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DEVELOPMENT, CHARACTERIZATION AND USE OF ANTI-ORPHAN CYTOCHROME P450 ANTIBODIES

Vývoj, charakterizace a použití protilátek proti orfanovým cytochromům P450

Diploma thesis

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Abstract

The cytochromes P450 (P450s) are important enzymes involved in metabolic pathways, which use exogenous and endogenous substances as their substrate for various enzymatic reactions. These enzymes can also use precarcinogens as their substrate and activate them into carcinogens, which leads to a cancer development. If the P450s are induced, the cancer risk increases. Some chemopreventive compounds may induce the P450s and thus be harmful to the human body. Therefore it is necessary to pay enough attention to a study of the mechanism of action of P450s and the influence of the chemopreventive compounds on the activity of cytochromes P450.

mRNA expression of most of the P450s isoforms is detected in a number of healthy (nontransformed) tissues, *viz.* liver, brain, heart, colon, kidney or placenta. Nevertheless there are a few P450s isoforms which mRNAs are expressed at relatively low levels in the nontransformed tissues, whereas the expression in the transformed tissues is significantly higher. One of these P450s is CYP2W1, which can be used as a prognostic marker for colorectal cancer - therefore it is useful to be able to detect a presence of this enzyme in various tissues. A detection of P450s can be accomplished by using a method Western blot. In this method, the immunodetection is achieved by using specific antibodies. The mammalian antibodies (IgG) are for this purpose used the most but also the antibodies isolated from egg yolk (IgY) are getting popular in the last years.

In this diploma thesis, two peptide sequences were chosen from the primary structure of CYP2W1 and conjugated to a keyhole limpet hemocyanin (KLH). After the immunization of hens by these immunogens the IgY were isolated by precipitation method with sodium chloride. The next step was purification by affinity chromatography, which led to obtain specific antibodies. At the end, the purified IgY were used for the immunodetection of CYP2W1 in cell lysates and their efficacy was compared to the efficacy of antibodies produced in rabbit.

Keywords: xenobiotics, cytochrome P450, immunodetection, chicken antibodies

Abstrakt

Cytochromy P450 jsou důležité enzymy účastnící se metabolických pochodů, které využívají exogenní i endogenní látky jako substráty pro různé enzymatické reakce. Tyto enzymy mohou také jako substrát využít prekarcinogeny a aktivovat je na karcinogeny, což může vést k rozvoji rakoviny. Některé chemopreventivní látky mohou zvyšovat aktivitu cytochromů P450, a tím i riziko rozvoje rakoviny. Proto je důležité věnovat náležitou pozornost studiu mechanismu účinku těchto enzymů a vlivu chemopreventivních sloučenin na jejich aktivitu.

mRNA exprese většiny isoforem cytochromů P450 je pozorovatelná ve zdravých (netransformovaných) tkáních, například játrech, mozku, srdci, tlustém střevě, ledvinách či placentě. Přesto existuje několik cytochromů P450, jejichž mRNA jsou v netransformovaných tkáních exprimovány málo, zatímco jejich exprese v transformovaných tkáních je znatelně vyšší. Jedním z takovýchto enzymů je CYP2W1, který může být použit jako prognostický marker kolorektální rakoviny - proto je užitečné mít schopnost detekovat přítomnost tohoto enzymu v různých tkáních. Detekce cytochromů P450 může být dosaženo metodou Western blot. V této metodě je imunodetekce provedena s využitím specifických protilátek. Pro tyto účely jsou nejčastěji používány savčí protilátky (IgG), ale protilátky izolované z vaječného žloutku (IgY) se v posledních letech stávají také populárními.

Během této diplomové práce byly z primární struktury CYP2W1 vybrány dvě peptidové sekvence, které byly navázány na keyhole limpet hemocyanin (KLH). Po imunizaci slepic tímto imunogenem byly izolovány IgY precipitační metodou s chloridem sodným. Dalším krokem byla purifikace afinitní chromatografií, kterou byly získány specifické protilátky. Nakonec byly purifikované IgY použity pro imunodetekci CYP2W1 v buněčných lyzátech a jejich účinnost byla srovnána s účinností králičích protilátek.

Klíčová slova: xenobiotika, cytochrom P450, imunodetekce, slepičí protilátky

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List of abbreviations

A2780	human ovarian carcinoma cell line
A375	human malignant melanoma cell line
A549	human lung adenocarcinoma epithelial cell line
AhR	aryl hydrocarbon receptor
APS	ammonium persulfate
Aspc	human pancreatic cancer cell line
AQ4	[1,4-bis{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxy--anthracene-9,10-dione]
AQ4N	[1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8--dihydroxyanthracene-9,10-dione]
Arnt	aryl hydrocarbon receptor nuclear translocator
BIS	*N*,*N'*-methylenebisacrylamide
BSA	bovine serum albumin
BT-474	human breast cancer cell line
BxPC3	human pancreatic cancer cell line
Cal51	human breast cancer cell line
CAPAN	human pancreatic cancer cell line
CAR	constitutive androstane receptor
CH	constant region of heavy chains
CL	constant region of light chains
CRCA	colorectal cancer cell line
CYP2S1	cytochrome P450 2S1
CYP2W1	cytochrome P450 2W1
DEA	diethylamine
DLD-1	colorectal adenocarcinoma cell line
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC	Enzyme Commission number
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay

ER	endoplasmic reticulum
Fab	fragment antigen-binding
Fc region	fragment crystallisable region
HAA	heterocyclic aromatic amines
HAMA	human anti-mouse antibody
HEK293T	human embryonic kidney 293T cells
HeLa	human cervical cancer cell line
HCT116	human colorectal carcinoma cell line
IgG	antibody (immunoglobulin) of class G
IgY	chicken antibody
kDa	kilodalton (1 Da ~ 1.66×10^{-27} kg)
MCF7	human breast cancer cell line
mcKLH	maleimide activated keyhole limpet hemocyanin
MDA-MB-231	human breast cancer cell line
MiaPaCa	human pancreatic cancer cell line
mRNA	messenger ribonucleic acid
NADH	reduced form of nicotinamide adenine dinucleotide
$NADP^+$	oxidized form of nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NC	nitrocellulose
NK	natural killer cells
P450	cytochrome P450
Panc	human pancreatic cancer cell line
PaTu	human pancreatic cancer cell line
PBS	phosphate buffered saline
pNPP	para-nitrophenyl phosphate
PPAR	peroxisome proliferator-activated receptor
PVDF	polyvinylidene difluoride
PXR	pregnane X receptor
RF	rheumatoid factor
RIA	radioimmunoassay
RPE	transformed human retinal pigment epithelial cell line

RXR	retinoid X receptor
Saos-2	human osteosarcoma cell line
SD	standard deviation
SDS	sodium dodecyl sulphate
Sulfo-SMCC	sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1--carboxylate
TEMED	*N*,*N*,*N'*,*N'*-tetramethylethylendiamine
Tris	tris(hydroxymethyl)aminomethane
U2OS	human bone osteosarcoma epithelial cell line
VH	variable region of heavy chains
VL	variable region of light chains
ZR-75-1	human breast cancer cell line
ZR-75-30	human breast cancer cell line

1 INTRODUCTION

1.1 Cytochrome P450

Cytochromes P450 (P450s) are enzymes which form one of the most important groups of oxygenases [1]. These enzymes are present in most Eubacter and Archaebacter species and function usually as monooxygenases [2]. The P450s participate in metabolism of endogenous substances as steroids [3], bile acids [4], arachidonic acid [5] or eicosanoids [6] but also in metabolism of xenobiotics (such as drugs, carcinogens or pollutants) [7]. Humans have 57 P450 genes, however, plants have significantly higher number of P450 genes [8]. The P450s were discovered in rat liver microsomes by Klingenberg in 1957 [9].

