2 mM

- Flavonoid stocks: prepared by dissolving the substances in DMSO to a concentration of 40 mM and stored at -20ºC. For the analysis was used 1:40 dilution (5 μL of 40 mM solution + 195 μL of PBS/ethanol 50:50) to use flavonoids of a final concentration of 1 mM.

Schematic protocol for measuring protein activity:

- Control: 1977.5 μL of buffer + 22.5 μL of protein ERp57 44.4 μM (final concentration 0.5 μM) + 40 μL Di-E-GSSG 7.4 μM (final concentration 150 nM) + 10 μL DTT 1 mM (final concentration 0.5 μM). Monitor fluorescence for 180 sec with excitation peak at 525 nm and reading performed at 545 nm at 25°C. Repeat measurement 3 times. Blank was obtained using the same solution except the addition of ERp57 (fluorescence increase was neglegible).

- Samples with flavonoids: 1937.5 μL of buffer + 22.5 μL of protein ERp57 44.4 μM (final concentration 0.5 μM) + 40 μL of flavonoid 1 mM (final concentration 20 μM). Incubation for 2 minutes. Add 40 μL Di-E-GSSG 7.4 μM + 10 μL DTT 1 mM. Monitor fluorescence for 180 sec with excitation peak at 525 nm and reading performed at 545 nm at 25°C. Repeat measurement 3 times.

3.5. Protein stability

Differential scanning fluorimetry (DSF) is a rapid and inexpensive screening method to indentify low-molecular–weight ligands that bind and stabilize purified proteins. The temperature at which a protein unfolds is measured by an increase in the fluorescence of a dye with high affinity for hydrophobic parts of the protein, which are exposed as the protein unfolds. DSF is best performed using a conventional real-time PCR instrument. Ligand from stock solutions are added to a solution containing protein and dye, distributed into the wells of the PCR plate and fluorescence intensity measured as the temperature is gradually raised.

The stability of a protein is related to its Gibbs free energy of unfolding (ΔG) which is temperature-dependent. The stability of most proteins decreases with the temperature; as the temperature increases, the ΔG decreases and becomes zero at equilibrium where the concentrations of folded and unfolded protein are equal. At this point, the temperature is considered as melting temperature (T_m). If a compound binds to a protein, the free energy contribution of ligand binding in most cases results in an increase in the T_m. It has been shown that the stabilizing effect of compounds upon binding is proportional to the concentration and affinity of the ligands. The fluorescence intensity is plotted as a function of temperature; this generates a sigmoidal curve that can be described by a two-state transition. The inflection point of the transition curve (Tm) is calculated using the Boltzmann equation:

$$y = LL + \frac{(UL - LL)}{1 + e^{Tm - \frac{x}{a}}}$$

where the LL and UL are the values of minimum and maximum intensities, respectively, and *a* denotes the slope of the curve within T_m. The simplest way to calculate T_m values is to determine the maximum of the first derivative; this possibility is offered by many software packages, such as Graph Pad Prism 5.

The dye with the most favorable properties for DSF is SYPRO orange, owing to its high signal-noise ratio. The SYPRO has excitation at 492 nm and emission at 610 nm (Niesen et al, 2007)
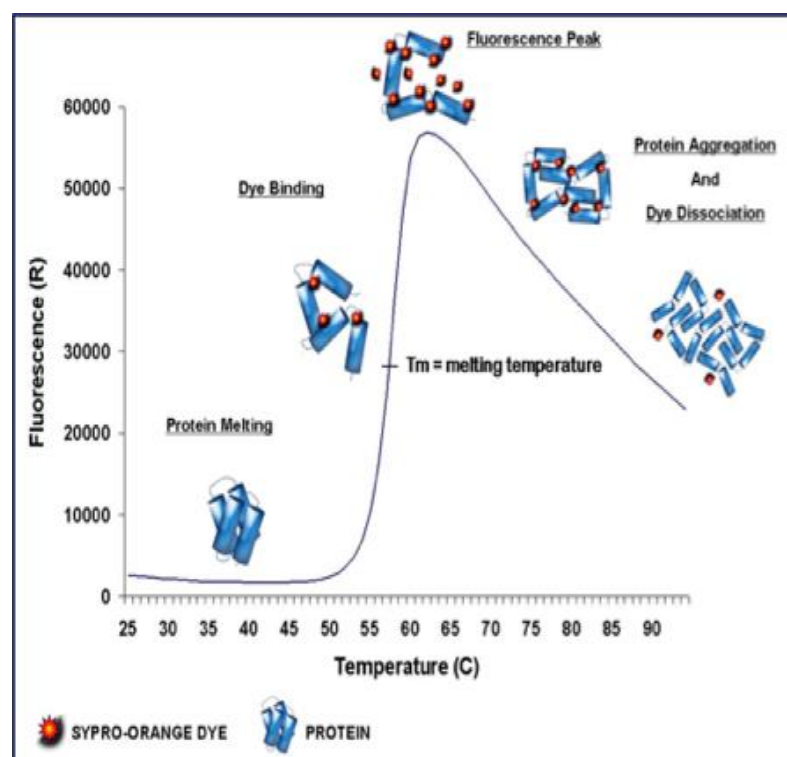

Figure 14. Representation of the process of protein denaturation and fluorescence variation of SYPRO

At the beginning when the protein is still in its stable conformation there is low fluorescence emission because the SYPRO hasn't bound the protein yet. As the temperature increases the protein begins to expose its hydrophobic sites that allow SYPRO to bind. This is pointed out by a significant increase in fluorescence at around 610 nm as a result of binding of the protein with SYPRO. Once the maximum peak intensity is reached, there is a gradual decrease in fluorescence that can be explained by the release of SYPROfrom the protein caused by the aggregation and precipitation of the latter.

Preparation of the oxidized protein for PCR-melting: protein ERp57 was diluted in buffer TBS (20 mM Tris/HCl, 150 mM NaCl) to yield the final concentration of 1 μM. Afterwards, the SYPRO orange commercial stock solution was added in a 1:1000 dilution.

Preparation of the flavonoids stock solutions:

1. From the initial solution of 40 mM in DMSO it was prepared 400 μM intemediate solution in buffer made of 4 parts TBS:EtOH (50:50)/6 parts TBS to have final concentration of 50 μM in the well.

2. From the intermediate solution of 400 μM it was prepared 160 μM solution in buffer made of 4 parts TBS:EtOH/6 parts TBS to obtain the final concentration of 20 μM in the well.

3. From the intermediate solution of 400 μM it was prepared 40 μM solution in buffer made of 4 parts TBS:EtOH/6 parts TBS to obtain the final concentration of 5 μM in the well.

4. From the 160 μM solution it was prepared 16 μM solution in buffer made of 4 parts TBS:EtOH/6 parts TBS to obtain the final concentration of 2 μM in the well.

5. From the 40 μM solution it was prepared 4 μM solution in buffer made of 4 parts TBS:EtOH/6 parts TBS to obtain the final concentration of 0.5 μM in the well.

Schematic protocol for loading PCR plate:

- In each well it was added 35 μL of stock protein + TBS buffer + SYPRO orange
- Afterwards, 5 μL of different intermediate solutions of flavonoids were added to each well to yield concentrations of 0.5 μM, 2 μM, 5 μM, 20 μM, 50 μM. In protein control test it was added 5 μL of buffer made of 4 parts TBS:EtOH/6 parts TBS.

3.6. EMSA (Electrophoretic mobility shift assay)

The gel electrophoresis mobility shift assay (EMSA) is used to detect protein complexes with nucleic acids. In the classical assay, solutions of protein and nucleic acid are combined and the resulting mixtures are subjected to electrophoresis under native conditions through polyacrylamide or agarose gel.It is based on the observation that the electrophoretic mobility of a protein-nucleic acid complex is typically less than that of the free nucleic acid (Hellman and Fried, 2007). A DNA fragment labeled with flourscein was used allowing its visuakization in absence of other dyes as ethidium bromide. Fluorescein emits fluorescence upon excitation with UV light or wavelengths in the blue spectra. If a protein binds to DNA segment its mass increases causing the delay in its electrophoretic migration compared to the free DNA fragment.

For the separation run it was used a 5% acrylamide gel which possesses mesh wide enough to allow migration of the free DNA and the DNA-protein complex.

Polyacrylamide gel 5%:

- Acrylamide-bisacrylamide (30%) 1.5 mL
- 10X TBE (Tris-Borate-EDTA) 0.3 mL
- Bi-distilled water 10.2 mL
- 10% APS (Ammonium persulfate) 100 μL
- TEMED (N,N,N′,N′-Tetramethylethylenediamine) 10 μL

Running buffer: TBE 0,025%

- 10X TBE 25 mL
- Bi-distilled water 975 mL

To test the effects of flavonoids on DNA-binding capability of ERp57, an 80 bp DNA fragment, AT rich, was analyzed in presence of the isolated *a′* domain of ERp57 (D4 domain) which is mainly responsible for the DNA-binding properties of ERp57.

