PALACKÝ UNIVERSITY IN OLOMOUC

Faculty of Science

Department of Biochemistry

Purification of recombinant proteins from yeast expression system

DIPLOMA THESIS

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Abstrakt	Cílem předkládané diplomové práce byla studie exprese a purifikace enzymu cytokinindehydrogenasy (CKX, EC 1.5.99.12). Prvním krokem bylo naklonování vybraného genu (AtCKX2) do vhodného expresního systému. Gen byl exprimován ve kvasince *Pichia pastoris* pomocí expresního vektoru pPIC9K umožňujícího integraci genu ve více kopiích. Již dříve byly u *P. pastoris* popsány případy zvýšení exprese požadovaného genu pomocí tohoto systému. Vektor pPIC9K umožňuje izolaci transformantů s různým počtem insertů za účelem ověření, zda vyšší počet kopií rekombinantního genu zároveň vede ke zvýšení exprese proteinu. Výsledná úroveň exprese se u jednotlivých klonů obsahujících několik kopií rekombinantního genu lišila. Výsledky screeningu aktivity enzymu u jednotlivých klonů ukázaly 2,5-krát vyšší aktivitu v porovnání s referenčním klonem, jenž exprimoval stejný protein pomocí vektoru pGAPZaA zajišťujícího integraci pouze jedné kopie genu. Rekombinantní proteiny byly navrženy tak, že obsahovaly histidinovou kotvu na různých místech v sekvenci. Proteiny byly purifikovány pomocí různých chromatografických metod - chromatografie na základě hydrofobních interakcí - Octyl Sepharosa, iontoměničové chromatografie - High Q Sepharosa a afinitní chromatografie - Ni-NTA Sepharosa, identifikovány pomocí SDS-PAGE a MALDI-TOF a též byly porovnány jejich aktivity.
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Abstract	In presented diploma thesis, the expression and purification of the enzyme cytokinin dehydrogenase (CKX, EC 1.5.99.12) was studied. The first step in the protein expression involved specific cloning of the target gene (AtCKX2) into an appropriate expression vector. The gene was expressed in the *Pichia pastoris* expression system in the pPIC9K vector allowing multicopy gene integration. Multiple copy integration of recombinant genes in *Pichia pastoris* has been previously demonstrated to increase expression of the desired gene in some cases. The vector pPIC9K allows isolation of transformants with different number of inserts, in order to test whether increasing the copy number of your recombinant gene will lead to a subsequent increase in secreted protein expression. The results considering increased expression in clones with multiple copy integration of recombinant genes were diverse. Screening of the enzyme activity for clones with multiple copy integrations indicated 2.5 times higher activity when compared with the activity of the same protein expressed in the pGAPZαA vector (a reference clone with single gene integration). Recombinant proteins were designed to contain polyhistidine tags with different locations in the protein sequence. Proteins were purified with different chromatography methods - hydrophobic interaction chromatography – Octyl Sepharose, ion-exchange chromatography – High Q Sepharose and affinity chromatography – Ni-NTA Sepharose, identified with SDS-PAGE and MALDI-TOF methods and their enzyme activity compared.
Keywords	*Pichia pastoris*, protein expression, cytokinin dehydrogenase, multiple gene insertion
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I herewith declare that this diploma work has been written only by me, suggested and advised by my advisor Marta Kowalska, MSc., and resourced from the competent literature. All sources are cited, described and included in the bibliography.

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In Tuzla, March 2010.

I would like to express my sincere gratitude to my advisor Marta Kowalska, MSc., from the Division of Molecular Biology, Department of Biochemistry, Palacký University in Olomouc, for her advices, patience and extraordinary assistance during my studies and laboratory work.

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Contents

1. Summary

In presented diploma thesis, the expression and purification of the enzyme cytokinin dehydrogenase (CKX, EC 1.5.99.12) was studied. The first step in the protein expression involved specific cloning of the target gene (*AtCKX2*) into an appropriate expression vector. The gene was expressed in the *Pichia pastoris* expression system in the pPIC9K vector allowing multicopy gene integration. Multiple copy integration of recombinant genes in *Pichia pastoris* has been previously demonstrated to increase expression of the desired gene in some cases. The vector pPIC9K allows isolation of transformants with different number of inserts, in order to test whether increasing the copy number of your recombinant gene will lead to a subsequent increase in secreted protein expression.

The results considering increased expression in clones with multiple copy integration of recombinant genes were diverse. Screening of the enzyme activity for clones with multiple copy integrations indicated 2.5 times higher activity when compared with the activity of the same protein expressed in the pGAPZαA vector (a reference clone with single gene integration).

Recombinant proteins were designed to contain polyhistidine tags with different locations in the protein sequence. Proteins were purified with different chromatography methods - hydrophobic interaction chromatography (HIC) – *Octyl Sepharose*, ion-exchange chromatography – *High Q Sepharose* and affinity chromatography – *Ni-NTA Sepharose*, identified with SDS-PAGE and MALDI-TOF methods and their enzyme activity compared.

2. Souhrn

Cílem předkládané diplomové práce byla studie exprese a purifikace enzymu cytokinindehydrogenasy (CKX, EC 1.5.99.12). Prvním krokem bylo naklonování vybraného genu (*AtCKX2*) do vhodného expresního systému. Gen byl exprimován ve kvasince *Pichia pastoris* pomocí expresního vektoru pPIC9K umožňujícího integraci genu ve více kopiích. Již dříve byly u *P. pastoris* popsány případy zvýšení exprese požadovaného genu pomocí tohoto systému. Vektor pPIC9K umožňuje izolaci transformantů s různým počtem insertů za účelem ověření, zda vyšší počet kopií rekombinantního genu zároveň vede ke zvýšení exprese proteinu.

Výsledná úroveň exprese se u jednotlivých klonů obsahujících několik kopií rekombinantního genu lišila. Výsledky screeningu aktivity enzymu u jednotlivých klonů ukázaly 2,5-krát vyšší aktivitu v porovnání s referenčním klonem, jenž exprimoval stejný protein pomocí vektoru pGAPZaA zajišťujícího integraci pouze jedné kopie genu.

Rekombinantní proteiny byly navrženy tak, že obsahovaly histidinovou kotvu na různých místech v sekvenci. Proteiny byly purifikovány pomocí různých chromatografických metod - chromatografie na základě hydrofobních interakcí (HIC) - Octyl Sepharosa, iontoměničové chromatografie - High Q Sepharosa a afinitní chromatografie - Ni-NTA Sepharosa, identifikovány pomocí SDS-PAGE a MALDI-TOF a též byly porovnány jejich aktivity.

3. Aims of the work

- Selection of conditions for the growth of *Pichia pastoris* in the expression system producing recombinant CKX (medium, cultivation period).

- Purification and identification of differently His-tagged CKX enzymes (*N*-terminus, *C*-terminus, both ends).

- Screening for the best clones of the recombinant gene multiple copy integration into *Pichia pastoris* genome.

I. Theoretical part

4. Enzyme activity

Enzymes are substances known as extremely selective biological catalysts, which have the ability to highly and efficiently accelerate chemical reactions. Each cell in the living world is equipped with its own genetically determined set of enzymes. Many regulatory mechanisms depend on the presence of the enzymes, because they allow adaptation on the conditions changes. It is defined that almost all enzymes are proteins, but in the cells are also present „ribozymes“, catalytically active ribonucleic acids (Koolman & Rohm 2005). Enzymes have remarkable ability to transform one form of energy into another one. To execute an active function, some enzymes require presence of *cofactors*, small organic molecules, which can be either tightly or loosely bound (Stryer *et al*. 2002).

Enzymes are subjects to several important aspects that differentiate them from ordinary chemical catalysts. Reactions catalyzed by enzymes have factors of 10^6 to 10^{12} greater than corresponding uncatalyzed reactions, and greater value than chemically catalyzed reactions. Enzymes requires restrained conditions such as temperatures bellow 100°C, atmospheric pressure, and nearly neutral pH's, in difference to the chemical catalysis, which often requires increased those conditions values. Enzymatic reactions have the specific attribute that they rarely give side products. Presence of other substances than substrates affects the catalytic activities of many enzymes. Enzyme activity regulatory processes show specific regulatory means, which include allosteric control, covalent modification, and variation of the amounts of enzyme protein synthesized (Voet & Voet, 1995).

Different factors, such as genetic defects, toxins or nutritional deficits can result in deficiencies in the quantity or catalytic activity of the enzymes. Genetic mutations or viral and bacterial pathogenic infections can produce defective enzymes. Usage of the pharmaceutical agents can inhibit specific enzymes, and in the combination with the gene therapy, may be used to treat deficits in enzyme level or functions (Murray *et al.*, 2003).

4.1 Enzymes as catalytic activity factors

Temperature

Most of the enzymes in human organism show maximum activity and optimum function at a temperature of approximately 37°C. An increase of the temperature in a range from 0°C to 37°C increases the rate of the enzymatic reaction by increasing the vibration energy of the substrates. Loss of secondary and tertiary structure of the enzymes occurs at higher temperatures (Smith *et al.*, 2004).

pH

Maximal activity of the enzyme is detected at optimal pH, which is generally close to the pH of the environment, in which the enzyme is normally found: at higher or lower pH, enzyme activity decreases (Nelson & Cox, 2004).

Increase of the enzyme reaction rate is in the cases, where the pH goes from a very acidic level to the physiologic range. On the other way, decrease of reaction rate occurs as the pH value goes from the physiologic range to a very basic range (Fig. 1) (Smith *et al.*, 2004).

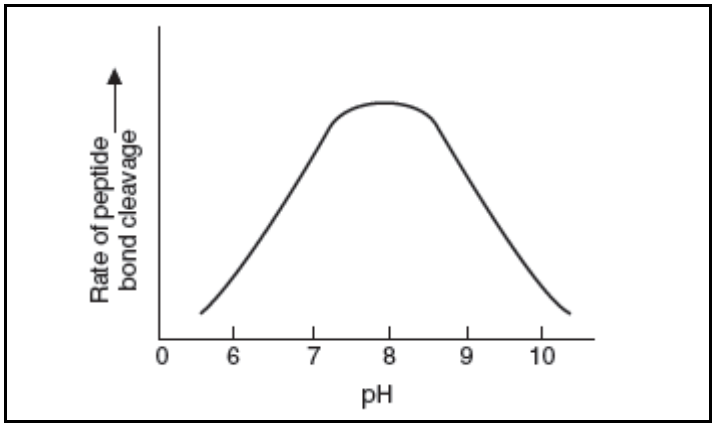

Figure 1. Relation between pH values and enzyme activity. Optimal pH value determines highest enzyme activity value (Smith *et al.*, 2004).

Enzyme inhibition

Enzymatic reaction rate can be changed and decreased with the compounds named *inhibitors*, the process being either reversible or irreversible. Group of the inhibitors, defined as mechanism-based inhibitors participate in an intermediate step of the enzymatic reaction. They can react irreversibly with the functional groups in the active site of an enzyme. Other group of inhibitors, covalent inhibitors covalently bond themselves with the functional groups in the active site. Transition state analogs, the specific groups of inhibitors have the ability to bind tightly to the enzyme than do substrates or products. Heavy metals, as inhibitors can be highly toxic, thanks to the tight binding to a functional group in the active site of an enzyme (Smith *et al.*, 2004).

Enzyme and substrate concentration

The reaction rates of all known enzymes are dependent on the concentration of the substrate. Some pathways are destitute of substrate in specific conditions such as starvation. In contrast, when more substrate is available, storage pathways (e.g. glucose conversion to glycogen in the liver) and toxic waste disposal pathways (e.g. the urea cycle, which prevents NH_4^+ toxicity by converting NH_4^+ to urea) are normally regulated in the way to speed up. Michaelis-Menten equation (Fig. 2) describes the response of an enzyme to changes in substrate concentration (Smith *et al.*, 2004).