Cytochromes P450 are so called because these enzymes can be quantitated by using the characteristic 450 nm absorption peak of the CO adduct with reduced P450. Thus the P450 comes from an abbreviation for **p**igments with an absorbance at **450** nm [10].

As mentioned previously, P450s catalyse the monooxygenase reactions during which one oxygen atom from oxygen molecule is reduced and forms a molecule of water while the second oxygen atom is inserted into a substrate. For this action cytochromes P450 use two electrons provided by NAD(P)H *via* cytochrome P450 oxidoreductase. The following equation describes the process:

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$$

where a hydrocarbon substrate (RH) forms with molecular dioxygen a hydroxylated metabolite (ROH) and one molecule of water.

1.1.1 Cytochrome P450 Structure

Cytochromes P450 are transmembrane metaloenzymes with structure similar to peroxidase and NO synthase. They can be found mainly in the smooth endoplasmic reticulum and mitochondrial membranes. The structure of cytochromes P450 contains heme b (Fig. 1.1) which is present also in hemoglobin or myoglobin. Heme b is composed of protoporfyrin IX with one central iron atom to which an apoprotein is bound *via* SH- groups of cysteine. The sixth coordination site contains a water molecule [1].

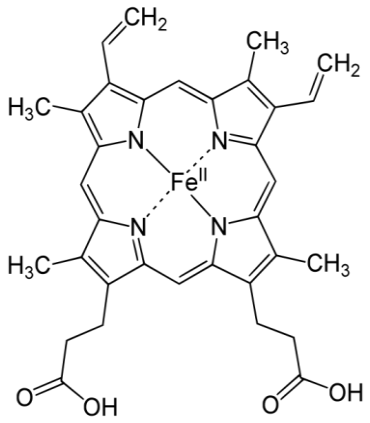

Figure 1.1: Structure of heme b

1.1.2 Nomenclature of Cytochromes P450

Since the first description of cytochromes P450, researchers in the field have used a variety of different nomenclatures for an increasing number of forms of P450 isolated in their laboratories. This plethora of names has become more and more confusing. Thus during the First International Workshop on "P450 Genes and Their Regulation" (April 1985 in Airlie, Virginia) a Committee on Standardized Nomenclature of the P450 Genes was formed. This committee has decided to sort P450s by using a global alignment and percentage of their similarity calculated by

the algorithm described by Lipman and Wilbur in 1983 [11] into families and subfamilies. Originally one gene family contained proteins at least 36% similar to proteins in any of the other gene families. Any proteins within the same subfamily were approximately 70% or more similar. The families were described by Roman numerals whereas the subfamilies were described by capital letters and individual genes by Arabic numerals [12].

Later it was established that to the same family of mammal P450 proteins belong those with at least 40% genetic similarity and to the same subfamily those P450 proteins with 55% or higher genetic similarity. An Arabic numeral was chosen to describe each of families [13].

Nowadays 18 cytochrome P450 families and 43 subfamilies are known, of which isoforms 1-3 participate mainly in metabolism of xenobiotics. More information about isoforms of P450 can be found in the internet database Cytochrome P450 Homepage [14].

1.1.3 The Catalytic Cycle of Cytochromes P450

Cytochromes P450 are mixed function oxidases (MFO) - enzymes which are able to transfer electrons to molecular oxygen and insert one of its atoms into a molecule of substrate. Cytochromes P450 are the terminal oxidases of the MFO system and cooperate with NADPH:cytochrome P450 oxidoreductase (EC 1.6.2.4) and optionally also with cytochrome b_5 and NADH:cytochrome b_5 oxidoreductase (EC 1.6.2.2) [1].

Two classes of human P450s concerning the redox partners involved in electron transport have been described: the adrenal mitochondrial P450 systems obtaining electrons from NADPH *via* adrenodoxin reductase and adrenodoxin [15], and the liver microsomal P450s obtaining electrons from NADPH *via* a FAD and FMN-containing oxidoreductase [16]. The first discovered bacterial P450 system was the camphor hydroxylase of *Pseudomonas putida*. It was found that this system is organized analogously to the mitochondrial one. Electrons are being transferred

from NADH *via* a FAD-containing reductase (putidaredoxin reductase) and an iron-sulfur protein of the [2Fe-2S] type (putidaredoxin) to cytochrome P450cam [17].

Figure 1.2 shows a catalytic cycle of P450s. In the first step of this cycle, a substrate binds to a ferric form of enzyme (1), which is followed by the first reduction of the heme group from the ferric to the ferrous state by an electron provided by NADPH *via* the accessory flavoprotein NADPH:cytochrome P450 oxidoreductase (2). The next steps are molecular oxygen binding to the ferrous heme center of P450 (3), transfer of a second electron to the system and a peroxo group formation (4). The second electron may come from NADPH *via* NADPH:cytochrome P450 oxidoreductase or, in some cases, *via* cytochrome b_5 [18; 19]. In the following steps the unstable peroxo (O-O) bond is cleaved (5), the substrate is oxygenated (6) and the final product is released (7). The second oxygen atom is reduced and forms a water molecule. Cytochromes P450 may also use a variety of peroxides or peroxo acids as the oxidizing agent [20].