In each well wereloaded 10 μL of solutions prepared as followed:

1. Free DNA: 1 μL DNA 5 ng/μL + 3 μL binding buffer 3X + 6 μL H_2O

2. DNA + D4 domain of ERp57: 1 μL DNA 5 ng/μL + 2 μL D4 81 μM + 3 μL binding buffer 3X + 4 μL H_2O

3. DNA + D4 domain of ERp57 in reduced form: 1 μL DNA 5 ng/μL + 3 μL binding buffer 3X + 2 μL D4 81 μM + 1 μL DTT 0.1 M + 3 μL H_2O

4. DNA + D4 domain of ERp57 + flavonoids in different concentrations: 1 μL DNA 5 ng/μL + 3 μL binding buffer 3X + 2 μL D4 81 μM + 1 μL of flavonoids in final concentrations of 25 μM and 50 μM

Following a pre-run of 20 minutes at 140 V, the solutions were loaded and electrophoresis was performed for 10 minutes at 80 V and then at 110 V for 20-25 min. Images were captured on Chemidoc MP ^{TM} Imaging System (BIO-RAD).

4.   Results and discussion

4.1. Purification of ERp57

ERp57 was cloned using PCR into the expression vector pET21,expressed in E.coli BL21, and later purified as described in Chapter 3.

The cells were lysed by sonication with Ultrasonic Sonicator and clarified lysate was fractioned by ammonium sulfate precipitation (30% and 75%). Samples taken during the purification were analysed by SDS-PAGE to ensure that the protein is actually present in the 75% saturation precipitate.
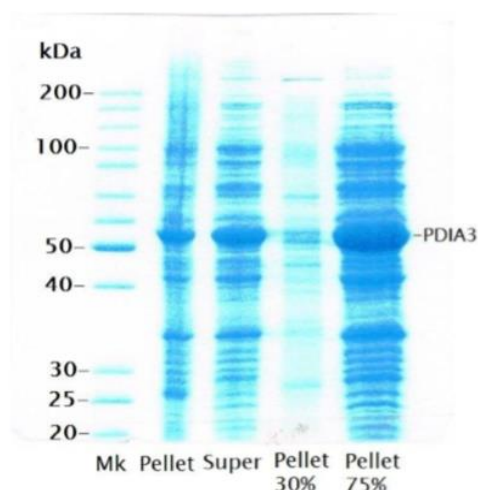

Figure 15. SDS-PAGE analysis (10% polyacrylamide gel) of fractions obtained after lysis and ammonium sulphate precipitation. P: unsoluble proteins, S: soluble extract, 30% and 75% ammonium sulfate precipitation, M: protein markers

Purification of the ERp57 protein required several passages through chromatographic column. Firstly, protein solution was loaded on the ion-exchange column Macro-Prep Q (Biorad), from which the greatest amount of ERp57 protein was elute in 150 mM NaCl.This eluate was then diluted with Tris HCl buffer to lower the salt concentration and loaded on Heparin Affi-Prep column. Proteins were eluted with a NaCl gradient from 20 mM to 1 M NaCl. The fractions collected were analysed by SDS-PAGE electrophoresis which showed that fractions 27 to 33 have the greatest amount of ERp57 (Figure 16.). Fractions were collected and dialyzed to lower salt concentration.
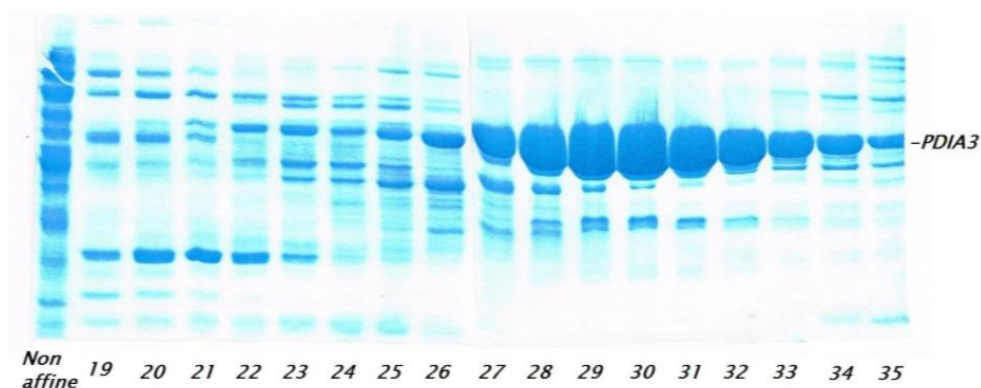
Figure 16. SDS-PAGE electrophoretic analysis (10%) of fractions eluted from the heparin column

Following dialysis, the solution was passed through another anion-exchange column and fractions obtained by passing through elution gradient of NaCl from 20 mM to 250 mM were analyzed by SDS-PAGE, as shown in Figure 17.
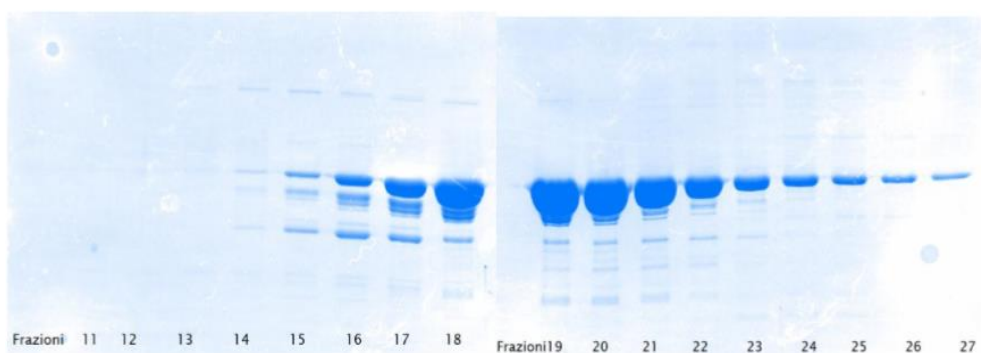

Figure 17. SDS-PAGE electrophoretic analysis (10%) of fractions eluted from the second anion-exchange column

Fractions 18-21 were collected, dialyzed and after spectrophotometric quantification used for further experiments (ε= 45000 M^{-1}cm^{-1}).

4.2. Purification of abb' fragment of ERp57

Protein corresponding to abb' domains of ERp57 was cloned using PCR into the expression vector pET21, expressed in E.coli BL21 and then purified as described in Chapter 3.

The cells were lysed by sonication using Ultrasonic Sonicatorand clarified lysate was fractioned by ammonium sulfate precipitation (50% and 75% saturation). Samples taken during the purification were analysed by SDS-PAGE to ensure that protein is actually present in the 75% saturation precipitate.
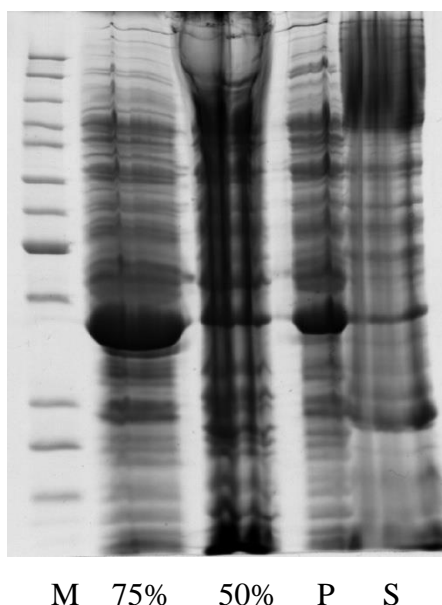

M   75%   50%   P   S

Figure 18. SDS-PAGE analysis (10% polyacrylamide gel) of fractions obtained after lysis. P: unsoluble proteins, S: soluble extract, 50% and 75% ammonium sulfate precipitation, M: protein markers

After resuspension of the pellet and dialysis, the solution was loaded onto the anion-exchange column Macro-Prep Q (Biorad), from which the greatest amount of abb' fragment was elute in 150 mM. Eluate was diluted with Tris HCl buffer to lower salt concentration and loaded on theAffi-Prep Heparin column which was then eluted with a NaCl gradient from 20 mM to 1 M in Tris HCl 20 mM pH 8.0 buffer.