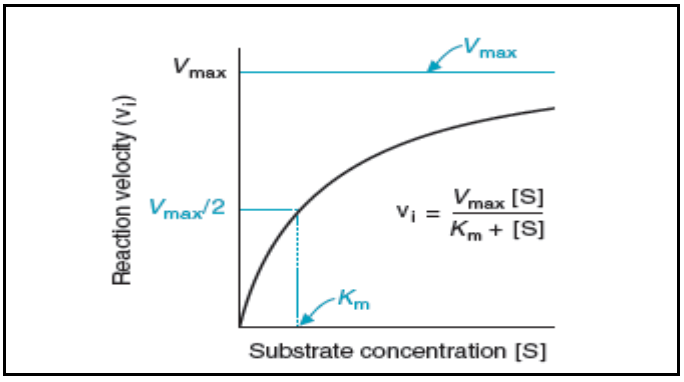

Figure 2. A graph of the Michaelis-Menten equation V_{max} (solid blue line) in the initial velocity extrapolated to infinite substrate concentration [S]. K_m (dashed blue line) is the concentration of [S] at which $v_i = V_{max}/2$ (Smith *et al.*, 2004).

Before the enzyme active sites are free to accommodate more substrate, the enzyme/substrate complex has to dissociate. In the conditions where the substrate concentration is high and temperature and pH kept constant, the rate of the enzymatic reaction is proportional to the enzyme concentration (Wrolstad *et al.*, 2000).

4.2 Enzyme activity assays

Enzyme assays are scientific procedures, based on the measurement of the rate of enzyme reactions. They usually follow changes in the concentration of either substrates or products to measure the rate of the reaction. There are many different laboratory and diagnostic methods of measurement, which have specific characteristics (Drobnič-Košorok, 1997).

Most of the enzyme assays give progress curves that remain nearly linear for only short periods of time. Very sensitive methods for detecting products are needed in these cases, such as spectrophotometric or fluorescence measurements, or the use of radioisotopically labeled substrates. One of the most sensitive approaches is to arrange the assay in the way that a product of the reaction serves as a catalyst for another enzymatic process, thus amplifying the amount of final product to be measured. Another approach is to measure rates of reactions in a very small volume, and continuously separate and measure products using capillary electrophoresis and fluorescence detection (Metzler, 2003).

Spectrophotometric assays are most eligible and most often used, since they allow the rate of the reaction to be measured and determined continuously. On the other side, radiometric assay are based on the removal and counting of labeled sample and have the ability to detect very low levels of enzyme activity. The most sensitive methods of enzyme analysis use specialized lasers, which detect changes in the fluorescence of cofactors during an enzyme's reaction mechanism. These modern methods give a new view of the kinetics and dynamics of single enzyme molecule. Enzyme assays characterized by typical sampling method are defined as *continuous assays* (continuous activity reading), and *discontinuous assays* (samples are taken, then the reaction is stopped and the concentration of substrates/products determined).

Fluorometric enzyme assay

For measuring the enzyme reaction, fluorometric enzyme assays use a characteristic difference in the fluorescence of substrate from product. These types of assays are in general more sensitive than spectrophotometric assays, but they can be affected by interference caused by contaminations, impurities and the instability of fluorescent compounds when they are exposed to the light. The examples of these assays are coenzymes NADH and NADPH. The reduced forms are fluorescent and the oxidized forms are non-fluorescent, therefore oxidation reactions can be followed as a decrease in the fluorescence and reduction reactions by an increase (Viht *et al.*, 2005).

Spectrophotometric enzyme assay

Spectrophotometric assays are characterized by measuring of the change in quantity of light that the specific assay solution absorbs. If this light is in the visible region (VIS) it is possible to detect a change in the color of the assay mixture (colorimetric assays). UV light is often used for the detection, since the common specific coenzymes NADH and NADPH absorb UV light in their reduced forms, but not in their oxidized forms. Many oxidoreductases that use NADH as a coenzyme are possible to assay by following the change in UV absorbance at a wavelength of 340 nm (Murray *et al.*, 2003), Fig. 3.

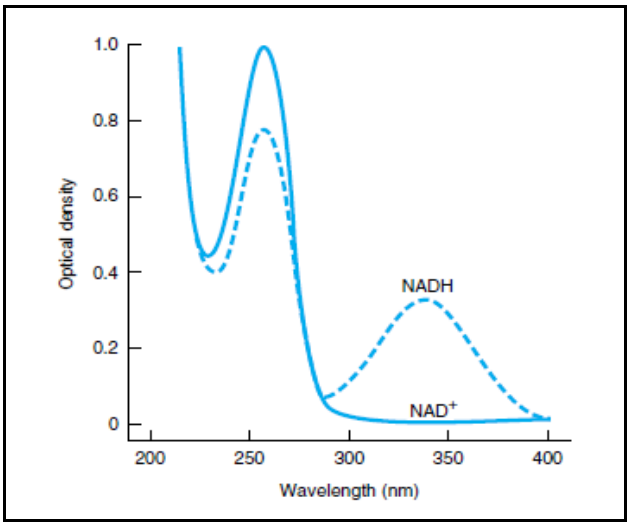

Figure 3. Absorption spectrum of oxidized (1) and reduced (2) form of coenzyme NAD^+. The reduced coenzymes NADH and NADPH absorb light at the wavelength of 340 nm, whereas their oxidized forms NAD^+ and $NADP^+$ do not (Murray *et al.*, 2003).

Calorimetric enzyme assay

Calorimetric assays are very general, because many reactions involve some change in heat and with use of a microcalorimeter, not much enzyme or substrate is required. Detection of the heat absorbed or released by chemical reactions, is the base of the enzyme assay. These assays are very useful, because they can be used to measure reactions that are impossible or inconvenient to treat in any other method. This assay is successfully used for penicillin G acylase, where the assay measure the formation of the acidic products of penicillin G hydrolysis by following the decrease in pH with indicator phenol red (Simmons & Gibson, 1999).

Chemiluminescent enzyme assay

This group of enzyme assays detects the emission of the light, caused by light-producing chemical reaction. Chemiluminescent type of assay can be highly specific and sensitive, since the produced light is captured by a photographic film or photomultiplier over short or longer period. These assays require specific separation of bound from unbound label and they are heterogenous. In contrast, a homogeneous assay requires the light-producing reaction to be affected with ligand binding to its receptor. On the other side, chemiluminescent assay can be more complicated to perform, comparing to the others types and enzymatic reaction can be hard to quantify, because not all the light released by a reaction will be detected.

Light scattering enzyme assay

As the assay procedure, this method is rarely used. Classical light scattering and dynamic light scattering are mostly used techniques, where classical method provides a direct measure of molecular mass and therefore is very useful for determining is the native state of a protein is a monomer or a higher oligomer. It is also useful for measuring the masses of aggregates or other non-native species. Dynamic method is useful for detection of the presence of very small amounts of proteins. Light scattering techniques are practical for the measurement of antigen-antibody reactions (Weetal & Gaigalas, 1993).

Radiometric enzyme assay

Radiometric enzyme assays determine characteristic incorporation of radioactivity into specific substrates, or the release of the radioactivity from them. The most commonly used radioactive isotopes in these types of assays are ^{14}C, ^{32}P, ^{35}S and ^{125}I. Radioactive isotopes have the characteristic phenomenon of the specific labeling of a single atom of a particular substrate. Usually, radiometric assay configuration depends on the availability of a satisfactory quantitative method of separating labeled product from unreacted substrate. That aspect makes these assays extremely sensitive, specific and free from interference. They are often the only way of measuring and determining a specific reaction in crude extracts (Eisenthal & Danson, 1992).

Chromatographic enzyme assay

One of the commonly used chromatographic methods, high-performance liquid chromatography (HPLC), is frequently used in laboratories for the determination of enzymatic activities. The product formed and leftovers of the substrate may be quantitatively determined by means of a specific internal standard. HPLC assay is selective and comparable with other traditional methods and the whole assay can performed in suitable time period (30 min or less per sample) (Pietta *et al.* 1987).

The usefulness of HPLC assay is not only in its power as a separation technique, but also in its diversity. The modes that are available, such as ion-exchange, and reversed and normal-phase, permit separation and measurement of the most complex samples. Therefore, HPLC has a tremendous potential for enzyme assays and therefore is often used in assays of various enzyme systems. One outstanding feature of the technique, in terms of its use for enzyme analysis, is that the change in substrate and product concentration can be measured concurrently (Pietta *et al.* 1987).

Polarimetric enzyme assay

Number of different types of enzymes and enzymatic reactions, including several peptidases, phosphohexose isomerase, glutamate decarboxylase, and

glutamate/alanine transaminases, can be detected and measured by polarimetry (Blass & Adams *et al.,* 1976).

The optical activity of complex molecules, such as biopolymers with multiple chiral and asymmetric centers, can be assayed with this method (Aktas *et al*., 2000).

Electrochemical enzyme assay

The electrochemical determination of dehydrogenase/oxidase activity has potential advantages over the conventional procedures: simplicity, economy, portability, speed, minimal sample volume and no requirement for secondary enzymes or toxic chemicals. Dehydrogenases/oxidases have widespread uses in different fields of science and technologies where their inherent biochemical selectivity have been combined with photometric (Kunst et al., 1983; Werner *et al.,* 1970) or electrochemical transduction systems (White et al. 1994, 1996; Nguyen *et al*., 1993) to develop highly reliable and sensitive assay procedures. In addition to their substrate specificity, these groups of enzymes are finding increasing uses as labels in immunoassay systems (Huet & Bourdillon, 1993; Skládal & Kaláb, 1995; Marco *et al*., 1995). Most current dehydrogenase/oxidase enzyme assay procedures rely on specific changes in the optical properties of the sample solution due to the oxidation of an added dye (Biozyme method, 1995). Oxidase enzymes usually conform to the following reaction pathway:

$$\text{substrate}_{ox} + H_2O + O_2 \xrightarrow{\text{oxidase enzyme}} \text{substrate}_{red} + H_2O_2$$

The use of a number of dyes whose redox state can be determined fluorimetrically has been evaluated (Guilbault *et al*.,1967). Hydrogen peroxide as a product provides electrochemical determination of the catalytic activity of many oxidoreductases. Monitoring of the current generated by the oxidation of hydrogen peroxide at the electrode poised at a suitable potential, the enzyme activity can be detected amperometrically. Guilbault (1976) has reviewed a number of electrochemical oxidase assay procedures, where most of them require the use of additional compounds other than substrate and oxygen.

Luong *et al.* (1994) have reported the use of a mediated system for the electrochemical sensing of glucose oxidase (GOX) in fermentation broths. The use of rhodinised-carbon electrocatalysts for the non-mediated detection of hydrogen

peroxide at low potentials is known (White *et al.,* 1994a, b). All these systems share common assay problems that arise from specific interactions of other redox components that may interfere (Kroger *et al.,* 1998).

5. Assays for oxidoreductase family of enzymes

Generally, the enzymes that have characteristic reaction of an electron transfer from one molecule to another are classified as *oxidoreductases.* These enzymes catalyze the oxidation reaction:

$$A + B^- \rightarrow A^- + B$$

Because of the presence of free electrons, most of the metabolic oxidation reactions involve removing hydrogen from the electron donor, so these enzymes are also defined as *dehydrogenases*. The term *oxidase* is used only for the enzymes, in which the oxidation reaction with molecular oxygen (O_2) participating as the electron acceptor occurs.

Enzyme Commision (EC) classifies oxidoreductases according to the substrate they utilize:

- EC 1.1 - Acting on the CH-OH group of donors
- EC 1.2 - Acting on the aldehyde or oxo group of donors
- EC 1.3 - Acting on the CH-CH group of donors
- EC 1.4 - Acting on the CH-NH_2 group of donors
- EC 1.5 - Acting on the CH-NH group of donors
- EC 1.6 - Acting on NADH or NADPH
- EC 1.7 - Acting on other nitrogenous compounds as donors
- EC 1.8 - Acting on a sulfur group of donors
- EC 1.9 - Acting on a heme group of donors
- EC 1.10 - Acting on diphenols and related substances as donors
- EC 1.11 - Acting on a peroxide as acceptor
- EC 1.12 - Acting on hydrogen as donor
- EC 1.13 - Acting on single donors with incorporation of molecular oxygen (oxygenases)

- EC 1.14 - Acting on paired donors, with incorporation or reduction of molecular oxygen
- EC 1.15 - Acting on superoxide as acceptor
- EC 1.16 - Oxidizing metal ions
- EC 1.17 - Acting on CH or CH_2 groups
- EC 1.18 - Acting on iron-sulfur proteins as donors
- EC 1.19 - Acting on reduced flavodoxin as donor
- EC 1.20 - Acting on phosphorus or arsenic in donors
- EC 1.21 - Acting on X-H and Y-H to form an X-Y bond
- EC 1.97 - Other oxidoreductases
- EC 1.98 - Enzymes using H_2 as reductant
- EC 1.99 - Other enzymes using O_2 as oxidant.