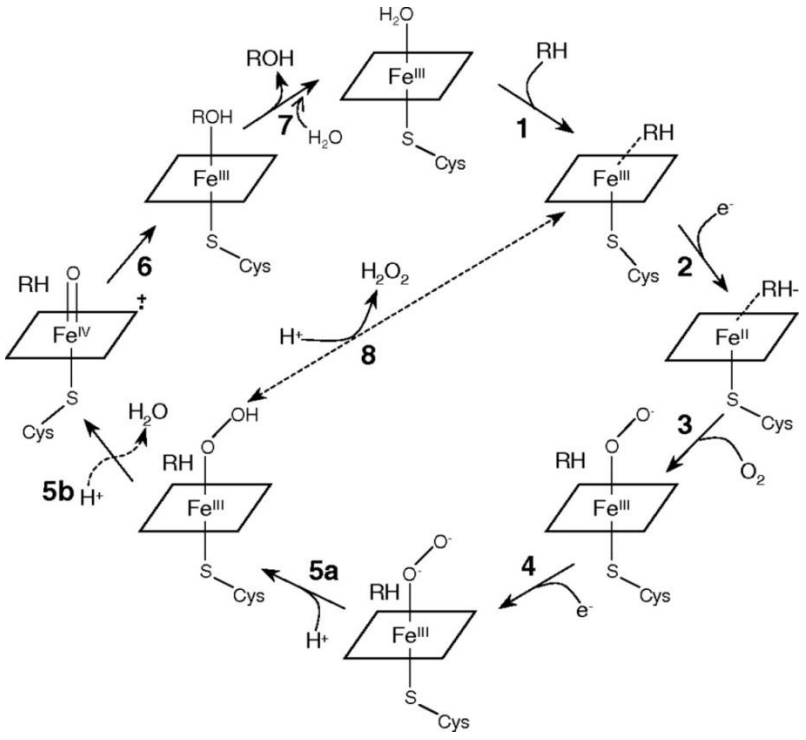

Figure 1.2: The catalytic cycle of cytochromes P450. RH – substrate, ROH – hydroxylated product, H_2O_2 – hydrogen peroxide, Cys – the conserved axial cysteine ligand. The figure was adapted from [21].

1.1.4 Inhibition of Cytochromes P450

Three steps in the catalytic cycle of cytochromes P450 are particularly vulnerable to inhibition: a) the substrate binding, b) the binding of molecular oxygen and c) the oxidation of substrate. P450 inhibitors may be divided into three classes [22; 23]:

I. *reversibly binding agents*: they compete with substrate for occupancy of the active site. The inhibitor may also bind to the prosthetic group of enzyme. A release of the inhibitor leads to the enzyme function recovery.

II. *agents forming quasi-irreversible complexes with the heme iron atom*: Compounds that undergo P450-catalyzed activation on reactive intermediates and quasi-irreversibly inactivate the enzyme responsible for their activation. These compounds can be displaced from the heme iron atom only under unique experimental conditions.

III. *irreversibly binding agents*: Compounds that are oxidatively activated and inactivate the enzyme by covalently binding to it – as sulfur compounds [24], halogenated structures as chloramphenicol [25], alkyl and aryl olefins and acetylenes [26], furanocoumarins [27] and carbamazepine [28] or tamoxifen [29].

Inhibitors that are affecting the enzyme before the first oxidation step are mostly reversible inhibitors. On the other hand, inhibitors affecting the enzyme meanwhile or after the first oxidation step are quasi-irreversible or irreversible inhibitors [22].

Some examples of P450s inhibitors are shown in Table 1.1 (page 17).

1.1.5 Induction of Cytochromes P450

Induction of P450s is a tightly regulated process controlled primarily at the level of gene transcription [30]. The regulation of the expression of CYPs is sensitive to xenobiotic exposure, which allows the cell to increase the level of CYP enzymes to facilitate elimination of a toxicant [31].

Some cytochrome P450 genes are expressed constitutively, while others are inducible. The inducers are often substrates for the induced enzymes, thus P450 activities remain elevated only as needed. The induction is mostly a protective mechanism, which enhances detoxification [1; 30; 32].

The CYP induction can proceed *via* the following intracellular receptors [33]:

- *Aryl hydrocarbon receptor (AhR)* – regulates the gene expression of CYP1 family [34]. AhR is a cytosolic receptor, which can be activated by inductors such as polycyclic aromatic hydrocarbons or polychlorinated dibenzofurans [31]. The AhR becomes activated by ligand binding in the cytosol. The activated receptor then translocates to the nucleus, where it forms a heterodimer with the nuclear factor Arnt. This heterodimer binds to DNA enhancer sequences ("xenobiotic-response elements") and stimulates target gene transcription.

- *Constitutive androstane receptor (CAR)* – a novel orphan nuclear receptor which mediates induction of CYP2B genes by phenobarbital and other similar lipophilic compounds.

- *Pregnane X receptor (PXR)* – also called PAR or SXR. Activates CYP3A genes in response to diverse chemicals.

- *Peroxisome proliferator-activated receptor (PPAR)* – mediates induction of the fatty acid hydroxylases of the CYP4A family by acidic chemicals.

CAR, PXR and PPAR are nuclear receptors that after activation by ligand binding form a heterodimer with retinoid X receptor (RXR). This heterodimer binds to DNA sequences responsible for target gene transcription [33].

Table 1.1 shows examples of P450s inducers.

Table 1.1: Examples of inhibitors and inducers of some cytochromes P450.

CYP	Inhibitor	Inducer
1A2	Fluvoxamine	Smokers vs. non-smokers
2B6		Rifampicin
2C8	Gemfibrozil	Rifampicin
2C9	Fluconazole, amiodarone	Rifampicin
2C19	Omeprazole, fluvoxamine	Rifampicin
2D6	Paroxetine, fluoxetine, quinidine	
2E1	Disulfiram	Ethanol
3A4/5	Atazanavir, clarithromycin, itraconazole, etc.	Rifampicin, carbamazepine

The table was adapted from [35].

1.1.6 Orphan Cytochromes P450

The human genome contains 57 P450 genes of which at least 13 can be considered "orphans" [36]. Table 1.2 (page 18) summarizes the current human orphan P450s and their substrates.

The orphan P450s are so called because their regulations, expression patterns and functional information are still largely unknown. The "orphans" are mostly found in CYP families 1-4 [37]. For some of the orphan P450s, information about their mRNA expression and possible substrates is available, while very limited information is available about the isoforms 3A43, 4A22, 4F22, 20A1 and 27C1 [36].

Table 1.2: Human P450 orphans.

P450	Expression site examples	Possible substrate
2A7	*	
2S1	Liver, skin, stomach, SI, spleen, lung, etc.	Retinoic acid
2U1	Thymus, brain	Arachidonic acid
2W1	Tumors	Carcinogens
3A43	*	
4A22	*	
4F11	Liver, kidney, heart, skeletal muscle, brain	Erythromycin
4F22	*	
4V2	Eye	Fatty acids
4X1	Trachea, brain, pancreas, lung, kidney, liver	Anandamide
4Z1	Breast, breast carcinoma, kidney, liver	Fatty acids
20A1	Liver, brain	
27C1	Liver, kidney, pancreas, spleen, others	

SI – small intestine. * - the expression is not clear. The table was adapted from [36; 37; 38].

1.1.6.1 CYP2W1

The CYP2W1 gene has 5 transcripts (splice variants) of which 3 are protein coding. The gene of CYP2W1 protein maps on chromosome 7, concretely 7p22.3 according to Entrez Gene [39]. The protein transcript used for the selection of peptide sequence for antibody production contains 490 amino acids and has got a molecular weight of 53.844 kDa. The other protein transcripts contain 408 amino acids (44.731 kDa) and 288 amino acids (30.631 kDa) [40].