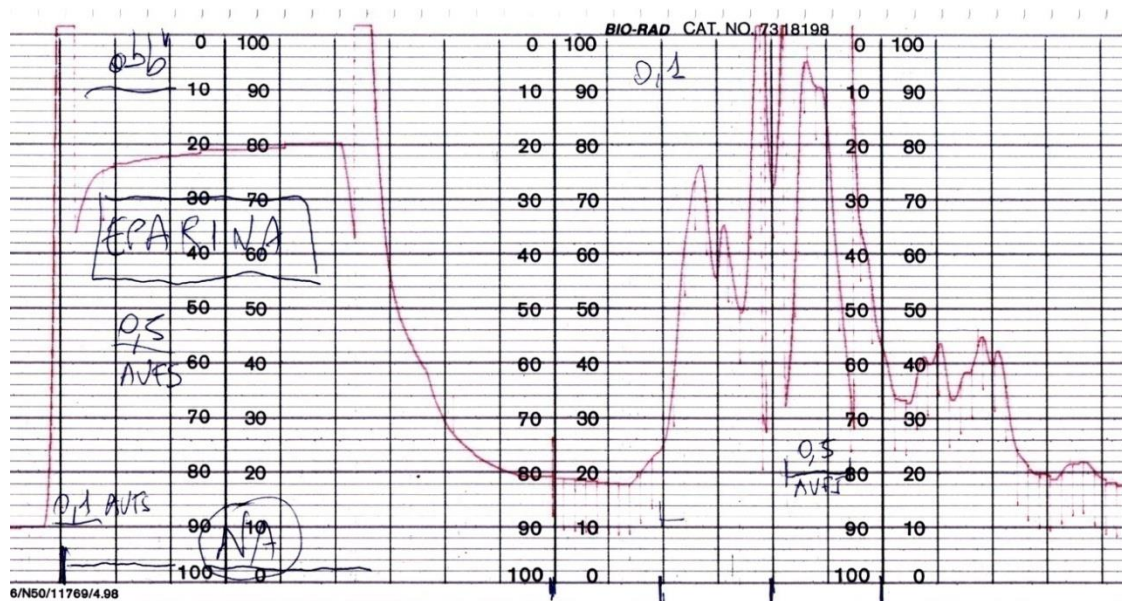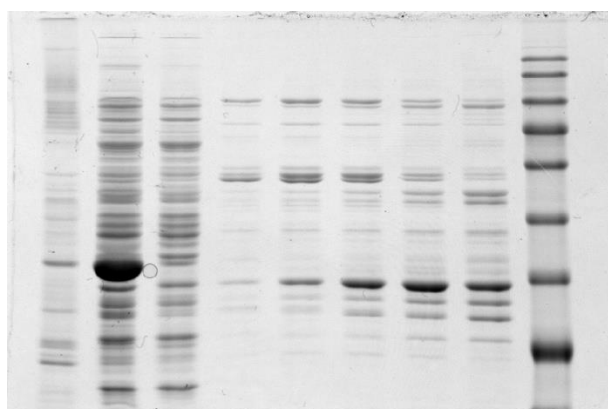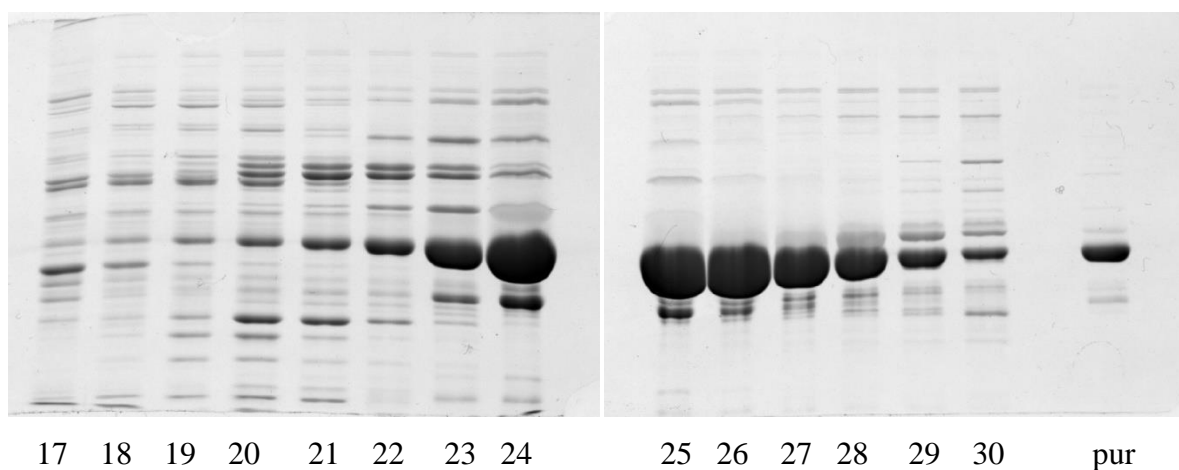Figure 19. Heparin chromatogram of elution of abb' fragment of ERp57

P   S   FT   12   13   14   15   16   M

17   18   19   20   21   22   23   24   25   26   27   28   29   30   pur

Figure 20. SDS-PAGE electrophoretic analysis (10%) of fractions eluted from the anion-
exchange column (P-pellet, S-supernatant, FT-flow through, pur-purified domain)

Electrophoretic analysis showed that the greatest amounts of protein corresponding to abb' fragment of ERp57 is bound to the heparin column and is eluted from fraction 24 to 28. These fractions were collected, dialyzed and after spectrophotometric quantification used for further experiments.

4.3. Purification of *a'* domain of ERp57

Protein corresponding to *a'* domain of ERp57 was produced by E.coli BL21 as described in Chapter 3. The cells were lyzed by sonication and the solution was precipitated in ammonium sulphate, first at 30% and subsequently at 75%.
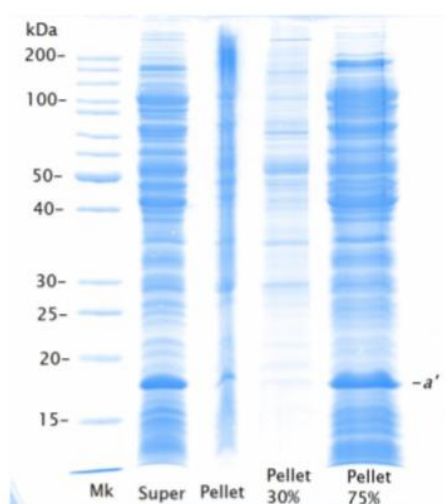

Figure 21. SDS-PAGE analysis (12% polyacrylamide gel) of fractions obtained after cell lysis and ammonium sulphate precipitation

In figure 21. it is demonstrated that the domain *a'* (which has a molecular weight of 18284 Da) is found in the pellet after precipitation at 75% ammonium sulphate. This pellet was resuspended in and dialyzed in the same buffer over the night to remove the ammonium sulphate. The dialyzed solution was centrifuged and the supernatant was passed on to anion-exchange Macro-Prep Q column.
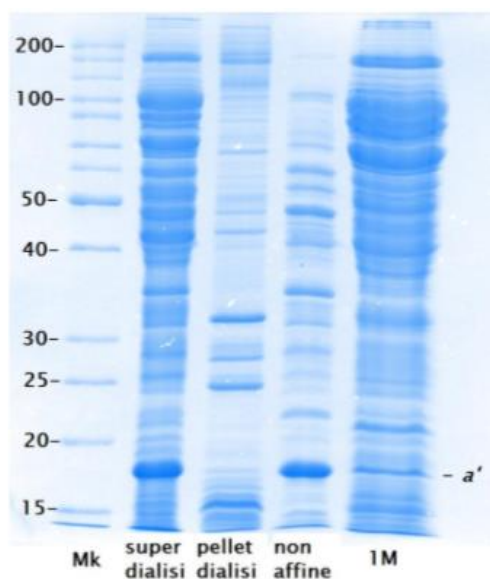
Figure 22.SDS-PAGE analysis after anion-exchange chromatography

As shown in Figure 22. the *a'* domain of ERp57 does not bind to the anion-exchange column so we find it in the flow through. The flow through is then passed over the Heparin Affi-Prep column and eluted with NaCl gradient from 20 mM to 1 M. The fractions were analysed by SDS electrophoresis.
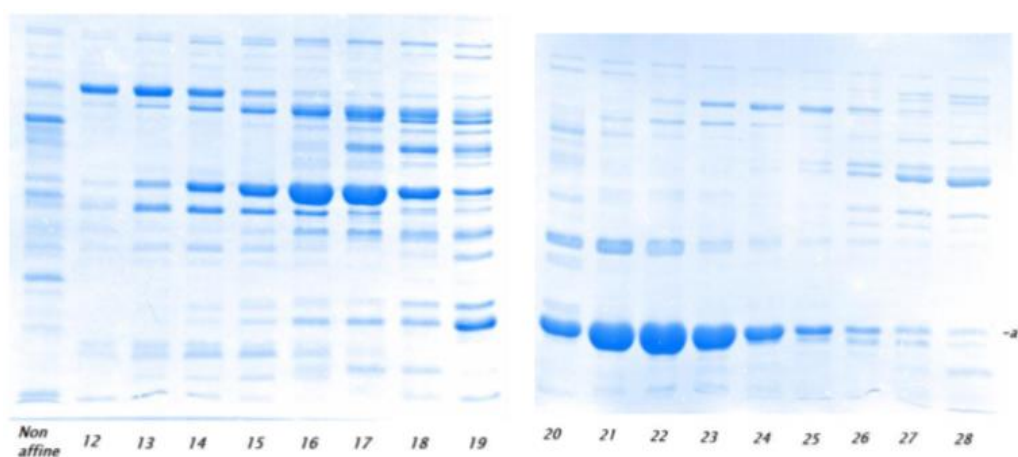

Figure 23. SDS-PAGE analysis (12% polyacrylamide gel after heparin column and gradient elution)

Electrophoretic analysis showed that fractions containing the highest amount of *a'* domain are 21, 22, 23. These fractions were collected, dialyzed and after spectrophotometric quantification($\varepsilon= 14565M^{-1}cm^{-1}$) used for further experiments.

4.4. Quenching effects of flavonoids in interaction with ERp57

To learn more about structural features relevant for interaction of flavonoids with ERp57, fluorescence spectroscopy analysis with ERp57 protein in reduced conditions was performed. To expand the analysis on flavonoids-ERp57 interaction already started in the laboratory, additional flavonoids which differs in size, substitutions and the presence of a sugar moiety were tested.

As mentioned in Chapter 3, ERp57 protein has an intrinisc fluorescence due to its three tryptophan residues (one in each redox active a and a' domains and one localized in the b' domain). Therefore fluorescence quenching studies may be used to determine the possible interactions between protein and other molecules. Moreover, to better analyze the interaction and obtain some quantitative parameters, fluorescence quenching of the protein was followed using different concentrations of flavonoids.