Dehydrogenase catalytic mechanism

Dehydrogenases transfer protons to a specific acceptor or coenzyme, small organic molecule, involved in the enzymatic catalysis, such as nicotinamide adenine dinucleotide (NAD^+ or NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$ or NADPH), flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). The wide diversity of dehydrogenases does not allow developing of the uniform catalytic mechanism for all cases. For each new dehydrogenase structure, the specific reaction mechanism should be solved (Murray *et al.*, 2003).

Continuous assays

Continuous assays give a continuous reading of a value that is proportional to the enzyme activity. The most used and applied continuous assays are spectrophotometric, fluorometric, calorimetric, chemiluminescent and light-scattering assays. Enzyme-catalyzed reaction is monitored as it proceeds, in difference over one, in which the enzyme reaction is run for a fixed time and then stopped before measuring formed products.

Discontinuous assays

Discontinuous types of assays are characterized by specific procedure: first, the samples are taken and the reaction is stopped and then the concentration of substrates/products is determined. Samples may be taken from an enzyme reaction at specific intervals and the amount of substrate or product consumption measured. Most used discountinuous assays are spectrophotometric, radiometric and chromatographic assays.

Radioactive assay

These assays are extremely sensitive and specific, because of the specific labeling of a single atom of the substrate. Typical assays of this type have been developed for enzymes acting on plant hormone cytokinin and auxin, and their antagonists (Kamínek *et al.,* 1997). Radioisotopic method often requires chromatographic separation of reaction products and residual substrates (Laloue & Fox, 1989).

Methods for conducting assays for enzyme activity on protein microarray-related applications

This modern procedure relates to methods of using modern protein chips to assay various characteristics of enzymes such as presence, amount, activity and/or function of enzymes present in a protein sample on a protein chip. The method uses a microarray, in which the protein and the substance are immobilized on the surface of a solid support in such a proximity to each other sufficient for the occurrence of an enzymatic reaction (Zhou & Schweitzer, 2005).

5.1 Specific examples of oxidoreductase assay

Alcohol dehydrogenase assay

Alcohol dehydrogenases (EC 1.1.1.1), Fig. 4, are the group of enzymes that enable the interconversion between alcohols and aldehydes or ketones with the reduction of NAD+ to NADH. In organism, they serve to break down alcohols, which could otherwise be toxic. In yeast and many bacteria, some alcohol dehydrogenases catalyze the opposite reaction as a part of fermentation process (Hammes-Schiffer & Benkovic, 2006).

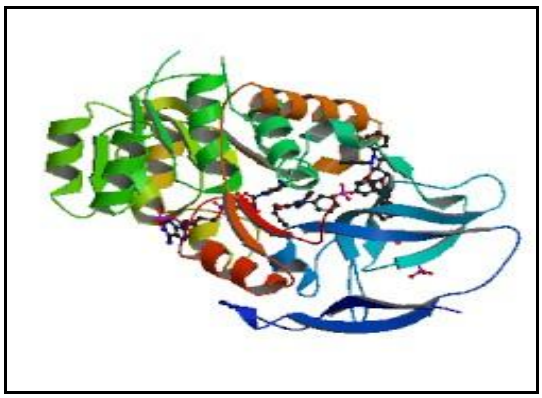

Figure 4. Crystal Structure of Human Zinc-Binding Alcohol Dehydrogenase 1 (PDB structure 2W98)

The reaction rate is often determined by the method of Vallee and Hoch (1955), in which the rate of absorbance at 340 nm resulting from reduction of NAD^+ is measured. One unit of the enzyme activity reduces one micromole of NAD^+ per minute at 25°C under the specified conditions.

Aldehyde dehydrogenase assay

Aldehyde dehydrogenases (E.C. 1.2.1.3), are enzymes that catalyze the oxidation (dehydrogenation) of aldehydes. Typical example is a liver aldehyde dehydrogenase (Fig. 5) responsible for the oxidation of aldehydes to carboxylic acids, which then leave the liver to be further metabolized in muscles and heart (Wierzchowski *et al.* 1996). In mammals, different classes of aldehyde dehydrogenase

enzyme exist: class 1 - cytosolic, class 2 - mitochondrial, and class 3 – less specific, expressed in tumors, stomach and cornea. All these enzymes are present in many tissues of the organism, but the highest concentration is in the liver (Crabb *et al.* 2004).

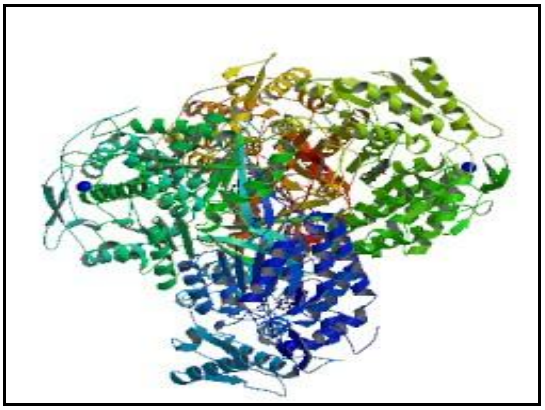

Figure 5. Human Mitochondrial Aldehyde Dehydrogenase complexed with agonist Alda-1 (PDB structure 3INJ)

Reaction mechanism:

$$\text{Acetaldehyde} + NAD^+ \xrightarrow{\text{Aldehyde dehydrogenase}} \text{Acetic acid} + NADH$$

A typical method of the assay is based on continuous spectrophotometric rate determination of the conversion of NAD^+ to NADH at 340 nm (Bostian & Betts, 1978).

Cytokinin dehydrogenase assay

Cytokinin dehydrogenase (CKX), also known as cytokinin oxidase (CKO), plays an important role in regulating the levels of cytokinins, plant hormones, which affect diverse processes such as cell differentiation, apical dominance, and senescence (Schmülling *et al.,* 2003).

Cytokinin dehydrogenase (EC 1.5.99.12) is classified as the enzyme that catalyzes the reaction shown in Fig.6 (Frébortová *et al*., 2004).

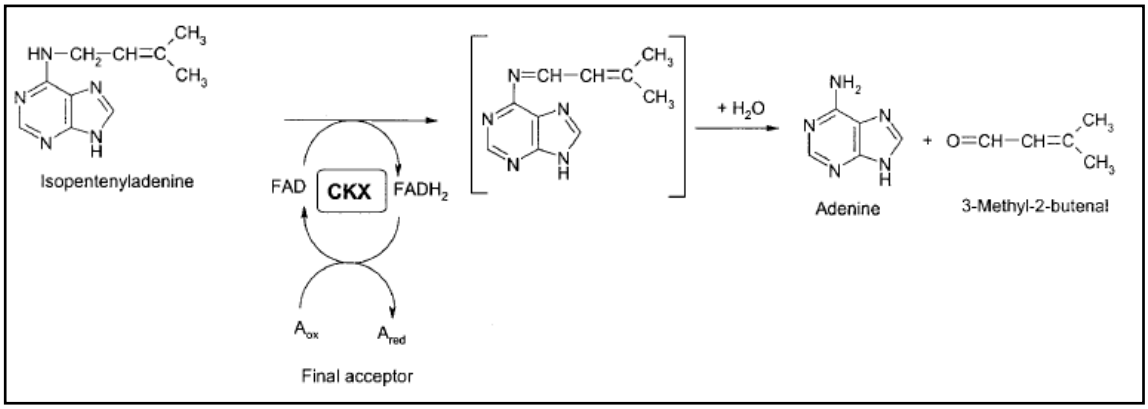

Figure 6. Chemical reaction catalyzed by cytokinin dehydrogenase (Frébortová *et al*., 2004).

CKX is a flavin adenine dinucleotide-containing oxidoreductase that specifically cleaves unsaturated N6-side chains from isopentenyladenine, zeatin and their derivatives. CKX is responsible for the metabolic inactivation of cytokinins in all plant species (Mok & Mok, 2001).

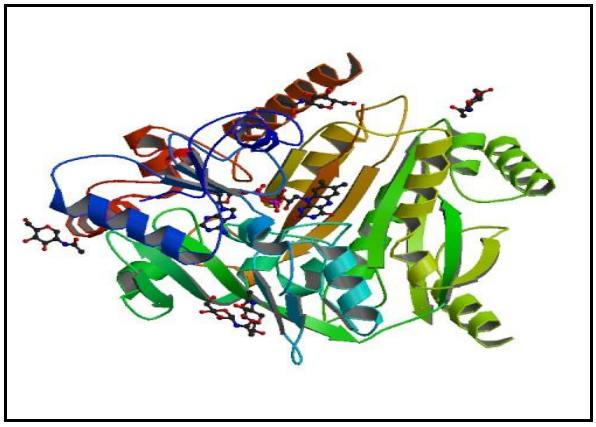

Figure 7. Structure of native cytokinin dehydrogenase
(PDB structure 1W1O)

Radioisotopic assay

Classical assay involves radioisotopic method, which requires chromatographic separation of reaction products and residual substrates (Laloue & Fox, 1989). The method was further optimized by Motyka and Kamínek (1994). The assay of CKX activity is based on the conversion reaction of [2,8-'H]-isopentenyladenine to adenine. Separation of the substrate from the product is possible by thin-layer chromatography

(TLC) on microcrystalline cellulose plates developed with 4 : 1 : 2 (v/v/v) mixture of ethylacetate : *n*-propanol : water (Motyka & Kamínek, 1994).

Libreros-Minotta and Tipton colorimetric assay

It is a simple and sensitive colorimetric assay for cytokinin dehydrogenase with Cu^{II}/imidazole as the electron acceptor suitable for assaying enzyme activity in crude plant cell extracts and at specific steps in protein purification. This assay is a typical spectrophotometric end-point method. The assay is based on the specific formation of a Schiff base between the reaction product 3-methyl-2-butenal and *p*-aminophenol added after stopping the enzymatic reaction with trichloroacetic acid. The assay is also effective in the submicromolar concentration range (Libreros-Minotta & Tipton, 1995).

Assays of cytokinin dehydrogenase use the 0.6 ml total volume solutions containing 100 mM imidazole, pH 6.5, 1 mM $CuCl_2$ and 40 µM isopentenyladenine. The catalytic reaction is started by addition of the enzyme, and the reaction is incubated at 30°C for 1h or less - depending on the activity of the enzyme preparation. Reactions stopped with the addition of 0.3 ml at 40% (w/v) trichloroacetic acid (TCA) and 0.2 ml *p*-aminophenol dissolved in 6% TCA. The reaction mixture is incubated at room temperature for 10 minutes, centrifuged, and the absorbance at 352 nm measured against blank that excludes substrate (Libreros-Minotta & Tipton, 1995).

Modified method of Libreros-Minotta and Tipton and continuous assay

The above widely used assay for cytokinin dehydrogenase enzymes can be modified for other substrates that isopentenyl adenine that give different products with *p*-aminophenol (Frébort *et al.*, 2002).

When other electron acceptors than Cu^{II}/imidazole are used, they allow measuring the activity in other buffers at variable pH values. The dyes 2,6-dichlorophenol indophenol (DCPIP) and phenazine methosulfate/MTT were first used for both the monitoring the enzymatic reaction continuously by the color change (Bileyu *et al*. 2001; Frébort *et al.* 2002) or assaying the reaction product discontinuously with *p*-aminophenol (Frébort *et al.* 2002). Another described continuous assay uses phenazine methosulfate coupled to nitroblue tetrazolium (NBT) as electron acceptor (Mahesh, 2001).

Another commonly used electron acceptor that works very well with the *p*-aminoaldehyde assay is 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0) (Frébortová *et al.* 2004).