CYP2W1 is expressed at relatively low levels (mRNA) in the human adult nontransformed tissues, whereas the expression in various tumors (mainly in colorectal cancer tissues) is significantly higher (at both – mRNA and protein levels). Approximately 30% of human colorectal specimens have been found to express CYP2W1 in high amounts [41; 42]. Recent study suggests that the extent of CYP2W1 expression in colorectal cancers might be used as a prognostic marker for malignancy and survival [43]. Furthermore it was found that the CYP2W1 mRNA is expressed in mouse embryos but not in adult animals [44].

A number of carcinogens were shown to be activated to genotoxic forms by heterologously expressed P450 2W1 which may be of particular relevance in light of the localization of this form of P450 in tumor tissues [45].

Gomez et al. have reported that a fraction of CYP2W1 expressed in human embryonic kidney 293 cells is glycosylated and localized on the lumenal site of the endoplasmic reticulum (ER), suggesting that it might not interact well with NADPH:cytochrome P450 oxidoreductase. Despite the reverse orientation in the ER membrane, CYP2W1 in HEK 293 cells was active in the metabolism of indoline substrates and was able to activate aflatoxin B1 into cytotoxic products [46]. Furthermore it has been demonstrated that CYP2W1 is able to catalyze benzamphetamine N-demethylation [47] and fatty acid oxidation [41]. The anticancer agent AQ4N [1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8--dihydroxyanthracene-9,10-dione] is converted to the topoisomerase II inhibitor AQ4 [1,4-bis{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxy-anthracene-9,10--dione] by reduction of *N*-oxides to dimethylamino substituents. This reductive reaction under hypoxic conditions can also be catalyzed by CYP2W1 and CYP2S1, which have been reported to be even better catalyst of this reaction than all previously examined P450 enzymes [48]. Another reaction that may be catalyzed by cytochrome 2W1 is the activation of duocarmycin analogues into cytotoxic substances [49]. The high expression of these forms of P450 together with their high catalytic activities for AQ4N or duocarmycin analogues activation suggest that they may be used for the targeted activation of anticancer prodrugs.

Degradation of the CYP2W1 protein was shown to be enhanced by N-linked glycosylation of the protein. The CYP2W1 protein expression is regulated by methylation of a DNA region, where a cytosine nucleotide is located next to a guanine nucleotide (CpG island) of CYP2W1 gene. It was observed, that colon tumour tissues that over-express CYP2W1 have significantly lower DNA methylation levels when compared to their corresponding nontransformed tissues [50].

1.1.6.2 CYP2S1

The CYP2S1 gene maps on chromosome 19, concretely 19q13.1 according to Entrez Gene [51] and encodes a protein containing 504 amino acids with a molecular weight of 55.817 kDa.

The mRNA expression has been detected in human skin and liver, but there are also reports of expression in trachea, lung, spleen, stomach and small intestine [52]. The CYP2S1 gene is reported to be regulated by the aryl hydrocarbon receptor (AhR) [53; 54]. Even though CYP2S1 is inducible *via* the AhR, no carcinogens have been found to be its substrates [47].

The catalytic specificity of CYP2S1 is uncertain. Smith et al. reported that retinoic acid is a substrate for CYP2S1 [55]. Since then, the oxidation of retinoic acid has not been repeated in any other laboratory attempt [47; 48]. Bui and Hankinson demonstrated that CYP2S1 could not be reduced by NADPH:cytochrome P450 oxidoreductase and that several catalytic activities of this form of cytochrome P450 could be observed if reactions were supported by oxygen surrogates (e.g. hydroperoxides) [56; 57; 58]. Nishida et al. reported that CYP2S1 (as well as CYP2W1) is capable of catalyzing the reduction of AQ4N, a topoisomerase-inhibiting anticancer drug [48].

Wang et al. tested the hypothesis that CYP2S1 is involved in the reductive biotransformation of known carcinogenic aromatic amines and heterocyclic aromatic amines (HAAs). The *N*-hydroxylamines of 2-naphtylamine, 4-aminobiphenyl and 2-aminofluorene were synthetized and found to be reduced by CYP2S1 under both anaerobic and aerobic conditions. Some nitroso and nitro derivatives of the arylamines could also be reduced by cytochrome P450 2S1. None of the amines tested were oxidized by this form of P450. The results suggest that CYP2S1 may be involved in the reductive detoxication of some activated products of HAAs and carcinogenic aromatic amines [59].

1.1.7 Chemoprevention

Chemoprevention or prophylaxis is defined as a use of drugs, chemicals, vitamins or other substances in the diet to prevent or decrease the incidence of a disease [60]. The ideal chemopreventative agent should have the following properties: a) little or no side effects, b) high efficacy, c) capability of oral administration, d) a known mechanism of action and e) low cost. It is difficult to respect all the mentioned properties as the precise mechanism of action of many chemopreventative agents is still unknown. Knowledge of the mechanism of action of the prospective chemopreventative agent would decrease the possibility of untoward interactions with other dietary constituents or administrated drugs [61].

Due to the causes of death in the Czech Republic, where the formation of neoplasms is the second most frequent cause [62], tremendous efforts are being made to prevent and treat cancer. A target group for prophylactic therapy are people exposed to carcinogens, with unhealthy lifestyle, genetic predispositions or patients who underwent cancer in past. Chemopreventive substances avoiding the cancer development are commonly contained in food (especially in vegetable diet) and can be divided into a few groups based on their structure as shown in Table 1.3 (page 23) [63].

Chemopreventive agents can also be divided by the mechanism of action and according the time period that agents appear to have activity in animal models of carcinogenesis. On this base, the three major types of chemopreventative agents are inhibitors of carcinogen formation, "blocking" agents and "suppressing" agents [61]:

- *Inhibitors of carcinogen formation*: act to prevent the formation of nitrosamines from secondary amines and nitrite in an acidic environment. Compounds that inhibit nitrosamine formation are: ascorbic acid, phenols or sulfhydryl compounds [64]. Proline and thioprolines scavenge nitrites by reacting with it to form non-mutagenic nitrosamines [65]. A potential use of these compounds would be to supplement the diet for populations with suspected high rates of endogenous formation of nitrosamines.

- *"Blocking" agents*: genotoxic carcinogens must first be metabolically activated to electrophilic forms that can damage DNA. This initiation phase of carcinogenesis may be blocked by the "blocking" agents. These agents can be divided into five major cathegories, namely: a) inhibitors of cytochromes P450, b) inducers of cytochromes P450, c) inducers of phase II enzymes, such as glutathione S-transferase, d) nucleophilic compounds that can act as scavengers or electrophiles and e) inducers of DNA repair.
- *"Suppressing" agents*: inhibit the promotion or progression phase of carcinogenesis. Many current "suppressing" agents can be cathegorized as a) inhibitors of polyamine metabolism, b) inhibitors of arachidonic acid metabolism, c) protease inhibitors, d) inducers of differentiation, e) inhibitors of oncogene expression, f) inhibitors of protein kinase C and g) inhibitors of oxidative DNA damage.