The quenching effect on protein intrinsic fluorescence was calculated using the following equation:

$$Q = 1 - \frac{F}{F_\circ} \times 100\%$$

Q: percentage of quenching

F: intensity of fluorescence in presence of quencher (flavonoids)

F_0: intensity of fluorescence in absence of quencher

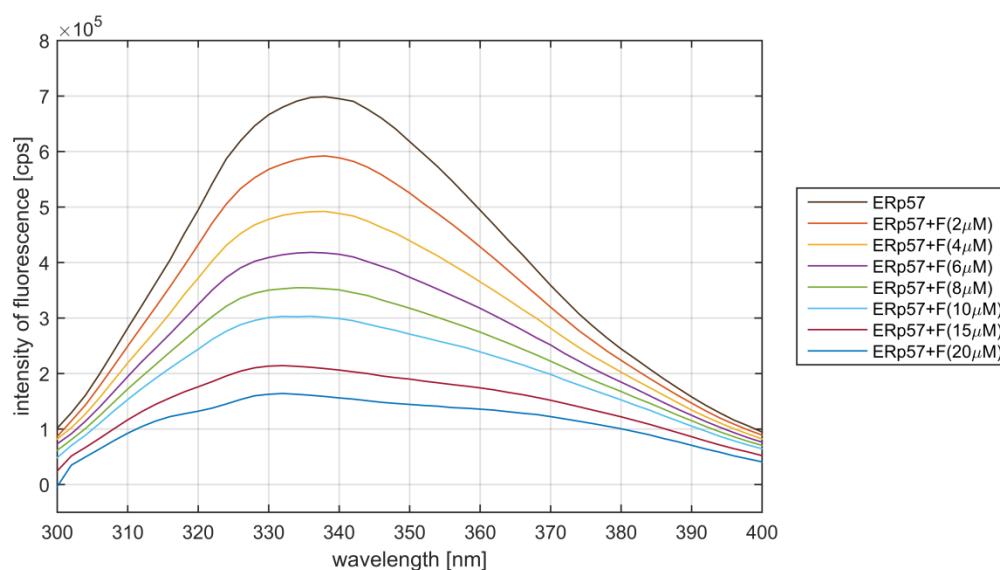
Figure 24. Decrease in fluorescence intensity of ERp57 with increasing concentrations of eupatorin (2, 4, 6, 8, 10, 15 and 20 μM).

In figure 26. it is demonstrated how increasing concentrations of eupatorin reduce the fluorescence intensity of the protein ERp57, highlighting in this way the existence of an interaction between the protein and the molecule. The same fluorescence analysis using increasing concentrations has been performed with all other flavonoid molecules.
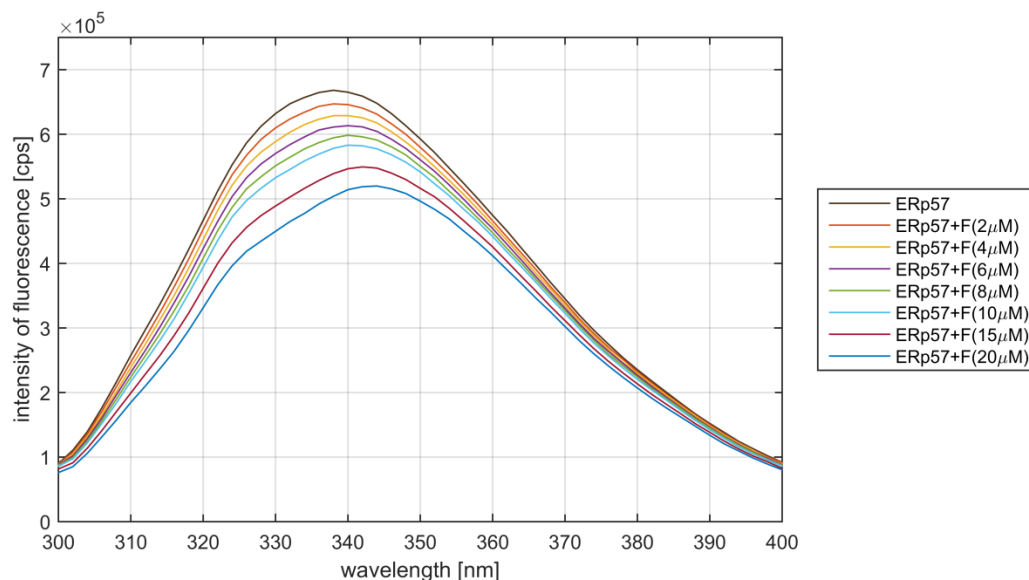

Figure 25. Decrease in intrinsic fluorescence intensity of ERp57 in presence of increasing concentrations of naringenin (2, 4, 6, 8, 10, 15 and 20 μM).

In figure 25. it is shown the effect of naringenin on fluorescence intensity of ERp57. It is apparent that naringenin has much less quenching effect.
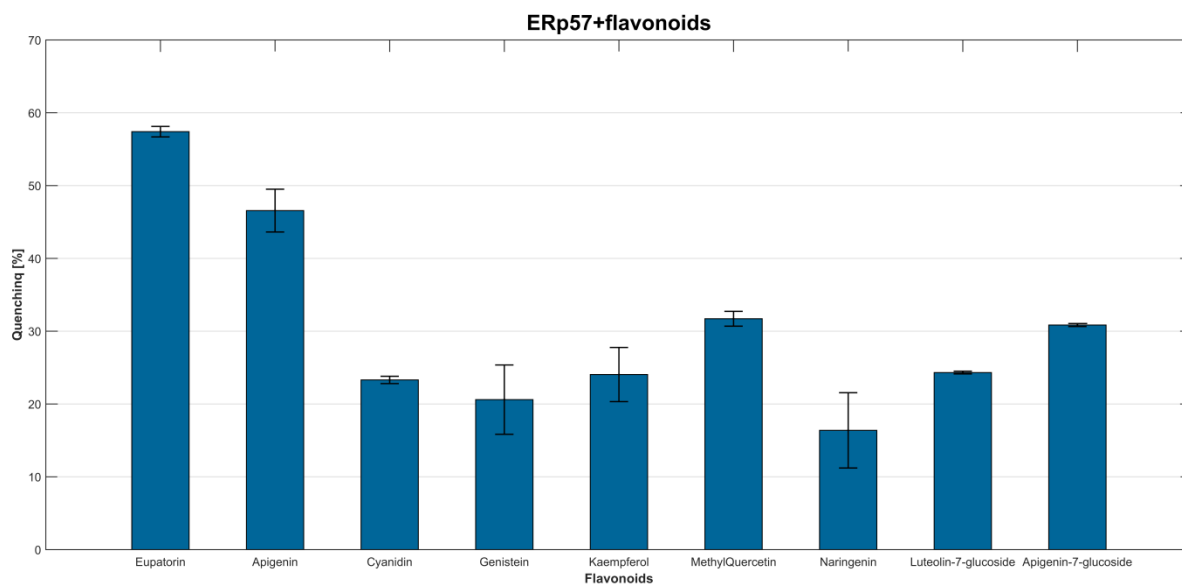

Figure 26. Comparison of quenching effect of different flavonoids (all at 20μM concentration) interacting with ERp57 (average of three measurements with error bars).

More specific data that quantify the interaction between the flavonoids and the whole protein ERp57 may be obtained by the Stern-Volmer equation, obtaining the **K$_{sv}$**(Stern-Volmer quenching constant), and by using a different derived equation that allows us to estimate the **K$_d$** (dissociation constant).

Stern-Volmer equation:

$$\frac{F_\circ}{F} = 1 + \text{Ksv } [Q]$$

[Q]: quencher concentration

F: intensity of fluorescence in presence of quencher (flavonoids)

F$_0$: intensity of fluorescence in absence of quencher
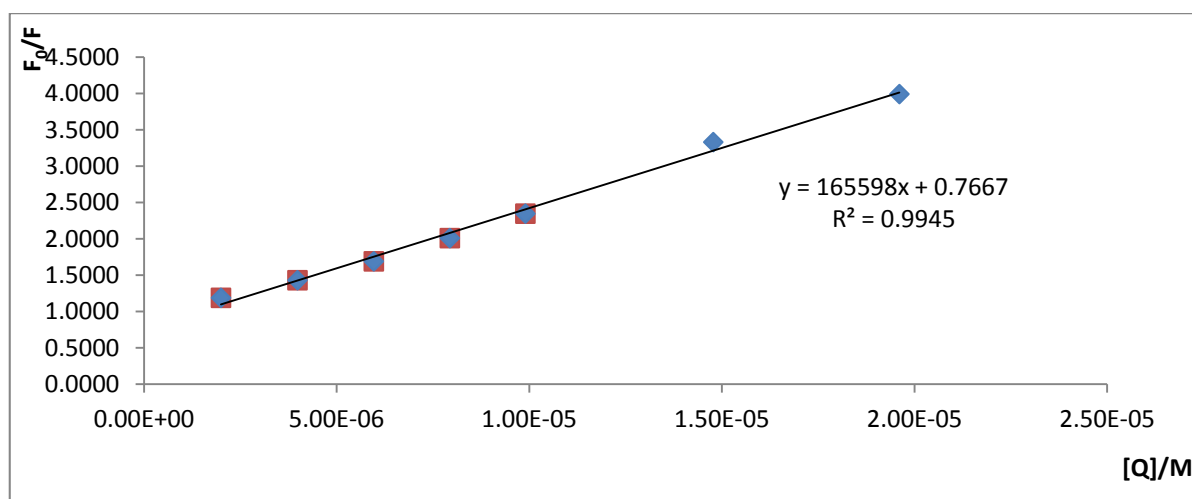
K$_{sv}$: Stern-Volmer quenching constant

Figure 27. Graphic analyses of data based on the quenching effect of eupatorin at increasing concentrations on ERp57 for calculating K$_{sv}$.