Glutamate dehydrogenase assay

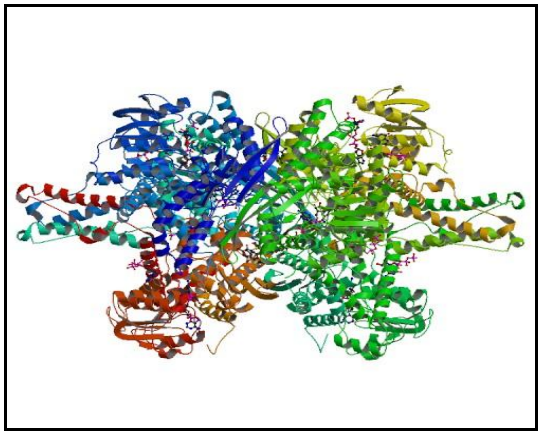

Figure 8. Structure of glutamate dehydrogenase complexed with bithionol (PDB structure 3ETD)

Glutamate dehydrogenase (EC 1.4.1.2, GMD), Fig. 8, is an enzyme, typically present in the mitochondria of eukaryotes. It is essential for urea synthesis, specifically converting the glutamate to α-ketoglutarate. The produced ammonia is usually routed to the urea cycle. The enzyme is abundant in liver, heart, and kidney. It catalyzes the following reversible reaction:

$$\text{2-oksoglutarate} + \text{NADH} + NH_4^+ \rightleftharpoons \text{L-glutamate} + NAD^+ + H_2O$$

Although normally the activity is low in serum, it is increased in various forms of liver damage and the measurement has useful and important diagnostic potential. Serum GMD activity can be measured by NADH formation at 340 nm. ADP or L-leucine increase activity of the enzyme. Decrease in activity at acidic pH was observed (Ellis & Goldberg, 1972).

Glycerol-3-phosphate dehydrogenase assay

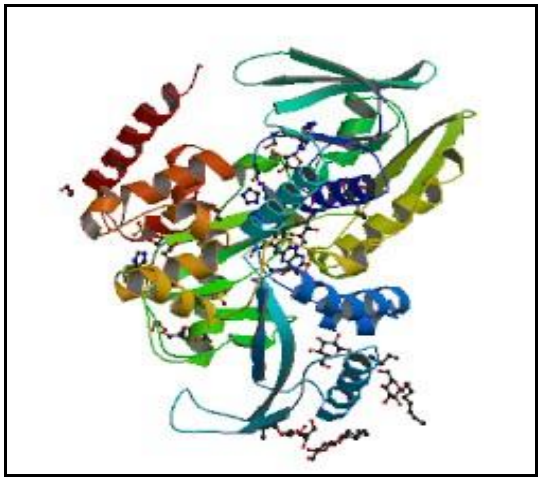

Figure 9. Crystal structure of Glycerol-3-phosphate dehydrogenase from E. coli (PDB structure 2QCU)

Glycerol-3-phosphate dehydrogenase (GPDH - EC.1.1.1.8), Fig. 9, is an enzyme that catalyzes the oxidation of dihydroxyacetone phosphate to glycerol 3-phosphate in following reaction:

$$\text{dihydroxyacetone phosphate} + \text{NADH} + H^+ \rightarrow \text{glycerol-3-phosphate} + NAD^+$$

Glycerol 3-phosphate in the liver, is created by direct phosphorylation of glycerol, in the muscles and adiopocytes being generated by reducing dihydroxyacetone phosphate, intermediate product in the glycolytic pathway, with NADH (Kozak & Birkenmeier, 1983).

Isocitrate dehydrogenase assay

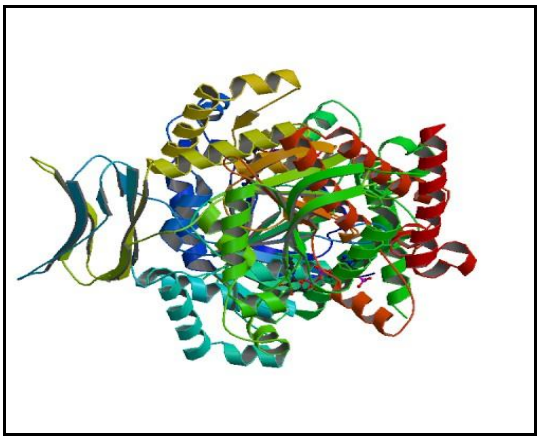

Figure 10. Crystal structure of human cytosolic NADP(+)-dependent isocitrate dehydrogenase in complex NADP (PDB structure IT09)

Isocitrate dehydrogenase (EC 1.1.1.42; EC 1.1.1.41, IDH), Fig. 10, is a ubiquitous enzyme found in all living organisms and having the function in citric acid cycle. This enzyme has two specific catalytic activities: hydrogens removal from its substrate isocitrate in addition to removal of the CO_2 from the six-carbon substrate to generate a five-carbon product α-ketoglutarate (Kornberg, 1995) (Fig. 11).

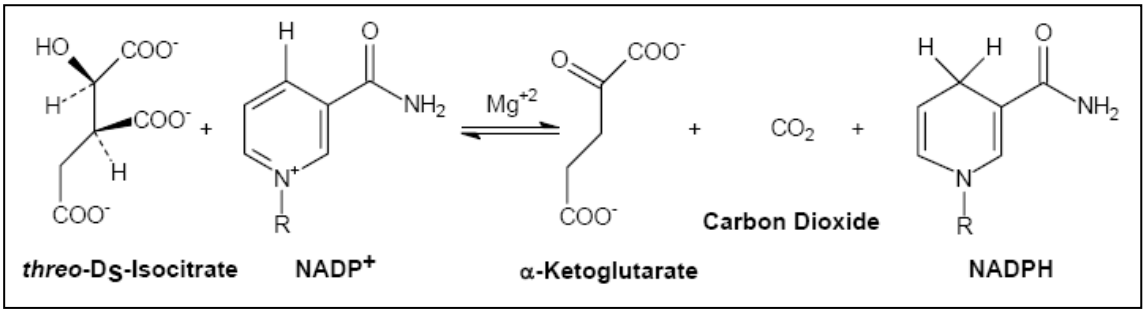

Figure 11. Reaction mechanism of the decarboxylative oxidation of *threo*-D_S-isocitrate (2*R*-3*S*-isocitrate) by $NADP^+$ (Kornberg, 1995).

The $NADP^+$-dependent form of IDH is found in bacteria and cyanobacteria, and in higher organisms, this form appear in all organs and tissues. The NAD^+-dependent form of IDH is limited to mitochondria of eukaryotic organisms. Both forms require a divalent metal ion (Williamson & Campbell, 1999). Typical activity assay (Kornberg

assay) is based on the increase in absorbance at 340 nm associated with the reduction of $NADP^+$ to NADPH (Kornberg, 1955).

Lactate dehydrogenase assay

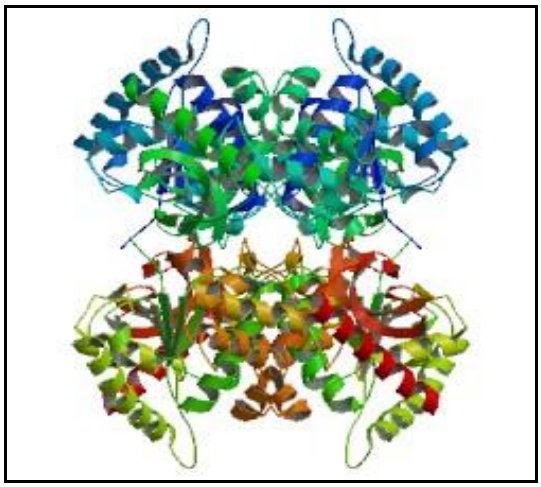

Figure 12. Crystal structure of Lactate dehydrogenase from *Staphylococcus aureus* (PDB structure 3D0O)

Lactate dehydrogenase (EC 1.1.1.27, LDH) catalyzes an interconversion of pyruvic acid and lactic acid, and it is found in many types of cells. Different specific enzyme forms of LDH are known:

- LDH-1 (4H) - in the heart
- LDH-2 (3H1M) - in the reticuloendothelial system
- LDH-3 (2H2M) - in the lungs
- LDH-4 (1H3M) - in the kidneys
- LDH-5 (4M) - in the liver and striated muscle.

An assay method is again based on the reaction rate determination by a decrease in absorbance at 340 nm resulting from the oxidation of NADH (Pagana, 1998).

Malate dehydrogenase assay

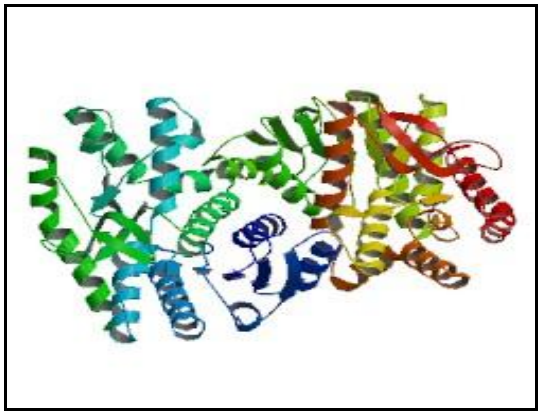

Figure 13. Structure of Malate dehydrogenase open conformation (PDB structure 3HHP)

Malate dehydrogenase (MLD) is present in all eukaryotic cells as two specific isozymes: mitochondrial (mMLD) and cytoplasmic (sMDH). In difference, prokaryotes contain only a single form of MDH. MDH activity is usually assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. The pH optimum for the reaction is 7.4-7.5 (Hayer-Hartl, 2000).

Pyruvate dehydrogenase assay

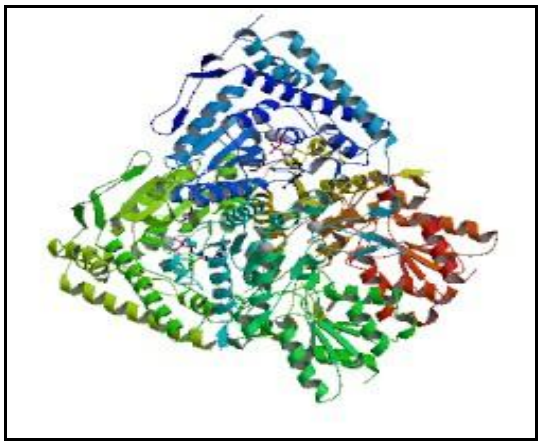

Figure 14. Crystal structure of the pyruvate dehydrogenase (E1p) component of human pyruvate dehydrogenase complex (PDB structure 3EXE)

Pyruvate dehydrogenase, (E1) is one of the components of pyruvate dehydrogenase complex (PDC). The pyruvate dehydrogenase complex is responsible for transformation of pyruvate into acetyl-CoA by a specific process defined as the pyruvate decarboxylation (Schwab, 2005).

A spectrophotometric assay is based on the measurement of the NADH production by coupling it to the reduction of a tetrazolium dye via an intermediate electron carrier (Hinman, 1981). The transfer of reducing equivalents from NADH to the dye under the condition when the intermediate electron carriers are diaphorase or phenazine methosulfate is rapid and effectively irreversible, even in the presence of lactate dehydrogenase and pyruvate.

A standard NADH assay which measures formation of NADH at 340 nm is usualy used to measure pyruvate dehydrogenase complex in mitochondrial preparations (Hinman, 1981).

Succinate dehydrogenase assay

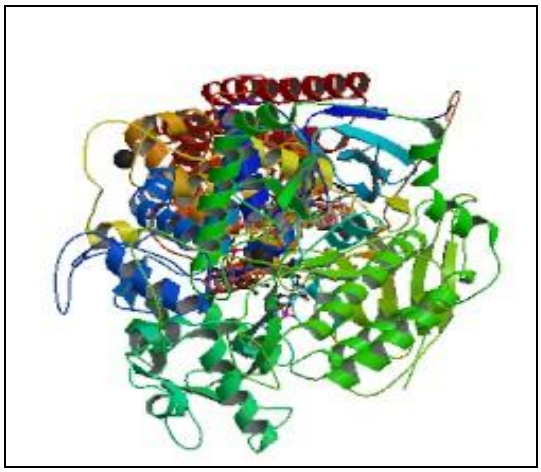

Figure 15. The crystal structure of human SSADH (Succinic semialdehyde dehydrogenase) in complex with SSA (PDB structure 2W8Q)

Succinate dehydrogenase (succinate-coenzyme Q reductase SQR, Complex II) is an enzyme complex specifically bound to the inner mitochondrial membrane of mammalian cells and many bacterial cells. Characteristically, it is the only enzyme that

participates in both - the citric acid cycle and the mammalian, mitochondrial, and bacterial electron transport chain (Oyedotun & Lemire, 2004).