Despite these beneficial effects, certain evidence proving chemopreventative agents to be ineffective or even detrimental exist. The clinical trials studying the influence of antioxidant food supplements in the years 2007-2008 resulted in showing no preventive effects and in some cases an increase in mortality after using vitamins A, C, E, selenium or ß-carotene [66].

Based on the mechanisms described above it is obvious, that the chemopreventive compounds are also able to affect the level of cytochromes P450 in body, which can influence the metabolism of other xenobiotics. The use of chemopreventive compounds may induce the activity of CYPs, which can cause the metabolic conversion of precarcinogens to carcinogens and thereby increase the risk of a cancer development [67]. Conversely the usage of substances, which may inhibit the activity of CYPs can cause an accumulation of unmetabolized substances in the body, which can further lead to serious health problems and even death [68].

Table 1.3: Chemopreventive agents divided according to their structure.

Structure	Representatives	Source
Carotenoids	lycopene, lutein, astaxanthin, zeaxanthin, cryptoxanthin, α-, ß-carotene	carrots, citrus fruits, peppers, tomatoes, salmon
Vitamins	vitamin C (ascorbic acid), vitamin E (alpha-tocopherol), vitamin A (retinol)	citrus fruits, strawberries, pineapples, broccoli, tomatoes, spinach, kale, green peppers, cabbage, nuts, marine foods
Polyunsaturated fatty acids	docosahexaenoic acid	vegetable-derived liquid oils, walnut oil, fish oil
Conjugated linoleic acid (CLA)	cis-9, trans-11 CLA and trans-10, cis-12 CLA	beef, cheese and whole milk
Organosulfur compounds	diallyl sulfide, N-acetylcysteine and S-allyl cysteine	garlic and onion oil
Phenolic compounds	flavonoids, polyphenols	plant seeds, fruit skin, peel, bark, and flowers
Curcumin	curcumin	turmeric (Curcuma longa)
d-Limonene	d-Limonene	citrus oils
Chlorophyll/Chlorophyllin	chlorophyll, chlorophyllin	higher plants, algae
Chitin and chitosan	chitin, chitosan	fungi, arthropods and marine invertebrate
Isothiocyanates	allyl isothiocyanate, sulforaphane	brassica vegetables
Dehydroepidandrosterone	dehydroepiandrosterone	adrenal hormone
Lactoferrin and its digested fragments	lactoferrin	milk

The data was adapted from [63].

1.2 Antibodies

Antibodies (also called immunoglobulins) are soluble glycoproteins that are capable of recognizing and specifically binding to antigens. Such recognition is an indication of the adaptive immune response of vertebrates. Immunoglobulins can be expressed as membrane-bound receptors on the surface of lymphocytes B (B cells), or as soluble molecules (secreted by plasma cells) present in serum or tissue fluids. Multiple non-covalent bonds mediate a specific interaction between an antibody and

its antigen. These attractive forces include hydrophobic forces, attractive forces between induced dipoles, hydrogen bridge formation and Coulomb forces [69].

The main tasks of antibodies in the body are: a) activation of the complement by classical pathway, b) opsonisation for phagocytosis, c) marking cells for recognition and killing by NK cells and d) sensitization of cells and induction of its apoptosis [69].

Antibodies are an essential tool in protein science. They are often used for detection and/or determination of various antigens making use of methods such as ELISA, Western blotting, affinity chromatography or immunodiffusion. Moreover, these immunoglobulins are widely used in clinical practice for determination of levels of either own antibodies (IgE, HIV test), low molecular weight compounds (progesterone) or proteins associated with various diseases (cancer markers) [70].

Antibodies may be sorted based on their origin into two groups [71]:

- *Polyclonal antibodies* - each of the polyclonal antibodies is produced by a different clone of the plasma cell. These antibodies are able to recognize several epitopes on an antigen, thus a further purification may be required to isolate the group of polyclonal antibodies or to isolate a specific antibody. The main source of the polyclonal antibodies is usually a serum of a previously immunized animal. Recently, antibodies isolated from chicken egg yolks are becoming more popular.

- *Monoclonal antibodies* - are usually produced by hybridoma cells, which are created by fusion of isolated plasma cell precursors with immortal cells. Each clone is grown from one identical parent cell separately, and therefore each clone can produce only one type of antibody. The high specificity of monoclonal antibodies is a significant advantage that allows their therapeutic applications. On the other hand, the process of preparation is expensive and demanding.

1.2.1 General Structure of Antibodies

Immunoglobulins have a structure similar to letter Y consisting of 4 polypeptides – two light chains (L) and two heavy chains (H), linked by disulphide bond. The light and heavy chains are aligned so that the N-terminal end of a single light and single heavy chain form an epitope-binding site, the so called Fab region and the C-terminal end forms the fragment crystallisable region (Fc). Each chain can be subdivided into a homologous region – constant (C) and variable (V) domains (see Fig. 1.3). The usual molecular weight of antibodies varies between 150 to 190 kDa, depending on its class and subclass [72]. The most common immunoglobulins belong to type G (IgG), next IgA, IgM, IgD and IgE.

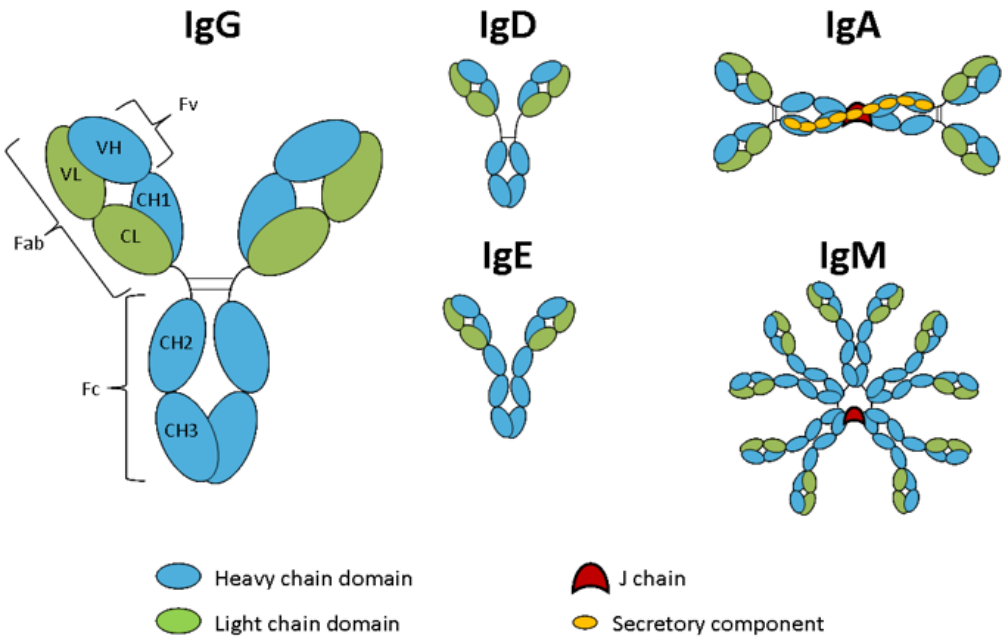

Figure 1.3: Schematic representation of the five immunoglobulin isotypes in mammals. VL – variable region of light chains, VH – variable region of heavy chains, CL – constant region of light chains, CH – constant region of heavy chains. The figure was adapted from [73].