Equation used to calculate the dissociation constant (K$_d$):

$$\log_{10} \frac{(F_0 - F)}{F} = n \log_{10} K_a - n \log_{10} \frac{1}{[Q_t] - (F_0 - F)\frac{[P_t]}{F_0}}$$

F_0: fluorescence intensity in the absence of quencher

F: fluorescence intensity in the presence of quencher

K_a: binding constant (reverse from K_d)

$[Q_t]$: final concentration of quenching agent

$[P_t]$: final concentration of protein
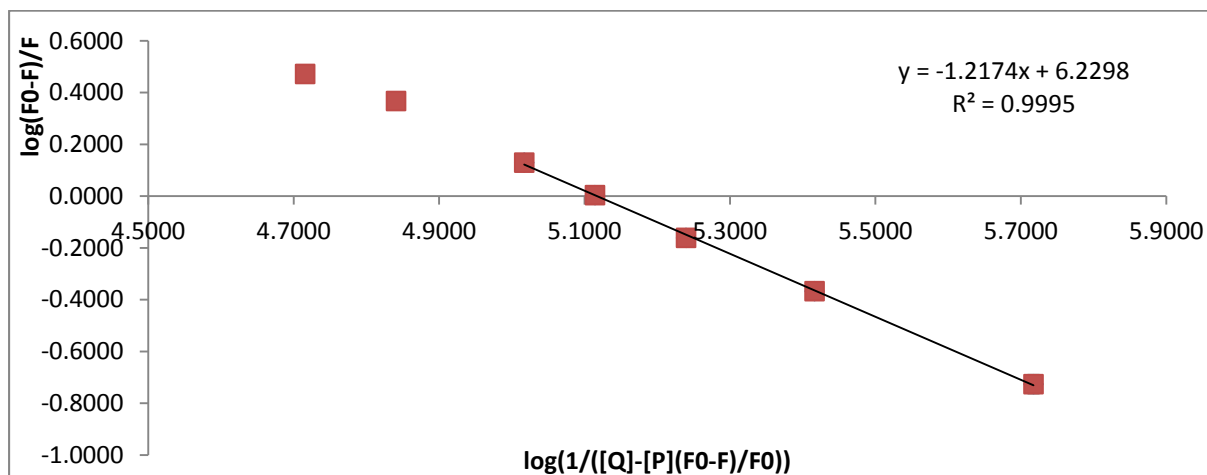
n: number of binding sites

Figure 28. Graphic analysis of data for calculating K_d

An important parameter that needs to be taken into consideration during this test is the concentration of the quencher. Some flavonoids show absorbance in the wavelength range used for quenching analysis. It is then possible that, at higher concentrations, they absorb part of light and/or emitted radiation and therefore alter the quantum of fluorescence and the shape of the spectrum. This phenomenon is due to the fact that the absorption occurs in the spectral range of overlap between the absorption and emission spectrum. To overcome this problem, solutions with absorbance less than 0.1 OD should be used. Therefore, the absorbance (at 290 and 338 nm) of flavonoids in highest concentration used in experiments (20 µM) was assessed. The obtained results showed that certain flavonoids (eupatorin, apigenin) at the highest concentration have absorbance values greater than 0.1 OD to at least one of two wavelengths. Thus, the value of the dissociation constant was calculated using only the first 5 concentrations of the molecules, that is up to 10 µM, where the absorbance of the flavonoid was lower.

Table 2. Summary table of binding parameters of ERp57 flavonoids interaction

	K_d (µM)	K_{sv} (M^{-1})	Quenching (%)
Eupatorin	7,6	16559	57,4
Apigenin	11,3	11893	46,6
Apigenin-7-glucoside	21,0	53477	30,9
3-O-MethylQuercetin	23,3	53821	31,7
Luteolin-7-glucoside	28,8	36634	24,3
Kaempferol	30,0	39349	24,0
Cyanidin	34,2	33682	23,3
Genistein	37,0	27326	20,6
Naringenin	48,2	19540	46,4

From obtained data arises that eupatorin and apigenin show the highest affinity for ERp57 (dissociation constant is the smallest) and naringenin shows the lowest affinity (dissociation constant is the highest).

4.5. Effects of flavonoids on protein activity

Catalytic activity of ERp57 requires binding and conformational changes of the substrate and all 4 domains of the protein are necessary to guarantee correct function of the protein. This process can be altered with the presence of small compounds such as flavonoids whose binding near the catalytic site can prevent correct interaction ERp57-substrate and that way reducing its activity.

Possible effect of flavonoids on reductase activity of the protein was tested using fluorogenic compound:dieosin glutathione disulfide. As described in Chapter 3, mixture consisting flavonoids and protein in buffer together with catalytic concentration of DTT and eosin-labeled glutathione was tested. Because of the reductase activity of ERp57 the disulfide bridge of glutathione is cleaved and this reduction causes an increase in fluorescence due to free eosin excitation at 525 nm.
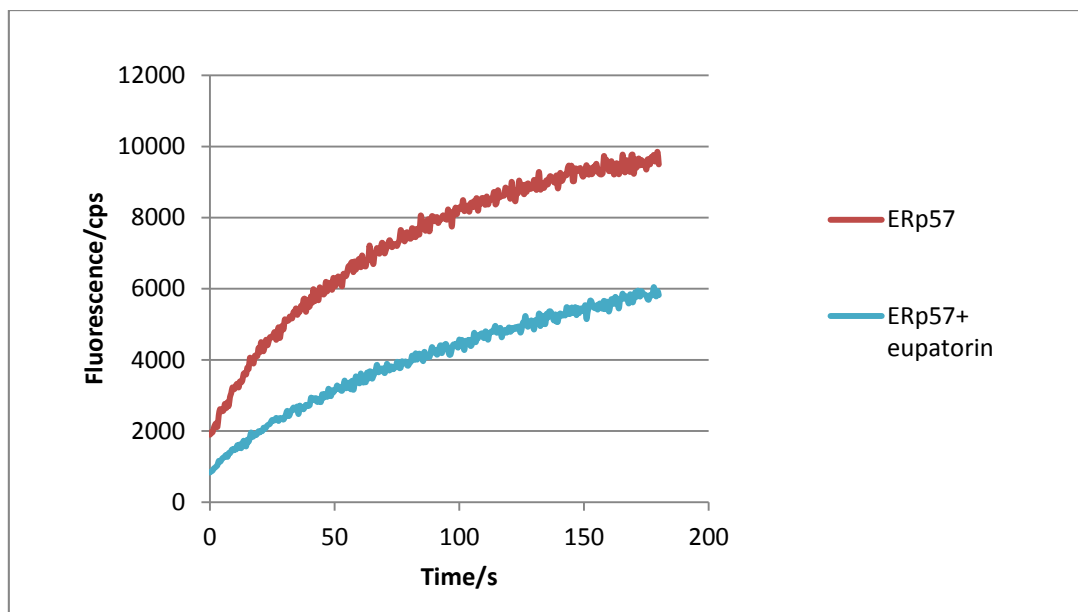
Figure 29. Decrease of fluorescence following the inhibiton of ERp57 reductase activity by eupatorin

It is evident that the rate of increase in fluorescence of dienosin glutathion disulfide in the presence of flavonoid in the incubation mixture is less than that observed for the protein ERp57 alone. This probably indicates that because of the interaction between flavonoid and the protein makes the latter is unable to reduce disulfide bond in the oxidized eosin-labeled glutathione and therefore the fluorescence emission due to the release of reduced glutathion linked eosin is lower. The increase of fluorescence is thus an indirect measure of the concentration of reduced glutathione which in turn is an indirect measure of the reductase activity of ERp57. Changes in fluorescence consequently indicate the possible effects, positive or negative, of the molecules on the catalytic activity of the protein.

The effects of flavonoids on the acitivity of protein ERp57 were tested at concentration of 20 µM for all molecules. The data were processed by extrapolating for each substance the slope of the tangent to the curve of fluorescence in a time interval from 0 to 15 seconds as shown in Figure 30.
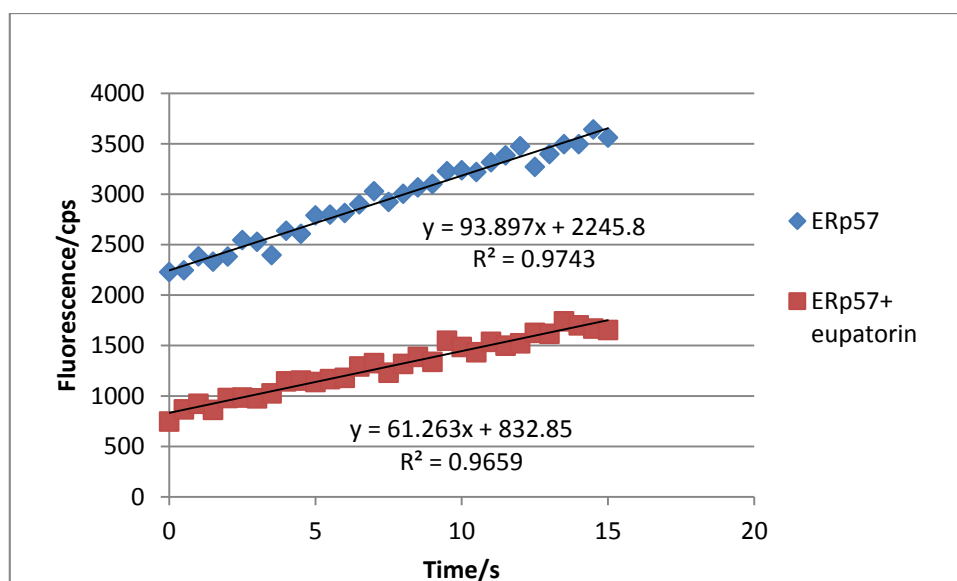
Figure 30. Linearization of the fluorescence curve in the first 15 seconds for the calculation of the slope of the tangent to the curve