There is one mitochondrial-specific property that is easily assayed - the ability of the enzyme succinate dehydrogenase to reduce the dye 2,6-dichlorophenol indophenol (DCPIP). Succinate oxidation to fumarate requires an active succinate dehydrogenase, located within the inner membrane. Normally, in the absence of exogenous dyes, the reaction occurs with a concurrent reduction of the coenzyme FAD. During an assay, an excess concentration of a dye (usually DCPIP) is used to observe its reduction. Reduction of a dye during the reaction specifically decreases the absorbance at 600 nm (it turns from blue to colorless) (Schnaitman & Greenwalt, 1968).

II. Experimental part

6. Materials and methods

6.1. Biological material

The following *Pichia pastoris* strains were used:

Pichia pastoris strains	Genotype
GS 115	*his4*
SMD 1168	*his 4, pepA*

Vectors and clones used:

1. pGAPZαA - reference clone used for cloning of *C-terminus* tagged CKX2 (CKX2-His) and both ends tagged CKX2(His-AtCKX2-His)
2. pPIC9K - possible multicopy gene integration, vector used for cloning of *N*-terminus tagged CKX2 (His-AtCKX2).

Yeast strains and expresssion vectors were obtained from Invitrogen (Carlsbad, CA, USA).

6.2. Chemicals

- Acrylamide, antifoam A, Coomasie Brilliant Blue R-250, bromphenol blue, formamid, sodium chloride, sodium hydroxide, *p*-aminophenol, glass beads (500 μm), ammonium peroxodisulfate, isopentenyladenine, isopentenyladenosine, mercaptoethanol, phenol-chloroform-isoamylalcohol 125:24:1 pH 8, TEMED, Triton X-100, yeast extract from *Sigma* (St. Louis, MO, USA),
- imidazol, potassium phosphate, Bradford reagent, Macro-Prep High Q Support from *Bio-Rad* (Hercules, CA, USA),
- sodium dihydrophosphate, acetic acid, ammonium acetate, trichloroacetic acid, sodium carbonate from *Lachema* (Brno, Czech Republic),
- chloroform, formaldehyde from *LachNer* (Munich, Germany),
- 2.6-dichlorophenol indophenol from *Loba Chemie* (Fischamend, Austria),
- dimethylsulfoxide from *Panreac* (Barcelona, Spain),

- ethidium bromide, Tris, Tween-20, silver nitrate from *NeoLab* (Heidelberg, Germany),
- agarose LMT from *Cambrex* (Charles City, IA, USA),
- ethanol, methanol, butanol, glucose, sodium chloride, potassium chloride, hydrogene peroxide, trichloroacetic acid, EDTA, glycine from *Penta* (Prague, Czech Republic),
- Page Ruler Protein Lader 10-200 kDa from *Fermentas* (Vilnius, Lithuania),
- peptone, Tris, from *Duchefa* (Haarlem, Netherland),
- yeast nitrogene base (YNB) from *Difco* (Lawrence, KS, USA),
- NiNTA agarose from *Qiagen* (Hilden, Germany).
- RNase from *Top-Bio* (Prague, Czech Republic).

6.3 Instruments

Centrifuges: Mikro 200R, Mikro 120, Rotanta 460R, Hettich Zentrifugen (Tuttlingen, Germany) and Centrifuge MR 23, Jouan (Saint-Herblain, France); shakers: E-class Thermo Scientific (Dubuque, IA, USA) and Orbital shaker 420 (ThermoForma, Marietta, OH, USA); R'ALF Plus Fermentor, Bioengineering AG (Wald, Switzerland); chromatography device, Biologic LP, Bio-Rad (Hercules, CA, USA); vortex Yellowline TTS 2, IKA Works Inc. (Staufen, Germany); Thermomixer Comfort, Eppendorf AG (Hamburg, Germany); electrophoresis accessories from Bio-Rad Laboratories, (Hercules, CA, USA) and Biometra - Analytik GmbH (Göttingen, Germany); spectrophotometers: Agilent 8453, Agilent (Palo Alto, CA, USA) and WPA Lightwawe II, Biochrom (Cambridge, England); camera C-5060 Wide Zoom, with AlphaDigiDoc™ software, Olympus (Tokyo Japan); magnetic mixer RH-KT/C, IKA Works (Staufen, Germany); pH/mV/ºC meter Cyberscan, Eutech Instruments pH 510 (Vernon Hills, IL, USA); Real-Time PCR system StepOnePlus™, Applied Biosystems (Foster City, CA, USA); Spectrum device "Minicros sampler", Barnant Co. (Barrington, IL, USA) and other standard laboratory equipment and tools were used.

6.4 Methods

6.4.1 Protein purification

The recombinant enzymes AtCKX2 (three differently His-tagged AtCKX2 enzymes - *N*-terminus, *C*-terminus, both ends) were produced in *Pichia pastoris* and purified from yeast culture media with a series of chromatographic steps. Cloning of the corresponding gene and selection of the production clones was done by my advisor Marta Kowalska (unpublished results). Protein purification procedure was similar for all three samples of the enzymes, with certain modifications during the process.

6.4.1.1 Fermentation process of *Pichia pastoris* for AtCKX2 production

The yeast cultures were grown in the R'ALF Plus fermentor (Fig. 16), in 7 l of 0.67% YNB medium and 2% glucose, with impeller speed 1000 rpm, air flow 300 l/h, pH 6.5 controlled by the cascade with 5 M KOH and with the antifoaming control. After 40 h, the culture was fed at 0.2 ml/min with 1 liter of 50% glucose containing 0.12 % of trace metal solution and 2.4 mg of biotin. The fermentation process was designed by Marta Kowalska.

Smaller volume aliquots were cultivated in the 500 ml Erlenmeyer flasks, in 200 ml YNB medium (prepared with the potassium phosphate buffer pH 7.2 and 2% glucose) with 10 µl of clone culture added and incubated at 28°C with shaking at 230 rpm. After 86 h, the culture was harvested and a cell free medium was obtained by centrifugation at 5000 g.

Figure 16. R'ALF Plus Fermentor.

6.4.1.2 Antifoam removal

Removal of antifoam was tried by several methods, but was largely ineffective. Antifoam prevented proteins from salting out by ammonium sulfate, probably due to antifoam adsorption on the proteins (Marta Kowalska, personal communication).

Several methods and substances were used for antifoam removal:

- Celite 545 chromatography (antifoam flow-through observed),
- Active carbon chromatography (antifoam flow-through observed, proteins were retained on the column, but with no possibility to elute),
- Hydroxyapatite chromatography (antifoam flow-through observed),
- Ammonium-sulfate precipitation at 70-90% saturation (no protein precipitation occurred),
- Chloroform extraction (antifoam stayed in aqueous fraction).

The only successful method for partial antifoam removal was *centrifugation* of the medium, several times at the maximum possible speed. The antifoam was removed after first chromatographic column, but with a great loss of the protein.

6.4.1.3 CKX activity assay

For CKX activity determination, the Libreros-Minotta and Tipton modified colorimetric assay was used (Libreros-Minotta & Tipton, 1995). Aliquots of different AtCKX enzyme samples were incubated in the reaction mixture (total volume of 0.6 ml), composed of 20 mM reaction buffer (McIlvaine buffer, pH of 5.0, 5.5, 6.5, and 7.0), 10 mM electron acceptor (DCPIP or Q_0) and 10 mM substrate (iP or iPR) for different incubation period, depending on the protein concentration (0.5 - 7 h) at 37°C. Specific assay conditions for different AtCKX enzymes are indicated in Table 1.

Table 1. AtCKX enzymes activity assay conditions used

Enzyme	pH (McIlvaine buffer)	Substrate	Electron acceptor
AtCKX1	5.0	iPR	Q_0
AtCKX2	6.5	iP	DCPIP
AtCKX3	5.0	iPR	Q_0
AtCKX4	6.5	iP	DCPIP
AtCKX5	5.0	iPR	Q_0

The reaction was stopped by adding 0.3 ml of 40% TCA and 0.2 ml of 2% *p*-aminophenol (dissolved in 6% TCA), and samples were centrifuged for 5 min at 10,000 *g*. The absorption at 300 – 450 nm was measured by spectrophotometer against blank obtained by performing the reaction without substrate addition.

The enzyme activity was calculated as follows:

$$a = \frac{A_{352} \cdot V}{\varepsilon \cdot t}$$

where:

A_{352} = the absorbance at 352 nm

ε = molar absorption coefficient ($l\,cm^{-1}\,mol^{-1}$)

t = incubation time (s)

a = activity (katal)

V = volume (ml)

6.4.1.4 Protein concentration procedure

The fermentation procedure usually gains a large volume of the protein containing solution (7-9 l) that needs to be concentrated to a smaller volume. With the concentration, unnecessary components are removed, such as water, growth medium, etc.. Concentration was done by different methods, and the culture medium reduced several times, from 2-9 l to the volume of 50 ml, and finally to 1 ml, depending on the used method.

First concentration method utilized Spectrum device “Minicros sampler”, with a hollow fibre filtrating column that separated larger molecules such as proteins from the solvent. Volume aliquot loaded into the device flask was around 2 l, so the procedure was run several times for the whole volume aliquot obtained after fermentation to be completed. The pressure (max. 5.0 – 6.0 psi) and flow rate (1 ml) were controlled. The method was successful and simple to use also for the preparations of samples for buffer exchange prior to the chromatography.

Another concentration method used Amicon centriprep tubes, with ultrafiltration membrane (cut-off 10 kDa) for concentration of the smaller volumes (3-10 ml). Protein samples were added in the tubes, and centrifuged at 4600 *g*, for 30 min.

The last concentration method utilized Microcon centrifugal device YM-10 (cut off 10 kDa) for small volumes of the samples (0.5 – 2.0 ml). Volume aliquot of 0.5 ml was loaded into the tube and centrifuged at 14000 *g*, for 10 min.

All concentrations were done at 4°C, repeated several times, and the samples were carefully collected.

6.4.1.5 Buffer exchange for protein purification by column chromatography

Before loading the sample onto a column, the sample and the column need to be prepared. Columns were equilibrated with the starting buffer, depending on the used chromatographic method. The buffers are shown in Table 2. Prior loading on the appropriate column, the samples were buffer-exchanged with the starting chromatographic buffer using the procedures as above.

Table 2. Starting buffers for particular columns

Column	Starting buffer
Octyl Sepharose	20% $(NH_4)_2SO_4$ / 0.05 M Tris – HCl, pH 8
High Q Sepharose	0.02 M Tris – HCl, pH 8
Ni-NTA Sepharose	0.05 M Na_2HPO_4 / 0.5 M NaCl, pH 7.4

This procedure was an important step before the chromatography itself, because the columns and the samples were prepared for the separation and purification procedure (concentration and buffer-exchanging procedure), the residual substances removed from previous column usage, and suitable conditions were provided for sample loading and following elution.

6.4.1.6 Chromatography methods

Chromatography procedure was the most important step in the protein purification. Three types of chromatography methods in different steps of purification were used: hydrophobic interaction chromatography (HIC) – *Octyl Sepharose*, ion-exchange chromatography – *High Q Sepharose* and affinity chromatography – *Ni-NTA Sepharose*.

Octyl Sepharose chromatography

Octyl Sepharose Chromatography was used in the beginning of the laboratory work, for first purification attempts, but abandoned due to use of High Q Sepharose chromatography as better choice (Marta Kowalska, personal communication). Octyl Sepharose is a separation medium, on which proteins are separated on the base of the different hydrophobicity. The procedure is applicable for elimination of unnecessary hydrophilic proteins and contaminants.

The buffers used for Octyl Sepharose were:

- starting buffer : 20% w/v $(NH_4)_2SO_4$ in 0.05 M Tris – HCl, pH 8.0
- elution buffer 1: 10% w/v $(NH_4)_2SO_4$ in 0.05 M Tris – HCl, pH 8.0
- elution buffer 2: 0.05 M Tris – HCl, pH 8.0

The column was equilibrated with the starting buffer, before loading the sample on the column. The sample was loaded at the flow rate of 1 ml/min and further washed with the starting buffer. The concentration of ammonium sulfate concentration was changed stepwise (20% - 10% - 0%). The fractions were collected according to protein absorption at 280 nm and conductivity changes.

High Q Sepharose chromatography

Ion exchange chromatography is one of the most widely used laboratory techniques for protein purification. High Q is a strong anion exchanger containing specific quaternary amine functional groups and is ideal for purification of acidic and neutral proteins and peptides.