1.2.2 Classes of Mammalian Immunoglobulins

The mammalian immunoglobulins may be sorted into five different classes, based on the differences in the amino acid sequences in the constant region of heavy chains (type of heavy chain is marked by letters of Greek alphabet) – IgG (γ), IgA (α), IgM (μ), IgD (δ) and IgE (ε).

1. **Class IgG**

   IgG is the most universal monomeric immunoglobulin, responsible for most of the ordinary adaptive immune response. It represents approximately 70-75% of the whole immunoglobulin pool and can be organized into 4 subclasses IgG1-IgG4. These subclasses are present in the blood serum in approximate percentage amounts of 66%, 23%, 7% and 4%. Individual classes differ in their functions: opsonisation, toxin neutralization, adaptive immune response and complement activation. The IgG structure is formed by two identical heavy chains containing four domains and two light chains containing two domains, which are assembled by disulphide bonds [69].

2. **Class IgA**

   Immunoglobulins IgA are found mainly on mucous surfaces and in mucous secretions and they represent approximately 15-20% of the serum immunoglobulin pool. The main function of IgA is to prevent infection agents from breaching the physical barrier of tissues. Their structure is similar to a structure of IgG, except that they form dimers connected by a joining chain (J-chain) [69].

3. **Class IgM**

   Immunoglobulins of the M class are mostly pentameric structures which occur in mammal serum and represent 10% of all antibodies. Individual monomers are linked together with covalent disulphide bonds. Each pentameric structure provides ten binding sites, possessing significant avidity for antigens.

After binding of an antigen on a pentameric IgM structure, the classical complement pathway is activated. However, certain amount of IgM can also occur in monomeric form, as an integral part of the B cell membranes (BCR – B cell receptor). In this form, they act as starters of the specific immune response [69].

4. **Class IgD**

Immunoglobulins of the D class exist as both forms – as secreted antibodies (1% of Ig pool) and also in the plasma membrane of B cells, as receptors. This class of immunoglobulins remained mysterious since their discovery in 1964 [74]. Recently it has been observed that circulating IgD bind to basophils. The formed complexes are then responsible for inducing antimicrobial, opsonizing, pro-inflammatory, and B cell stimulating factors such as cathelicidins and interleukins. Due to these effects it seems that IgD orchestrates an ancestral surveillance system at the interface between immunity and inflammation [75].

5. **Class IgE**

Isotypes E of immunoglobulins are monomers which are very scarce in the serum (represent less than 0,01% of the Ig pool). Their main function is to protect against parasitic worms and certain protozoan parasites. IgE also play an essential role in hypersensitivity type I, as they mediate the interaction between antigen and mast cells/basophils through specific Igε receptors. This interaction activates the mast cells which then release mediators from their granules, e.g. histamine or serotonin [69].

1.2.3 Chicken Antibodies

Three immunoglobulin classes – IgA, IgM and IgY have been shown to exist in chicken [76; 77; 78]. Chicken IgY is transported from the blood circulation of mother to the egg yolk and the egg yolk thus contains high concentrations of

chicken IgY [79]. Other Ig classes are present mainly in the egg white. Chicken IgY is functionally equivalent to mammalian IgG in birds but it differs in many functional aspects from mammalian IgG [80].

1.2.3.1 Structure of IgY

Chicken IgY consists of two light and two heavy chains and has got a molecular weight of approximately 180 kDa. The heavy chain consists of four constant regions and one variable region and the light chain is composed of one variable and one constant domain [79]. Comparisons of C-region sequences in IgY and IgG show that the Cγ2 and Cγ3 domains of IgG are the most closely related to the Cυ3 and Cυ4 domains of IgY [81; 82]. The Cυ2 domain of IgY is "condensed" in IgG and forms a hinge region, which provides flexibility to the Fab fragment (Fig. 1.4) [83; 84].

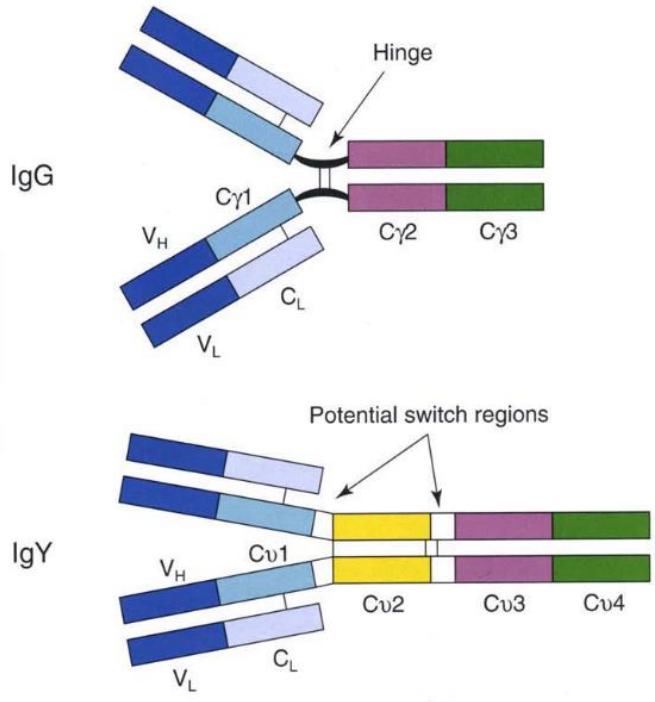

Figure 1.4: Structure of IgG and IgY. V – variable domain, C – constant domain, L – light chain, H – heavy chain. The figure was adapted from [80].

1.2.3.2 Advantages of Chicken Antibodies

Recently, chicken antibodies are gradually becoming popular due to many biochemical advantages over mammalian antibodies. A good immune response in the chicken can be obtained with small amounts of mammalian antigen (0.1–1.0 μg) [85]. Moreover, chicken can produce an unusually high avidity response after a single immunization [86].

Another indisputable advantage of chicken antibodies consists in their production. The concentration of IgY in chicken egg yolks is higher than its concentration in the chicken serum. Moreover, an egg collection is not as labour intensive as a blood sampling. Therefore, the sampling method is non-invasive and does not cause any stress to the animals [87]. Although the purification of yolk IgY is slightly more complicated than the simple serum recovering from blood, many inexpensive procedures of isolation were described. These isolation procedures usually consist of precipitation and centrifugation steps [88].

A hen lays about 250 eggs (approximately 4000 g of native egg yolk) per year. The serum to be obtained from a rabbit is roughly 40 ml. A single gram of the egg yolk was estimated to contain about 10 mg of IgY while 1 ml of a rabbit serum contains about 35 mg of IgG. Hence, all the eggs laid by an immunized hen in one year yield circa 40 g of IgY, while the serum of an immunized rabbit gives only about 1.4 g of IgG. Therefore, the antibody productivity of a hen is estimated to be nearly thirty times as much as of one rabbit [89].