From these data was calculated percentage of protein activity and reported in histograms with error bars.
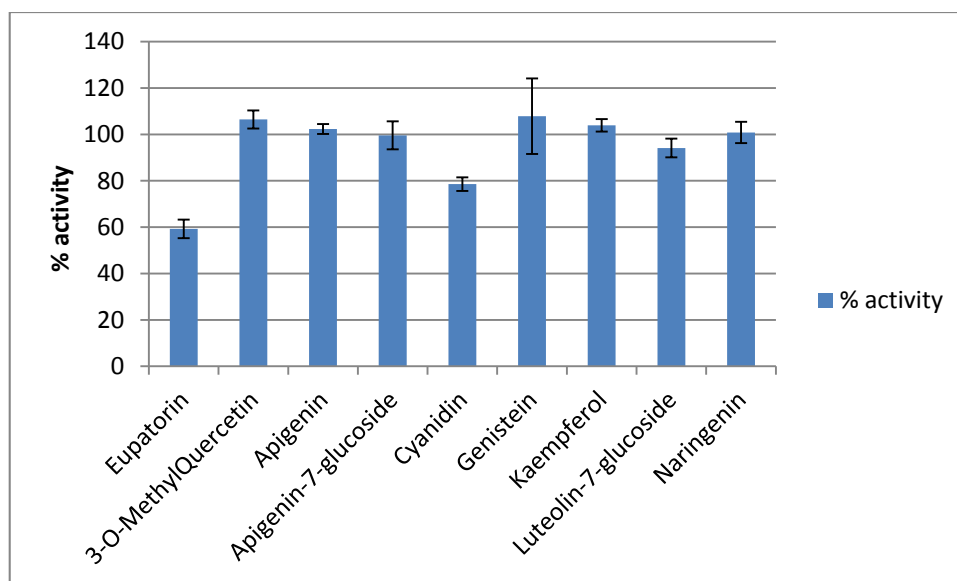

Figure 31. Comparison of flavnoid effect (20 μM) on ERp57 reductase activity

Unlike the quenching, in this case high concentrations of flavonoids did not interfere with the fluorescence analysis since data were recorded in visible range (in this case particulary at 525 and 544 nm), wavelengths at which these molecules don't give phenomenon of absorption. For this, no precautions were used during the calculation of the reductase activity.

4.6. Effects of flavonoids on protein stability

Another test carried out was to examine the effect of flavonoids on the protein stability. According to protocol given in Chapter 3, ERp57protein was treated with increasing concentrations of flavonoids in the presence of a fluorescent compound (SYPRO orange). The temperature at which a protein unfolds is measured by an increase in the fluorescence of a dye with the affinity for hydrophobic parts of the protein, which are exposed as the protein unfolds.

Temperature at which there is half of fluorescence increase is the melting temperature (T_m), which corresponds to the inflection point of the transition curve. T_m value can be calculated fitting data with the Boltzmann equation. An increase in the T_m was observed for ERp57 protein incubated in the presence of selected flavonoids, and the effect seems to be related to the flavonoid concentration. This observation support a stabilization effect of these substances on ERp57.
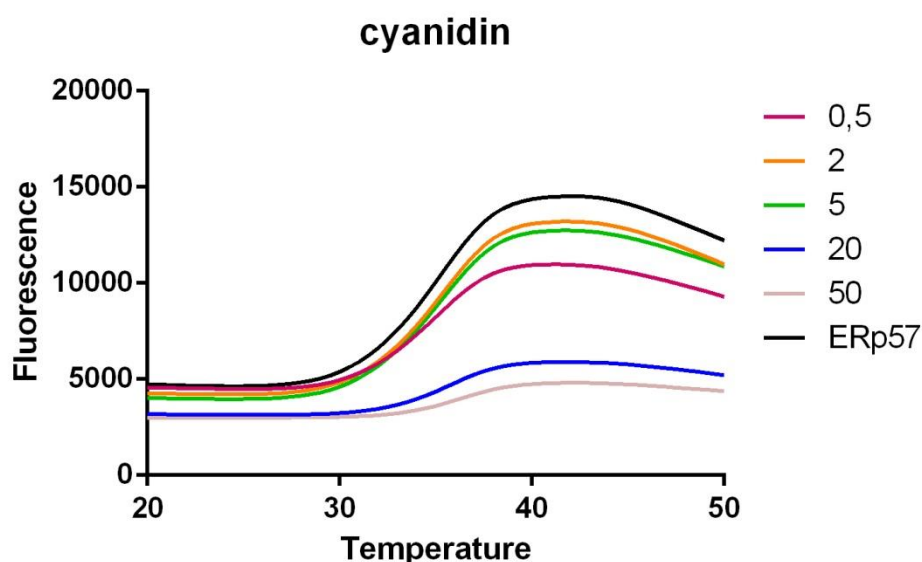

Figure 32. Changes in SYPRO orange fluorescence with the increase in temperature observed for the protein alone and in presence of different flavonoid concentrations (0.5, 2, 5, 20, 50 μM)

By analyzing data obtained in the test we can detect that some molecules show also a change in maximum fluorescence intensity when used in higher concentrations. We can hypothesize that these molecules could interact with multiple binding sites and that some of these sites are also close to or are the binding sites for the SYPRO. In this way SYPRO appeared unable to bind the protein and therefore the emission fluorescence appears reduced. However, further analysis of that phenomenon isn't possible because we don't have an estimate number of binding sites and their relative affinity.
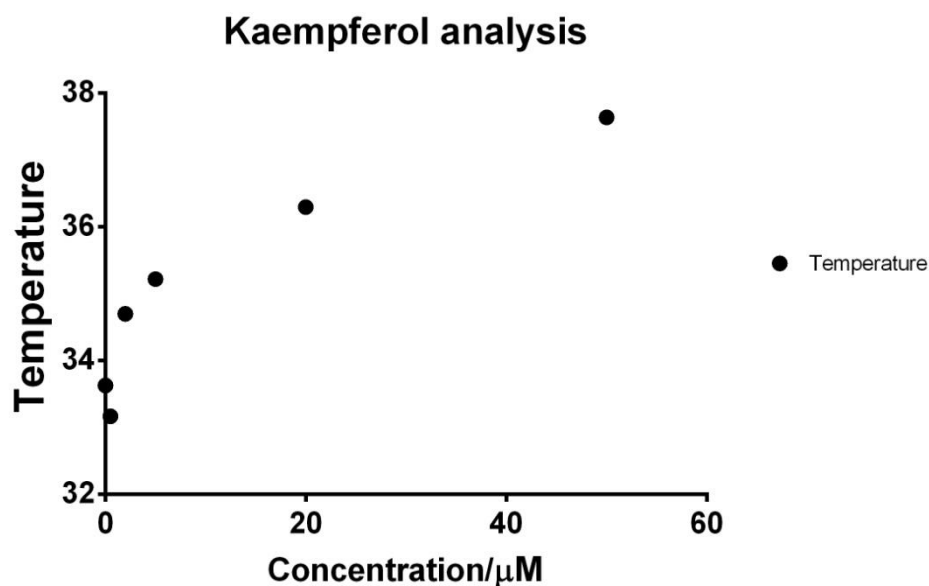
Figure 33. Increase in melting temperature (T_m) of ERp57 by increasing concentrations of kaempferol

Variations in melting temperature of ERp57 due to the effects of flavonoids are shown in Figure 34.
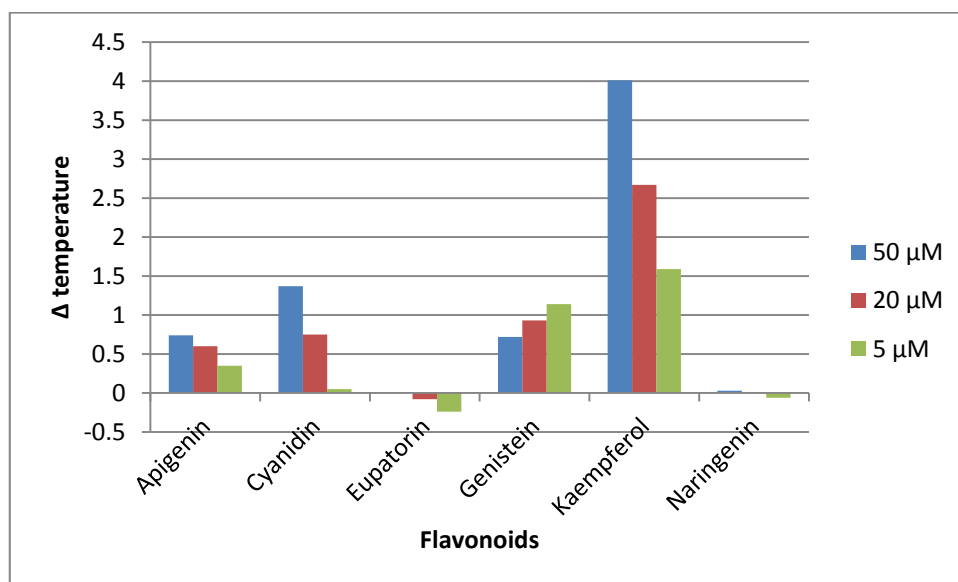