High Q Sepharose chromatography was performed at the same way for all three samples of the enzyme (His-CKX2, His-CKX2-His, CKX2-His).

After concentration, the sample was prepared for the chromatography column loading. Two types of columns were used in the experiment. Smaller column (15 ml of sorbent volume) worked effectively for loading of 1 ml samples, but was inappropriate for larger volume aliquots (5 ml), because the column capacity was too low and proteins had flowed through without retention.

Samples were loaded with the starting buffer (20 mM Tris – HCl, pH 8.0) and eluted with 40 ml of linear gradient (0-100%) of the elution buffer (20 mM Tris – HCl / 1 M KCl, pH 8.0) at a flow rate of 1 ml/min, with absorbance at 280 nm and conductivity monitoring.

For the larger samples (5-15 ml), the larger column (≈60 ml of sorbent) demonstrated as better choice. The proteins remained on the column after the loading and washing with the starting buffer and were eluted with 150-200 ml of the gradient as above.

Ni-NTA Sepharose chromatography

The Ni-NTA purification system (Fig. 17) is specially designed for purification of *His-tagged* recombinant proteins expressed in different expression systems. The Ni-NTA protein purification system is based on the remarkable selectivity of patented Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of six consecutive histidine residues — the 6 x His tag.

This technology allows one-step purification of many His-tagged protein under native or denaturing conditions. NTA, which has four chelating sites for nickel ions, binds nickel more tightly than metal-chelating purification systems that only have three sites available for interaction with metal ions. The extra chelating site prevents nickel-ion leaching and results in a greater binding capacity and protein preparations with higher purity than those obtained using other metal-chelating purification systems.

The Ni-NTA system can be used to purify His-tagged proteins from any expression system including baculovirus, mammalian cells, yeast, and bacteria.

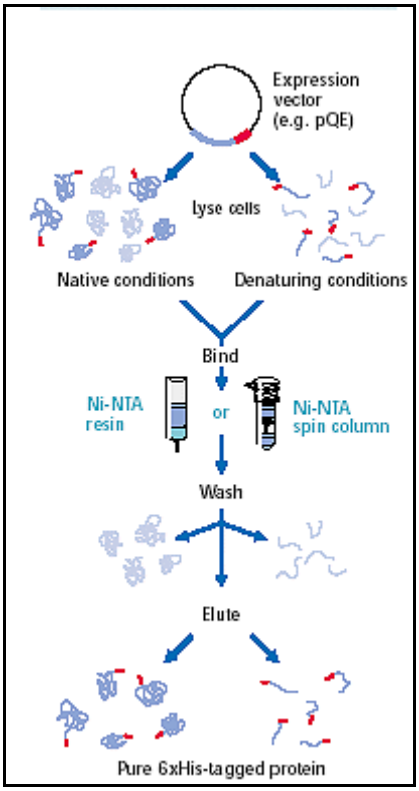

Figure 17. Protein purification with the Ni-NTA purification system (Qiagen).

Ni-NTA Sepharose chromatography was performed at the same way for all three samples of the enzyme (His-CKX2, His-CKX2-His, CKX2-His). A modified "circulation method" was used – first fraction was collected in the flask (after the volume flow through the system (≈80 ml), the flask connected again to the column at the starting buffer inflow. Gradient for this method was variable, and often missing, so the method needed to be modified during the whole procedure. Sample was loaded on the column several times (3-4), where the first fraction treated with the "circulation method". Addition of the starting buffer manually direct to the column (≈2ml) was necessary. The chromatography then continued with a regular flow, without the circulation.

Samples were loaded with starting buffer (50 mM Na_2HPO_4 / 0.5 M NaCl, pH 7.4) and eluted with a linear gradient of 500 mM imidazole in the same buffer.

After the procedure, the protein was washed with 20 mM Tris/HCl pH 8.0 for stability purposes, because it was indicated that the high concentration of imidazole decreases the enzyme activity (Marta Kowalska, personal communication).

6.4.1.7 Protein quantitation

For the protein quantitation, Bradford assay was used (Bradford, 1976). Samples were diluted in 1 ml of the Bradford reagent and incubated for 5 min at the room temperature. Volume of taken samples was different, depending on the concentration of protein samples (from 0.5 µl – 200 µl). Depending of used protein volume, Bradford solution for blank sample was diluted with the same volume of the water. Protein concentration (µg/µl) was estimated from the absorbance at 595 nm using a calibration curve with BSA.

6.4.1.8 Electrophoresis and immunoblotting

Electrophoreses were used for separation of isolated proteins, their molecular weight determination and evaluation of purification success. Two types of electrophoresis were applied – classical SDS-PAGE and Bis-Tris Tricine SDS-PAGE.

SDS-PAGE

Protein samples were mixed with the loading buffer (1% (v/v) 0.75 M Tris/HCl, pH 6.8, 5% (v/v) bromophenol blue, 10% (w/v) SDS, 5% (v/v) β-mercaptoethanol), heated at 99°C for 5 min, cooled on the ice for 10 min and spined down. Sample aliquots (20 µl) were loaded onto the gel that consisted of a stacking gel (30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, 0.75 M Tris/HCl, pH 6.8, 10% (w/v) SDS in H_2O, 0.1% TEMED, 0.1% APS) and running gel (30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, 2.25 M Tris/HCl, pH 9.2, 10% (w/v) SDS in H_2O, 0.1% TEMED, 0.1% APS). The gel was run in the running buffer, that consisted of 0.025 M Tris/HCl, pH 8.3, containing 0.192 M glycine and 0.1% SDS, at 30 mA for 20 min and then at 50 mA for approximately 60 minutes.

Bis-Tris Tricine SDS-PAGE

Protein samples were prepared as above. The gel was run with two different buffers; cathode buffer (0.1 M (w/v) tricine, 0.1% (w/v) SDS, pH 8.25) and anode buffer (0.2 M (w/v) Tris-HCl, pH 8.9). at 30 mA for 20 min and then at 50 mA for approximately 60 min.

Western blotting (immunostaining)

PVDF membrane was immersed in the methanol for 30 s, placed on the top of the developed SDS-PAGE gel and stacked into a frame along with paper filters. The assembly was inserted into a blotting chamber (Bio-Rad) filled with adequate volume of running buffer (2 mM Tris/HCl, pH 8.5, 1.2 mM glycine and 20% methanol) and run at 100 V for approximately 2 h.

After the electrophoresis process, PVDF membrane was blocked with 3% milk in TBS buffer (20 mM Tris/HCl, pH 7.5, 500 mM NaCl) for 1 h. The membrane was then rinsed twice in 10 ml of TBS-Tween (20 mM Tris/HCl, pH 7.5, 500 mM NaCl, 0.05 % Tween-20) for 10 min, put into 10 ml of 1% milk in TBS-Tween containing 0.1% of the primary antibody (anti-HvCKX2, Petr Galuzska, manuscript in preparation) and incubated overnight. After the incubation, the membrane was rinsed twice in 10 ml of TBS-Tween for 10 min and incubated in 10 ml of secondary antibody solution (anti-rabbit IgG conjugated with alkaline phosphatase, Sigma) for 2 h. The membrane was then rinsed twice in TBS-Tween for 10 min and incubated 10 min in a substrate buffer for alkaline phosphatase (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The membrane was then incubated in a staining solution prepared by adding 45 µl of 0.07% nitroblue tetrazolium in 70% dimethylformamide and 70 µl of 0.025% BCIP (5-bromo-4-chloro-3-indolyl phosphate) in 100% dimethylformamide to 10 ml of the substrate buffer for alkaline phosphatase for 1 h, then rinsed in water. All incubations were done at the room temperature with careful shaking.

7. Results and discussion

7.1 Enzyme activity screening of *Pichia pastoris* clones with a multiple copy integration of *AtCKX* genes

The genes *AtCKX3*, *AtCKX4* and *AtCKX5* were expressed in the *Pichia pastoris* expression system in the pPIC9K vector allowing multicopy gene integration. It has been demonstrated that multiple copy integration of recombinant genes in *Pichia pastoris* in some cases increase expression of the desired gene (Cregg et al., 1993).

The used vector pPIC9K gives the yeast resistance to Geneticin (G418 sulfate) to screen for possible multicopy inserts. The goal of the work was to find the *Pichia* clone with the highest increase of CKX activity when compared to the activity of the same gene product expressed in pGAPZαA vector, a single copy clone used as a reference. The activity was measured using the modified end-point method with *p*-aminophenol (Chapter 6.4.3.1). Protein content was determined by Bradford method.

Results of specific activity are given in the figures 18-21. Every sample of every AtCKX enzyme was independently measured four times and data was calculated as average value from those independent measurements. Conditions of the measurement were the same for each sample of specific related enzyme.

Cytokinin dehydrogenase 3 (AtCKX3)

Specific conditions for the enzyme activity assay were pH 5.0, iPR as the substrate and Q_0 as electron acceptor, incubation period 3 or 4 h. In the screening of AtCKX3 (Fig. 18), all clones exhibited higher activity than the reference one. The clone 5 was the most efficient one, showing 2.66 times higher specific activity than the reference.

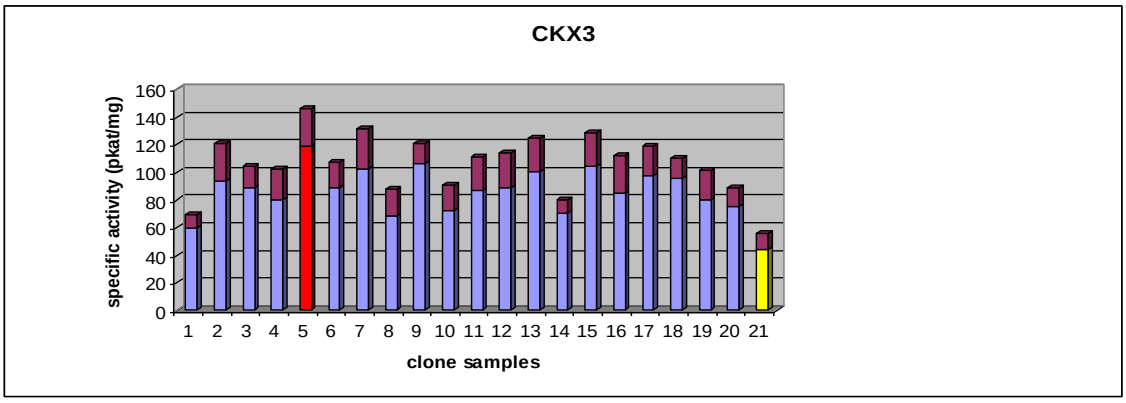

Figure 18. Specific activities of AtCKX3 clones. Clone 5 was the most efficient one (118.9 pkat/mg) comparing to the reference clone 21 (44.6 pkat/mg).

Cytokinin dehydrogenase 4 (AtCKX4)

Specific conditions for enzyme activity assay were pH 6.5, iP as the substrate and DCPIP as electron acceptor, incubation time 5 h. Two expression conditions were with different *P. pastoris* strains tested, 2-day (strain GS 115) and 4-day growth period (strain SMD 1168). The first experiment (Fig.19) showed increased activity values with the clone 3 being the most efficient (1.93 times higher specific activity than the reference).

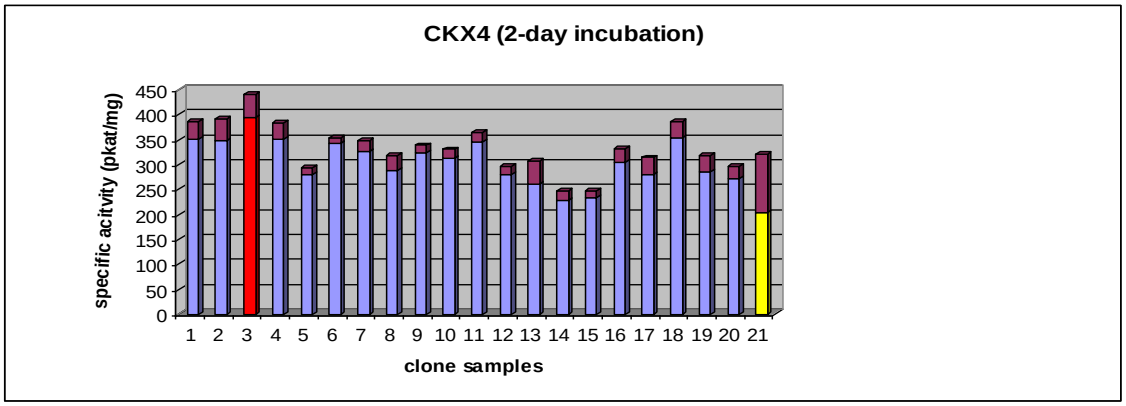

Figure 19. Specific activities of AtCKX4 clones (2-day incubation). Clone 3 was the most efficient clone (393.2 pkat/mg) comparing to the reference clone 21 (202.7 pkat/mg).