Other advantages are connected with immunological assays. Serum samples and lithium-heparin plasma are the clinically most widely used samples. These samples often contain interfering endogenous compounds which may lead to erroneous results. The complement system in both these samples can be activated by immune complexes if the samples are analysed within a few hours. The activated complement components bind to the antibodies and thereby partly block the antibody binding epitopes. This results in decrease of absorbance to about half value [90]. One of the main advantages of using chicken antibodies is the fact, that IgY does not activate the human complement system [91].

Chicken antibodies also offer other advantages to IgG. Due to a large evolutionary difference, IgY reacts with more epitopes on a mammalian antigen, which results in amplification of the output signal [90].

Chicken antibodies also find a use to avoid interferences caused by rheumatoid factor, human anti-mouse antibodies or Fc-receptors:

- Rheumatoid factor (RF) is an autoantibody that reacts with Fc region of IgG. RF occurs in red blood cells of healthy individuals but also in patient serum during some diseases (e.g. rheumatoid arthritis) and thus it is one of the major sources of interference in immunoassays. While performing a "sandwich" ELISA, where RF is present in the sample and reacts with the capture and detection antibody, false-positive results may be obtained [92].
- Human anti-mouse antibodies (HAMA) may be found in serum of many patients who have received mouse monoclonal antibodies for therapy or diagnosis. These antibodies give false-positive results in "sandwich-type" assays (e.g. RIA or ELISA) [93].
- It is well known that the Fc region of IgG strongly reacts non-specifically with protein A produced by *Staphylococcus aureus* and protein G produced by *Streptococcus* species. Both of these proteins aid bacterial survival and virulence. Chicken immunoglobulin, unlike mammalian, does not react with these proteins because it possesses a different structure of the Fc region [94; 95].

Due to all these advantages IgY offer a convenient alternative to the mammalian polyclonal antibodies.

2 AIMS AND OBJECTIVES OF THE THESIS

The aim of this thesis was to prepare and characterize chicken antibodies distinguishing CYP2W1. For this purpose, it was necessary to choose a suitable antigen for chicken immunization, extract antibodies from egg yolks, separate specific antibodies using affinity chromatography and prove their specificity using immunological methods.

The other objectives of this thesis were to detect CYP2W1 and CYP2S1 in various cell lines and other biological specimens and compare the detection efficacy of these chicken antibodies with antibodies produced in rabbit.

3 MATERIAL AND METHODS

3.1 Used Material

Bio-Rad, UK

Precision Plus Protein™ Unstained standard

Fluka, Switzerland

2-mercaptoethanol, acrylamide, *N,N'*-methylenebisacrylamide (BIS), p-nitrophenyl phosphate (pNPP), sodium dodecyl sulfate (SDS) tris(hydroxymethyl)aminomethane (Tris)

GE Healthcare, USA

CNBr-activated Sepharose^{TM} 4B

Lach-Ner, Czech Republic

acetic acid (CH_3COOH), ammonium persulfate (APS), disodium phosphate (Na_2HPO_4), ethylenediaminetetraacetic acid (EDTA), glycine, hydrochloric acid (HCl), L-cysteine hydrochloride, magnesium chloride ($MgCl_2$), methanol (MeOH), monosodium phosphate (NaH_2PO_4), phosphoric acid (H_3PO_4), potassium hydroxide (KOH), potassium phosphate (KH_2PO_4), sodium acetate (CH_3COONa), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), sodium chloride (NaCl)

Millipore Corporation, USA

Immobilion - NC transfer nitrocellulose membrane

Penta, Czech Republic

ethanol (CH_3CH_2OH), glycine, sodium azide (NaN_3), sodium hydroxide (NaOH)

Protein Milk Lactose company (PML), Czech Republic

non-fat dried milk - Laktino

Rockland, USA

bovine serum albumin - fraction V

Santa Cruz Biotechnology, USA

human CYP2W1 transfected 293T cell lysate

Serva, Germany

adenosine-diphosphate (ADP), Coomassie brilliant blue R-250 (CBB), dialysis tubes, *N,N,N',N'*-tetramethylethylendiamine (TEMED), sodium dodecyl sulfate (SDS), Tris/HCl, Triton X-100, Tween-20

Sigma-Aldrich, USA

alkaline phosphatase-conjugated goat anti-rabbit IgG, alkaline phosphatase-conjugated rabbit anti-chicken IgG, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (BCIP/NBT), diethylamine (DEA), guanidine hydrochloride, Tris hydrochloride

Thermo Fisher Scientific Brand, USA

Imject Maleimide-Activated KLH, Microplate F16 MaxiSorp NUNC-IMMUNO MODULE, SulfoLink^{TM} Coupling Resin, Microplate BCA Protein Assay Kit - Reducing Agent Compatible, PageRuler Prestained Protein Ladder

Whatman, USA

Whatman paper, No. 3

3.2 Used Devices

Analytical balance

Discovery - Ohaus, USA

Balance

Kern EW 600 - 2M, Kern & Sohn GmbH, Germany

Centrifuges

Centrifuge 5415 R, Eppendorf, Germany

Centrifuge 5418, Eppendorf, Germany

Centrifuge K70 D, MLW, Germany

Cooker

Cooker ETA, Czech Republic

Dispenser repeater

Dispenser Repeater®, Eppendorf, Germany

Electrophoresis equipment

Mini-PROTEAN Tetra System, BioRad, UK

Electrophoresis power supply

EPS 301 power supply, Amersham Biosciences, USA

Incubator

IR 1500 Automatic CO_2 Incubator, Flow laboratories, UK

Magnetic stirrer

KMO 2 basic IKA® - IKA®-Werke GmbH & Co., Germany

Microliter syringe

Microliter syringe 702 RN, Hamilton, Switzerland

Microplate reader

Microplate absorbance reader Sunrise, TECAN, Switzerland

Microplate washer

HydroFlex™, Tecan, Switzerland

pH meter

HI 2211, HANNA Instruments, USA

Pippetes

Multi pipette Proline, Biohit, Finland

Nichipet EX, Nichiryo America, Inc., Japan

Protein detection system

SNAP i.d.®, Millipore corporation, USA

Shaker

Shaker, Vývojové dílny, Czechoslovakia

Reax 2, Heidolph, Germany

Sonicator

Elmasonic E30H, P-Lab, Czech Republic

Spectrophotometer

SpectroMOM 195 D, MOM, Hungary

Agilent 8453 Diode Array Spectrophotometer, Hewlett-Packard, USA

Vortex

MS 1 shaker, IKA®, Germany

Water purification system

Milli-Q® Simplicity 185, Millipore corp. USA

Western blot equipment

TransBlot® Turbo™ Transfer System, Bio-Rad, UK

3.3 Methods

3.3.1 Peptide Selection

The successful production of anti-peptide antibodies is often determined by the prediction of the location of certain peptide sequence in the three-dimensional structure of the protein. For such analysis, protein prediction programmes are available. Other important factors to consider are protein hydrophobicity and flexibility.