Figure 34. Effects of flavonoids in different concentrations on protein ERp57 stability

As demonstrated in diagram, kaempferol has the highest stabilizing effect, followed by cyanidin and apigenin. Naringenin and eupatorin didn't show stabilizing effect, furthermore, eupatorin even showed a slight destabilizing effect on the protein. Genistein is the only exception were melting temperature decreases with the increase of flavonoid concentration.
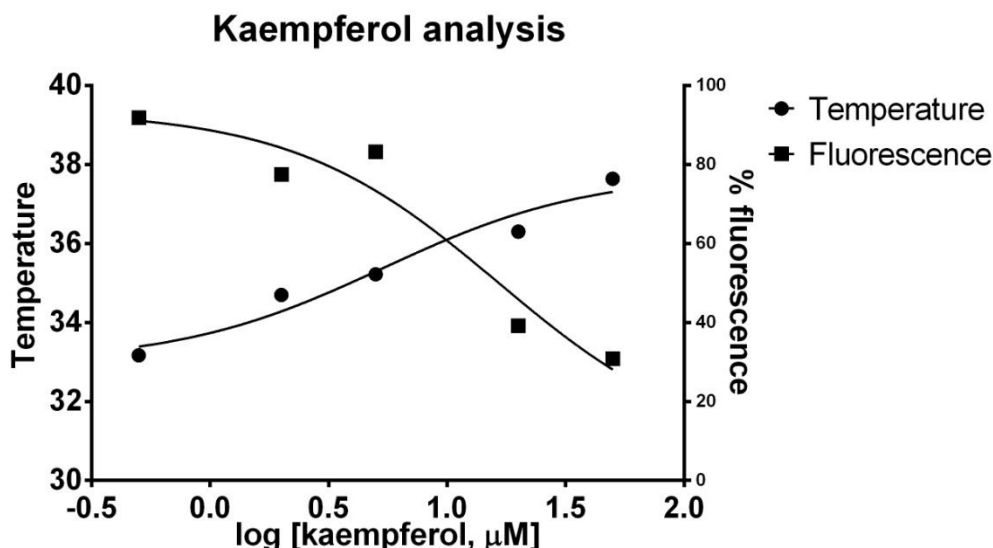
41

Figure 35.Comparison of temperature and fluorescence dependence on kaempferol concentrations

Looking into temperature and fluorescence trends gives better inside to flavonoid and SYPRO binding. Fitting with a log-dose response curve the observed melting temperature obtained from stability assay against the flavonoid concentration is possible estimate a $K_d$value that needs to be taken with caution for two reasons. First is that we don't know the exact number of binding sites for each flavonoid on the protein. We could guess from the fluorescence quenching data analysis but the equation assumes the same affinity for each binding site. Secondly, it is observed maximum fluorescence decreasing with the increasing of flavonoid concentrations which could mean that the flavonoids and SYPRO share the same binding sites on the protein.

Table 3. Comparison of K_d obtained by the DSF (K_{d1}) and quenching fluorescence (K_{d2})

	K_{d1} (µM)	K_{d2} (µM)
Apigenin	2,6	11,3
Cyanidin	43,0	34,2
Kaempferol	5,4	30,0

Moreover, fluorescence quenching assay experiments were performed at a constant temperature while the increase in the temperature in DSF analysis will result in a protein conformational change and new binding sites could be introduced. In any case, kaempferol not only stabilizes the protein but also interferes with SYPRO binding. In addition, Kd values

estimated from the DSF data analysis are in the same range as those obtained from fluorescence quenching data.

4.7. Effects of flavonoids on protein-DNA binding

The interaction between protein ERp57 and an 80 bp DNA fragment in the presence of all tested flavonoids was studied by EMSA as described in Chapter 3. The 80 bp DNA fragment was previously produced and its final concentration after purification was 50 ng/µL. 5 ng of DNA were used for each 10 µL incubation mixture, to yield a final concentration of 0.5 ng/µL, which was then loaded on a 5% polyacrylamide gel.

To analyze the interaction of the protein with DNA, it was carried out a test comparing the mobility of a free DNA solution with a DNA solution containing the a' domain, which contains the DNA binding site. To evaluate which molecules interfere with the DNA-binding activity of the protein, EMSA test with all molecules at 20 µM and then 50 µM was carried out.

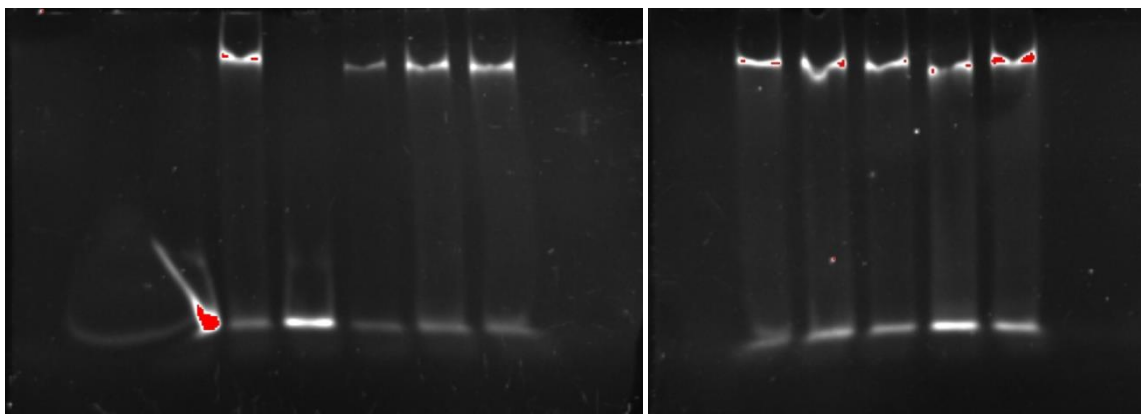Flavonoids tested were: genistein, naringenin, kaempferol, cyanidin, apigenin-7-glucoside, luteolin-7-glucoside.

Figure 36. Image obtained from EMSA assay. All flavonoids are in 20 µM concentrations. In order from the left to right are: DNA, DNA+a', DNA+a'+DTT, DNA+a'+genistein, DNA+a'+naringenin, DNA+a'+kaempferol, DNA+a'+cyanidin, DNA+a'+apigenin-7-glucoside, DNA+a'+luteolin-7-glucoside, DNA+a'+EGCG, DNA+a'+ellagic acid

As controls were used DNA fragment alone, to have a signal without shift due to protein binding, and the DNA fragment with the protein, to obtain a signal of DNA completely bound to the protein. For negative control, the protein was treated with DTT since protein reduction

abolishes its DNA binding activity. As it was previously demonstrated by Trnková at al. (2013), epigallocatechin gallate (EGCG) is able to interfere with DNA-domain a' interaction, with an 80% inhibition in the DNA binding activity, so EGCG was used as a positive control.

None of the tested flavonoids showed a significant DNA-binding inhibiton, and consequently a quantitave analysis wasn't performed. Knowing from previous research (Grillo et al, 2002) that binding site for DNA is localized on a' domain of the protein (a β-sheet before the active site), we can exclude this part of the protein as a binding site for the flavonoids.

5. Conclusion

In this thesis the importance of orientation of the B ring was tested. Apigenin has the B ring binded on C2 position while in genistein it is on C3. Also, it was tested the importance of free rotation of the B ring. In naringenin B ring has free rotation while it is blocked in cyanidin and genistein (cyanidin also has different C ring arrangement). Naringenin showed the highest dissociation constant (48 µM) and lowest binding affinity to ERp57. Genistein showed a slightly better K_d (37 µM) as well as cyanidine (34 µM). From these data we can conclude that a free rotation of the B ring, as well as a different position on the C ring, has a bad influence on binding of flavonoid to the protein. Apigenin is proved to be the molecule with the best binding abilities, with K_d=11 µM, and for this reason we can conclude that a B ring linked to C2 position and the absence of the free rotation are essential for binding to ERp57.

Furthermore, influence of additional hydroxyl group in position C3 was tested. As already mentioned, apigenin has a dissociation constant of 11 µM in contrast to kaempferol which showed 30 µM dissociation constant. This result confirms previous conclusion made in this laboratory that optimal number of hydroxyl groups for a better binding is 3.

Additionally, the importance of different methyl position was tested. Eupatorin, molecule with the lowest dissociation constant (7 µM) and best binding affinity has 2 hydroxyl- groups and 3 methoxyl- groups while 3-O-methylquercetin has 4 hydroxyl- and 1 methoxyl- showed a higher K_d (23 µM). From previous research quercetin (which has 5 hydroxyl- groups) showed a K_d equal to 23µM, so we can conclude that addition of onemethoxyl- group does not increase the binding affinity to ERp57. Probably the higher affinity observed for eupatorin is related to the presence of more than one methoxyl-group.

Finally, it was tested presence of a sugar moiety on A7 position. We previously observed that the addition of a sugar moiety on C3 position (rutin, isoquercetin and quercetin) does not have an effect on binding properties as compared to the corresponding un-glycosilated flavonoid, quercetin. In this case, apigenin-7-glucoside (K_d=21 µM) showed lower binding affinity than apigenin (11µM) while the affinity of luteolin-7-glucoside was even worse (29 µM). This can be explained by the fact that glucose, which is hydrophilic and rich in hydroxyl- groups, has a negative effect on flavonoid binding to the ERp57 protein. Considering that

luteolin-7-glucoside has one more hydroxyl- group than apigenin-7-glucoside, this is an agreement with the lower affinity observed for this molecule (29µM vs. 21 µM).