The other experiment (Fig. 20) showed the values of specific activity higher than the reference clone, the highest being the clones 3 and 17 with a double increase.

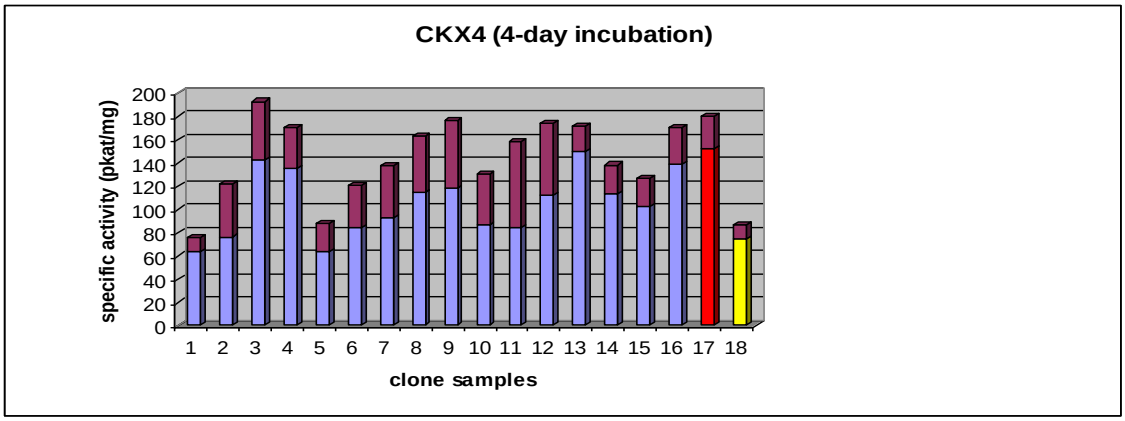

Figure 20. Specific enzyme activities of AtCKX4 clones (4-day incubation). Clone 17 was the most efficient (151.8 pkat/mg) compared to the reference clone -18 (74.8 pkat/mg).

Values of the activity for 4-day period with SMD 1168 strain showed less than one half of the values of the activity detected in 2-day period with GS 115 strain.

Cytokinin dehydrogenase 5 (AtCKX5)

Specific conditions for enzyme activity determination were: pH 5.0, iPR substrate as the substrate, Q_0 as electron acceptor, incubation time 4h and 5h. All but one of the ten samples demonstrated lower activity values then the reference clone (Fig. 21). It is not known, why the lower enzyme activity in newly cloned samples occurred, it may have been caused by unwanted gene silencing.

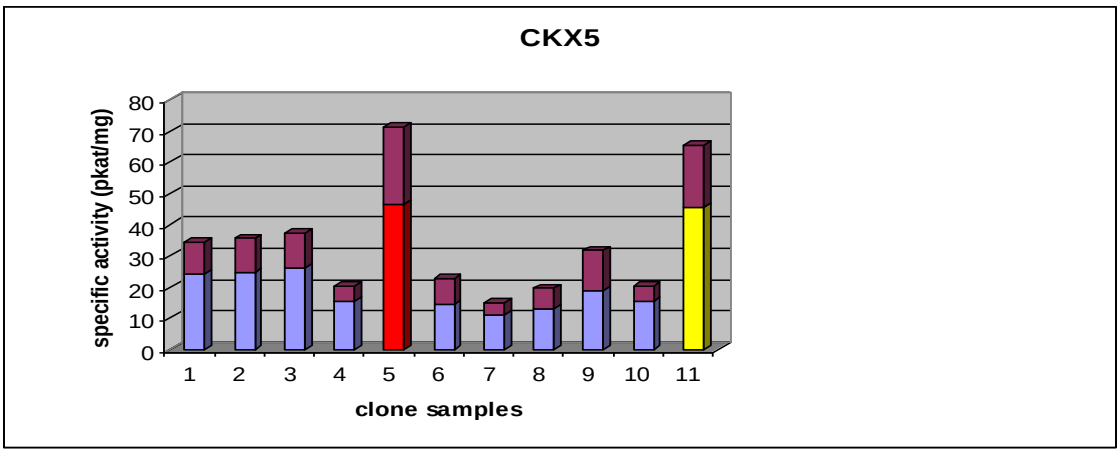

Figure 21. Specific enzyme activities of selected AtCKX5 clone samples. Clone 5 is the most efficient (46.5 pkat/mg) compared to the reference clone 11 (45.6 pkat/mg).

Activity screening of cytokinin dehydrogenases from *Arabidopsis thaliana* was done. Proteins were expressed with the use of a novel vector pPIC9K in *Pichia pastoris*. The vector enables multicopy integration of the gene of interest into *Pichia* genome what should result in increased expression level of the protein and therefore higher activities were expected. This appears not to be true in the case of AtCKX5 when all but one of the ten samples demonstrated even lower activities. All of 20 screened AtCKX3 samples had higher activities and clone 5 was chosen to be the most efficient. When it comes to AtCKX4 the screening was very diverse, revealing both lower and higher activities in 35 samples and finally, clone 3 from two day incubation growth period was chosen as the most efficient.

7.2 AtCKX2 enzyme purification

Purification of AtCKX2 enzyme achieved in this work was done on the base of the published information and data. Previously, small quantities of CKX enzymes were purified from wheat and barley grains (Galuszka *et al*. 2000). Larger quantities of the enzyme were obtained after the cloning of ZmCKX1 gene and expressing in yeast host (Bilyeu *et al.* 2001, Kopečný *et al*. 2005). It was determined that secreted AtCKX2 enzyme is probably the most active protein from seven *Arabidopsis thaliana* CKX enzymes (Galuszka *et al*. 2007). Recombinant AtCKX2 enzyme was purified from *Saccharomyces cerevisiae* yeast system (Frébortová *et al.* 2007).

In this work, the three differently His-tagged AtCKX2 enzymes were purified by several purification steps from batch cultures obtained in fermentor. The most complicated procedure was the removal of antifoam used in the fermentor. The antifoam was treated by several procedures, but all the treatments were unsuccessful, except the centrifugation at high speed. With this method, larger part of the antifoam was eliminated. The medium was concentrated by ultrafiltration. Enzyme activity was measured through every step of the purification.

All the steps were continuously controlled, but some quantity of the samples was lost during the procedures, mostly during the antifoam removal and concentration procedures.

Octyl-Sepharose chromatography

AtCKX2 activity was detected in the first and second fraction. Fractions were concentrated and applied to SDS-PAGE. Active fractions were pooled and used for the next purification step. First fraction showed more purified sample, and the mixture of fraction 1 and fraction 2 showed additional proteins that contaminate the sample.

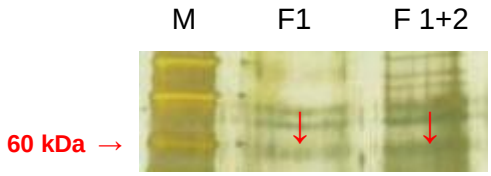

Figure 22. SDS-PAGE of the Octyl–Sepharose fractions 1 and 1+2.

For further purification attempts High Q Sepharose chromatography in expectance of purer sample.

High Q Sepharose chromatography

Ion exchange chromatography procedure was the first step in chromatographic methods. Macro-Prep High Q Sepharose proved as excellent choice for rapid purification and elimination of unwanted contaminants.

Smaller volume column (15 ml) showed a limitation of sample loading volume (not more 1 ml, otherwise pass through occurred). Bigger volume column (60 ml) was loaded with increased sample volume (2-5 ml) twice for each CKX sample that resulted in elution of the antifoam and part of the AtCKX2 enzyme in the first fraction. This probably happened due to a partial sorption of the enzyme to the antifoam, which was unfortunately unavoidable. This fraction was loaded in repeated procedure, in tendency to purify all present AtCKX2 from the sample.

AtCKX2 was eluted with a linear gradient of 1 M KCl in the starting buffer, and fractions with activity were collected and concentrated. For all three AtCKX samples (His-AtCKX2-His; His-AtCKX2; AtCKX2-His), the elution conditions were similar (Fig. 23), except for His-AtCKX2-His sample, which was prepared by fermentation in flask without the antifoam. During the concentration procedure, the samples were treated with the starting buffer for Ni-NTA chromatography, as a preparation for next purification step.

High Q Sepharose chromatography was more successful for purification of His-AtCKX2-His enzyme, than for other two samples, considering increased elimination of the contaminants. Active fractions were concentrated by ultrafiltration and used for the next purification step.

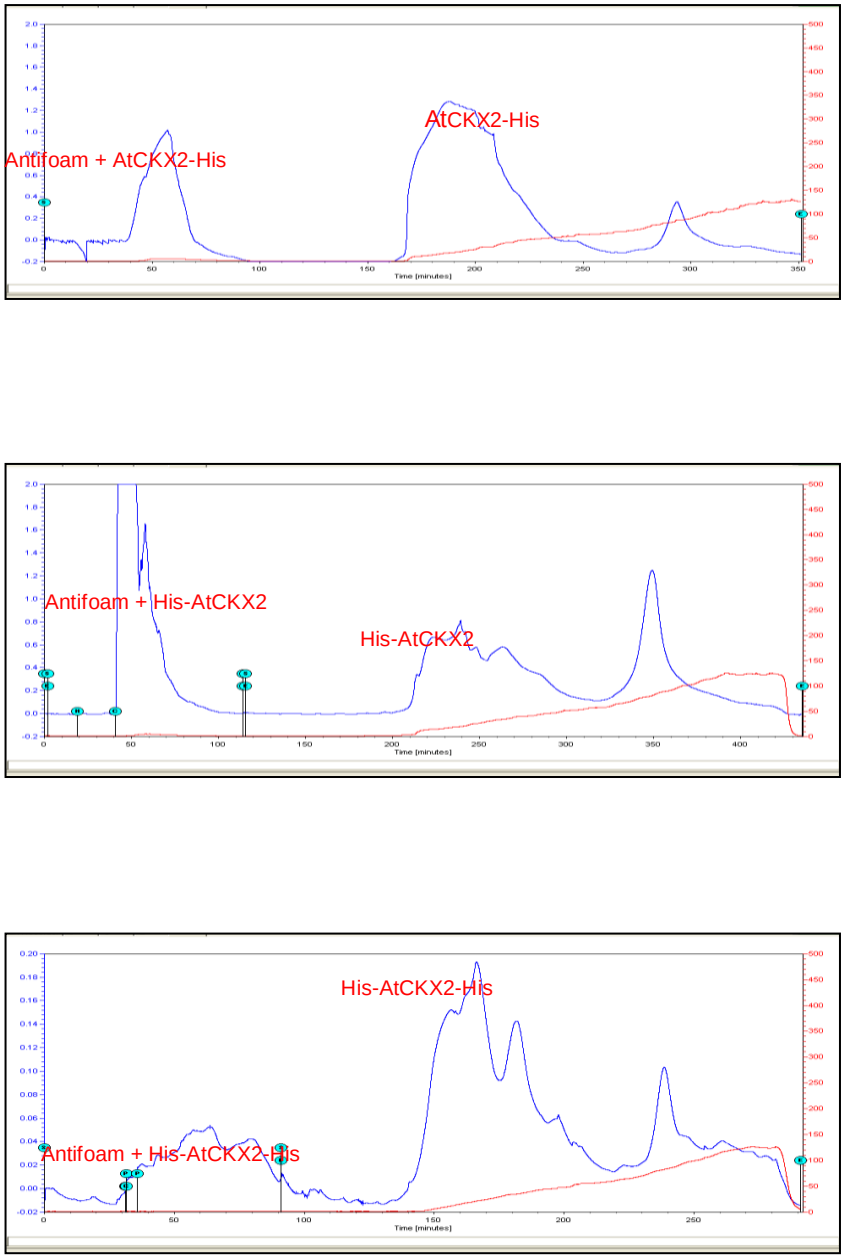

Figure 23. Purification of three His-tagged AtCKX2 enzymes on High Q Sepharose. The samples were loaded with 20 mM Tris/HCl pH 8.0 and eluted with 40 ml of 0-100% linear gradient of the same buffer containing 1 M KCl at a flow rate of 1 ml/min. All AtCKX2 enzyme samples (His-AtCKX2, AtCKX2-His, His-AtCKX2-His) demonstrated the same retention and elution with the buffer gradient.

Ni-NTA Sepharose chromatography

Affinity chromatography was the final step in AtCKX2 enzyme purification. Considering the fact that the enzymes were modified with one or two 6 x histidine tags, Ni-NTA was the best choice for final purification. The samples were loaded in 50 mM Na_2HPO_4 / 0.5 M NaCl, pH 7.4 and eluted with the same buffer containing 500 mM imidazole. Active fractions were collected as indicated in Fig. 24.

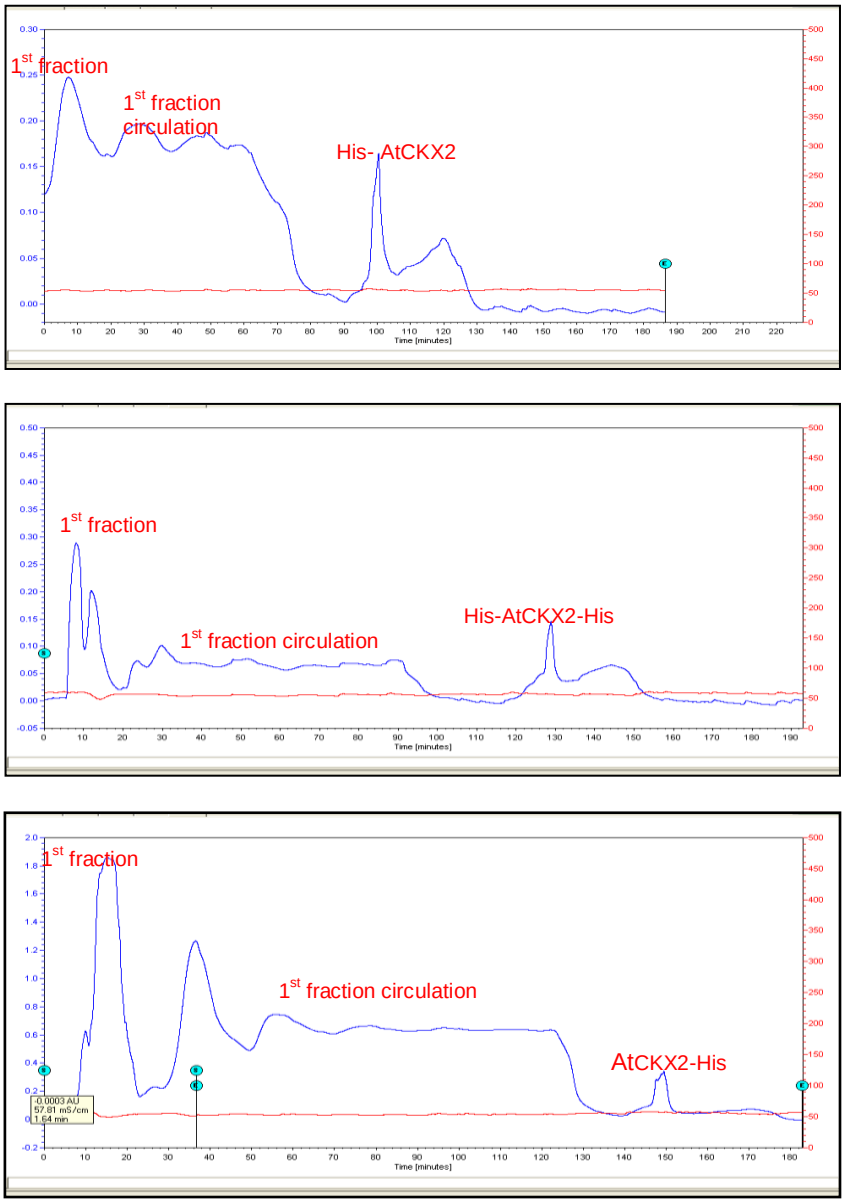

Figure 24. Purification of three His-tagged AtCKX2 enzymes on Ni-NTA Sepharose. The samples were loaded with 50 mM Na_2HPO_4 / 0.5 M NaCl, pH 7.4, and eluted with the same buffer containing 500 mM imidazole..

Purification progression

As indicated in Tables 3-5, the purification procedure was successful. Main complication was the sample loss during concentration procedure. All enzyme samples showed increased specific activity, followed with decreased protein content, which lead to the conclusion that the purification procedure was appropriately performed and controlled. N-terminus His-tagged AtCKX2 enzyme showed the highest specific activity (21.4 nkat/mg), comparing to the AtCKX2 sample with the protein C-terminus tag (8.7 nkat/mg).

Reason for almost three time lower specific activity of AtCKX2 enzyme with protein C-terminus histidine tag may be the deep burial of the C-terminus inside the protein and interference of His-tag with the active site (Mailto *et al.* 2004). Low protein content of His-CKX2-His enzyme (0.4 mg/ml) with both protein terminus tagged is probably due to the lower fermented medium volume and different fermentation conditions the other two samples.

Table 3. Purification procedure of His-CKX2-His tagged enzyme.

Fractions	Volume (ml)	Total protein (mg)	Activity (nkat)	Total activity (nkat/ml)	Specific activity (nkat/mg)	Purification (fold)	Yield (%)
Crude extract	1800	0.13	0.06	108	**0.5**	1.0	100
“Minicros sampler” ultrafiltration	10	2.6	3.1	30.8	**1.2**	2.5	28.5
High Q Sepharose	1.8	1.2	5.1	9.3	**4.1**	8.8	8.6
Ni-NTA Sepharose	0.5	0.4	7	43.5	**18.4**	39.4	3.2

Table 4. Purification procedure of His-AtCKX2 tagged enzyme.

Fractions	Volume (ml)	Total protein (mg)	Activity (nkat)	Total activity (nkat/ml)	Specific activity (nkat/mg)	Purification (fold)	Yield (%)
Crude extract	8500	0.3	0.2	1377	**0.5**	1.0	100
"Minicros sampler" ultrafiltration	15	6.09	23.2	347.4	**3.8**	7	25.2
High Q Sepharose	2.0	20	77.3	154.7	**3.9**	7.2	11.2
Ni-NTA Sepharose	1.2	2.3	49	58.8	**21.4**	39.6	4.3

Table 5. Purification procedure of AtCKX2-His tagged enzyme.

Fractions	Volume (ml)	Total protein (mg)	Activity (nkat)	Total activity (nkat/ml)	Specific activity (nkat/mg)	Purification (fold)	Yield (%)
Crude extract	8500	0.03	0.05	459	**1.8**	1.0	100
"Minicros sampler" ultrafiltration	25	7.1	17.7	444.3	**2.5**	1.4	95.3
High Q Sepharose	6.5	5.7	18	116.7	**3.1**	1.7	25
Ni-NTA Sepharose	1.9	3.4	32.6	62	**8.7**	4.8	12

SDS-PAGE electrophoresis

Purified proteins were analyzed with SDS-PAGE. As indicated in Fig. 25, the highest purity enzyme was obtained for His-AtCKX2. Purified enzymes aggregated with some remaining impurities, due to low polarity of AtCKX2 enzyme that probably resulted in fractional formation of an aggregate (Frébortová *et al.* 2007). The same aggregations were detected during the purification procedure of AtCKX1 (Marta Kowalska, unpublished results).

Results after Western blotting (immunoblotting) showed low quality of the image and they will not be included in the description.

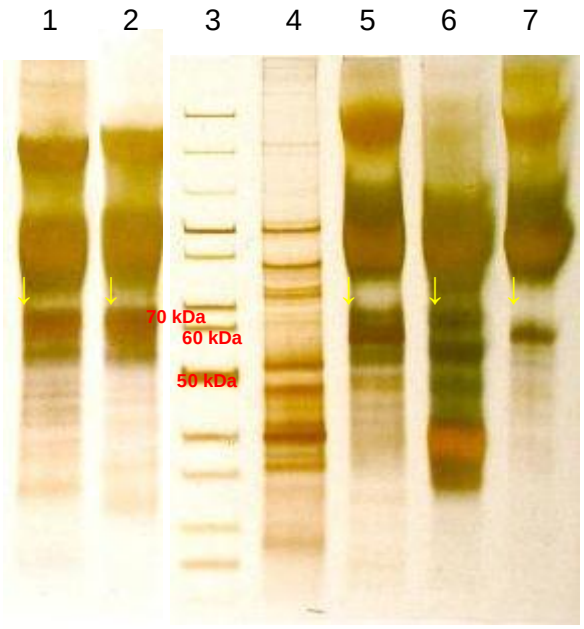

Figure 25. SDS-PAGE of purified recombinant AtCKX2 enzymes from *Pichia pastoris*: 1. His-AtCKX2-His sample after High Q Sepharose (1.25 µg of protein), 2. His-AtCKX2-His sample after Ni-NTA sepharose (0.4 µg of protein), 3. Marker (Page Ruler Protein Lader 10-200 kDa) (2 µl), 4. AtCKX2-His sample after High Q sepharose (5.72 µg of protein), 5. AtCKX2-His sample after Ni-NTA sepharose (3.73 µg of protein), 6. His-AtCKX2 sample after High Q sepharose (20 µg of protein), 7. His-AtCKX2 sample after Ni-NTA sepharose (2.3 µg of protein). The arrows show positions of AtCKX2 the protein band.

8. Conclusions

Activity of different AtCKX enzymes was screened (AtCKX3, AtCKX4 and AtCKX5), to select the best expressing *Pichia pastoris* clones with multiple gene integration pPIC9K vector, comparing to the reference clone with a single gene integration pGAPZαA vector. Expression conditions were identical for each enzyme and measurements done several times for each sample. The activity was measured using a modified end-point method. The pPIC9K vector enables multicopy integration of the gene of interest into *Pichia* genome what should result in increased expression level of the protein and therefore higher activities were expected. This was confirmed only in the case of AtCKX3, which expressed 2.5 times higher activity comparing to the reference clone. AtCKX4 and AtCKX5 showed diverse results, and even decreased activity value comparing to the reference clone. It is not determined why the diverse expression, decreased or almost equal, in newly cloned samples, comparing different number of integrated copies to the reference clone, was present. It was expected to obtain even higher activity value, comparing to the reference clone (Cregg *et al.* 1993).

Purification of differently His-tagged AtCKX2 enzymes was successful. AtCKX2 enzymes with histidine tags at N-terminus, C-terminus and both were partially purified and characterized. Specific activity of the purest AtCKX2 enzyme (histidines at N-terminus) was 21.4 nkat/mg protein with iP as the substrate and DCPIP as electron acceptor. On SDS-PAGE, the enzyme migrated as a band corresponding to the molecular mass of approximatively 60 kDa, followed by unidentified protein band from 80 kDa to 100 kDa.

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10. Abbreviations used

APS	Ammonium peroxodisulfate
BSA	Bovine serum albumine
CKX	Cytokinin dehydrogenase (EC 1.5.99.12)
DCPIP	2,6-Dichlorophenol indophenol
DMSO	Dimethylsulfoxide
E. coli	*Escherichia coli*
EC	Enzyme Commission)
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
GS115	*Pichia pastoris* strain which contains the gene for *AOX1*
H2	His-CKX2
2H	CKX2-His
H2H	His-CKX2-His
IMAC	Immobilized metal-affinity chromatography
iP	6(γ,γ′-Dimethylallylaminopurine riboside
iPR	6(γ,γ′-Dimethylallylaminopurine
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium chloride
OD	Optical density
PAF	*p*-Aminophenol
P. pastoris	*Pichia pastoris*
pGAPZα	Constitutive plasmid for *P. pastoris* with α-factor
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Q_0	2,3-Dimethoxy-5-methyl-p-benzoquinone
TBS	20 mM Tris, 500 mM NaCl, pH 7.5
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)aminomethane
ZmCKX1	Cytokinin dehydrogenase from *Zea mays* ecoded by the gene ZmCKX1 (GenBank accession no. Af044603)