In conclusion, apigenin and eupatorin remain the lead compounds with the best properties for binding to ERp57. Further experiments with additional compounds, as well as Quantitative Structure-Activity Relationship (QSAR) analysis on all tested molecules, will provide information to define the best properties for binding with high affinity to ERp57 and identify compound to be used as a selective inhibitor/modulator of ERp57 biological activities.

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7. Sažetak/Summary

7.1. Sažetak

UVOD

Protein PDIA3 odnosno ERp57 je protein iz obitelji protein disulfid izomeraza (engl. *protein disulfide isomerase*, PDI) koja se sastoji od različitih proteina s višestrukom organizacijom domena. Strukturu ERp57 obilježava prisutnost četiriju domena nazvanih *a*, *b*, *b'* i *a'*. Domene *a* i *a'* sadrže tioredoksinu sličnu sekvencu aktivnog mjesta dok su domene *b* i *b'* redoks inaktivne. Trodimenzionalna struktura ERp57 pokazuje da protein ima oblik slova U, s katalitičkim, tioredoksinskim domenama koje su okrenute jedna prema drugoj. Zbog svoje šaperonske, redoks i izomerazne aktivnosti sudjeluje u smanjenju štetnih posljedica u staničnom stresu.

Flavonoidi su skupina prirodnih spojeva sveprisutnih u našoj prehrani. Široko su rasprostranjeni u biljkama (voće, povrće, mahunarke, žitarice) te u pićima poput čaja, vina i piva. Prema kemijskoj strukturi flavonoidi pripadaju velikoj skupini prirodnih polifenolnih spojeva kojima je svojstvena prisutnost hidroksilnih skupina na aromatskoj jezgri (polihidroksi fenoli). Derivati su 1,3-difenilpropana (C6-C3-C6). Flavonoidi pokazuju spazmolitski, diuretski, protuupalni i dijaforetski učinak. Njihovo djelovanje može biti i antialergijsko, antimikrobno, antiviralno, estrogeno, antihepatotoksično, te trenutno najistraživanije-antioksidativno.

OBRAZLOŽENJE TEME

Protein ERp57 je povezan s raznim patološkim stanjima i oboljenjima te ima potencijal biti terapijskom metom, posebice imajući na umu da može stupiti u interakciju s polifenolima koji su poznati po svojim pozitivnim učincima na zdravlje.

Cilj ovog diplomskog rada bio je istražiti vezanje odabranih flavonoida (eupatorin, apigenin, apigenin-7-glukozid, kempferol, naringenin, 3-O-metilkvercetin, genistein, cijanidin, luteolin-7-glukozid) s proteinom ERp57. U tu svrhu korištena je fluorescencijska spektrofotometrija i učinak gašenja fluorescencije. Nadalje, istražen je utjecaj flavonoida na disulfidno reduktaznu aktivnost proteina, njegovu stabilnost te interakciju ERp57-DNA.

MATERIJALI I METODE

Transformirane bakterijske BL21 (koristeći plazmidni vektor pET21 i pET29) stanice upotrijebljene su za ekspresiju proteina ERp57 i njegove *a'* domene. Nakon rasta jedna kolonija je prenesena te je prema protokolu provedena ekspresija i purifikacija proteina ERp57, odnosno domene *a'*. Kvaliteta purifikacije provjerena je SDS elektroforezom, a količina proteina određena spektroskopski.

Kvantitativne analize potencijalne interakcije između pojedinog flavonoida i ERp57 izvedene su fluorimetrijskom titracijom pomoću učinka gašenja fluorescencije.

Reduktazna aktivnost proteina ERp57 i utjecaj flavonoida na aktivnost proteina testirana je koristeći dieozin glutation disulfid. Reduktaznom aktivnosti proteina ERp57 dolazi do redukcije molekule dieozin gluation disulfida te povećanja fluorescenije.

Ispitivanje stabilnosti proteina i utjecaj flavonoida na njegovu stabilnost provedena je pomoću diferencijalne pretražne fluorimetrije koristeći real-time PCR instrument.

Utjecaj flavonoida na interakciju ERp57-DNA ispitan je elektroforezom (EMSA) pomoću fluoresceina koji se veže na DNA.

REZULTATI I RASPRAVA

Eupatorin i apigenin pokazali su najveći afinitet vezanja za ERp57 (K_d= 7,6 i 11,3μM redom), dok je naringenin pokazao najmanji (K_d= 48,2 μM).

Interakcijom eupatorina, cijanidina i luteolin-7glukozida sa ERp57 dolazi do smanjenja reduktazne aktivnosti proteina (59%, 78% i 94%), dok ostali flavonoidi nemaju značajan utjecaj na njegovu aktivnost.

Apigenin i cijanidin imaju stabilizacijski efekt na protein te su dobivene konstante disocijacije otprilike u istom rangu kao i one dobivene quenching fluorescencijom.

Ni jedan od testiranih flavonoida nije pokazao značajnu inhibiciju vezanja proteina ERp57 na DNA.

ZAKLJUČAK

U ovom radu potvrđena je važnost hidroksilne skupine za interakciju flavonoida sa proteinom ERp57 (max. broj za optimalno vezanje je 3).Također je uočena važnost nedostatka slobodne rotacije B prstena flavonoida za vezanje s proteinom. Dodatak šećera na A7 poziciju djeluje nepovoljno na vezanje flavonoida i proteina ERp57.

7.2. Summary

The protein PDIA3 or ERp57 (Endoplasmic Reticulum protein 57) is a member of the family of disulfide isomerase (PDI). The protein is mainly localized within the endoplasmic reticulum and has an established function as a chaperon and disulfide-rearrangements enzyme.

Flavonoids are a broad class of molecules characterized by flavone core. They are found in leaves, cortex, seeds and flowers of plants where they hace various functions. Most of their health benefits are attributed to their anti-oxidant and chelating abitily.

ERp57 is involved in various diseases and has a potential to be a pharmacological target. The aim of this thesis was to reach a deeper understanding of the interaction between ERp57 and selected flavonoids (eupatorin, apigenin, apigenin-7-glucoside, kaempferol, naringenin, cyanidin, genistein, 3-O-methylquercetin, luteolin-7-glucoside). The goal is to understand how different substitutions on the flavonoid basic structure can modify the interaction with the protein.

Using fluorescence quenching of ERp57 by flavonoids it was calculated dissociation constant. Eupatorin and apigenin showed lowest Kd (7,6 and 11,3μM respectively) and therefore highest affinity to bind ERp57.

Eupatorin, cyanidin and luteolin-7-glucoside decreased reductase activity of the protein, while other flavonoids didn't show significant effect on it.

Apigenin and cyanidin showed positive effect on protein ERp57 stability.

None of the tested flavonoids showed a significant DNA-binding inbibiton.

In this thesis the importance of hydroxyl group for flavonoid binding to ERp57 is confirmed. Also, it is noticed the importance of the lack of free rotation of the flavonoid B-ring to bind with the protein. Addition of sugar moiety to A7 position adversely effects the binding of flavonoids and the protein ERp57. Taking into account fluorescence quenching results and the effects on protein activity and stability, we can conclude that eupatorin and apigenin the compound with the best properties for binding to ERp57.

Temeljna dokumentacijska kartica

INTERAKCIJA FLAVONOIDA S PROTEINOM ERp57

Ines Kanski

SAŽETAK

U okviru ovog diplomskog rada istražena je interakcija između odabranih flavonoida (eupatorin, apigenin, apigenin-7-glukozid, kempferol, naringenin, 3-O-metilkvercetin, cijanidin, genistein, luteolin-7-glukozid) sa proteinom ERp57 koji spada u skupinu disulfid-izomeraza (PDI). Za procjenu vezanja flavonoida sa proteinom ERp57 korištena je fluorescencijska spektrofotometrija i učinak gašenja fluorescencije, dok je za ispitivanje utjecaja flavonoida na vezanje ERp57-DNA korištena migracijska elektroforeza (EMSA). Također je testiran utjecaj flavonoida na aktivnost i stabilnost proteina.

Apigenin i eupatorin pokazali su najmanju konstantu disocijacije, odnosno najveći afinitet za ERp57 te ih to čini najboljim molekulama za vezanje na protein ERp57. Potvrđena je važnost hidroskilne skupine za interakciju flavonoida sa proteinom.

TESTING THE INTERACTION OF FLAVONOIDS WITH PROTEIN DISULFIDE ISOMERASE PDIA3 AND THE EFFECTS ON PROTEIN BIOLOGICAL PROPERTIES

Ines Kanski

SUMMARY

In this thesis, interaction between a protein that belongs to the family of protein disulfide isomerases (PDI)- protein ERp57, and chosen flavonoids- eupatorin, apigenin, apigenin-7-glucoside, kaempferol, naringenin, 3-O-Methylquercetin, cyanidin, genistein and luteolin-7-glucoside was tested. Interaction between ERp57 and flavonoids was assessed using fluorescence quenching method, while electrophorectic mobility shift assay (EMSA) was used to evaluate flavonoid effect on ERp57-DNA interaction. Flavonoid influence on protein activity and protein stability was also tested.

Apigenin and eupatorin showed the lowest dissociation constant and the highest binding affinity to ERp57, making them the best compounds to be used as selective inhibitors/modulators of ERp57 biological activities.