The length of chosen peptide is another parameter to consider. A peptide of approximately 10-15 residues is optimal for anti-peptide antibody production. Basically longer peptides are better since the number of possible epitopes increases with the peptide length. On the other hand the longer the chosen peptide is, the more expensive the synthesis is.

Based on the prediction programmes (http://blast.ncbi.nlm.nih.gov/, http://www.uniprot.org/, http://web.expasy.org/protscale/), two peptide sequences have been chosen and synthetized by biotechnology company VIDIA, Czech Republic.

3.3.2 Immunogen Preparation

Due to the low molecular weight of the peptide sequences, both chosen peptides had to be conjugated to a large carrier molecule to be able to elicit an immune response and antibody production against the haptens.

As the carrier molecule the KLH (keyhole limpet hemocyanin) was chosen. The KLH molecule was modified with Sulfo-SMCC (sulfosuccinimidyl-4-(*N*--maleimidomethyl)cyclohexane-1-carboxylate) crosslinker that contains an amine-reactive *N*-hydroxysuccinimide esther and a maleimide group. The maleimide group of mcKLH reacts with free sulfhydryl of cysteine and forms a stable thioether bond.

Conjugation of KLH and peptide *via* free sulfhydryls

The most reactive amino acid which occurs in proteins is the cysteine. The sulfhydryl group of cysteine may form a thioether bond with maleimide which is realized by addition to a double bond (Fig. 3.1) [96].

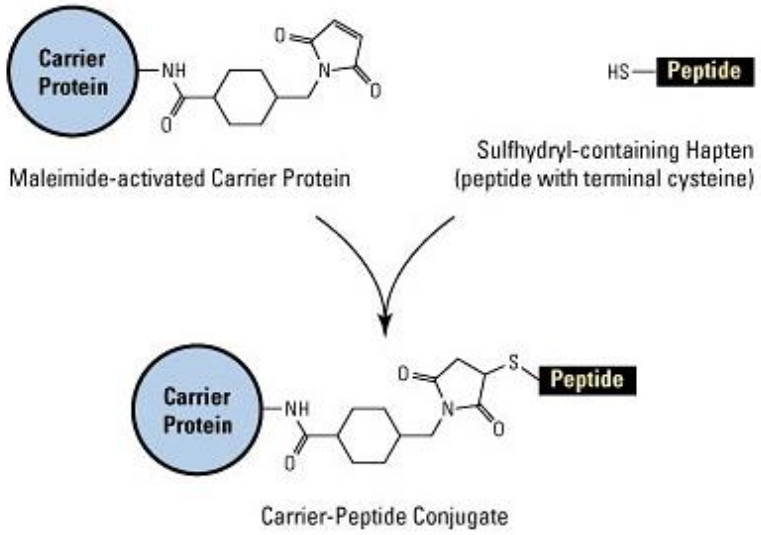

Figure 3.1: Conjugation reaction scheme of Imject Maleimide Activated mcKLH. The figure was adapted from [97].

At first, water solution of mcKLH (10 mg/ml) was prepared. Peptides were dissolved in conjugation buffer (phosphate-buffered saline - PBS; 0.15M sodium chloride, 0.1M sodium phosphate, pH 7.2) to prepare the same concentration as the mcKLH. Equal volumes of both solutions (250 µl of mcKLH + 250 µl of peptide solution) were mixed and reacted while stirring for 2 hours at room temperature. To the peptide conjugates, 2 ml of PBS were added to make the final concentration of peptides in solutions 1 mg/ml. The solutions were aliquoted (150 µl) into Eppendorf tubes and stored at -20°C.

3.3.3 Immunization of Chickens

The Leghorn chickens were bred in separate cages with a 12h day/night cycle with permanent access to water and food. Before the first immunization, 4-7 eggs of each chicken were collected. Subsequently, antibodies isolated from these eggs were used as negative control samples for the future experiments.

The immunization was performed intramuscularly in three weekly doses of 100 µl of KLH peptide conjugate (100 µg of peptide). The first dose was administrated in complete Freund's adjuvant, the two following doses were administrated in incomplete Freund's adjuvant. The eggs which were collected after the immunization were stored at 4°C.

3.3.4 Antibody Isolation

The immunoglobulin fraction (IgY) was isolated from pooled chicken egg yolks according to Hodek et al. [98]. For the isolation of specific antibodies, eggs collected one week after the last immunization were used. Egg yolks were separated from egg whites, washed with tap water and pooled into a calibrated cylinder. The pooled volume of egg yolks was diluted with tap water in a ratio 1:7 and pH of this solution was adjusted to pH 5.0 using 0.5 M HCl. The suspension of diluted yolks was frozen at -20°C.

The following day, a water-soluble fraction was separated from lipids during spontaneous thawing through a filter paper at room temperature. The filtrate was precipitated by addition of solid NaCl to a final concentration of 8.76% and pH was adjusted to 4.0. The mixture was stirred for 30 minutes and then incubated for additional 90 minutes at room temperature. The precipitate was collected by centrifugation at 2700×g for 20 minutes at 4°C (Janetzki K70). The supernatants were discarded and the pellets were resuspended in about 6 ml PBS (containing 0.1% sodium azide). The obtained solutions were stored in tubes at 4°C.

Protein concentration was detected by measuring absorbance of the samples at 280 nm (SpectroMOM 195 D) using PBS (containing 0.1% sodium azide) as a reference sample, according to formula:

$$c = A_{280} \cdot f \cdot n,$$

where A_{280} is an absorbance at 280 nm, f is the empiric factor = 1.094 and n is the dilution of sample.

3.3.5 Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) was used to determine the specificity of isolated antibodies. The quantification of specific primary antibody in this method is based on a colour change given by reaction of enzyme bound to a secondary antibody with its suitable substrate. At first, each well of microtitre plate is coated with an antigen. After that, a primary antibody is added, followed by incubation with a compatible secondary antibody conjugate. Each step is succeeded by series of buffer rinses to remove all unbound proteins. The final step is the addition of the enzyme substrate and the production of coloured product with enzyme bound to the secondary antibody. When the reaction is complete and terminated, the entire plate is placed into a plate reader and the optical density of each well is determined. The amount of coloured product is proportional to the amount of primary antibody bound to the antigen.

Reagents:

Immobilization buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$; pH 9.6

Washing agent (PBS-Tween 20): 13.4 mM NaCl, 1.8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 0.1% (v/v) Tween 20; pH 7.2

Blocking buffer: 2% (w/v) egg white solution in PBS-Tween 20

PBS: 13.4 mM NaCl, 1.8 mM Na_2HPO_4, 1 mM NaH_2PO_4; pH 7.2

STOP solution: 3M NaOH

Substrate solution: 1 mg/ml p-nitrophenyl phosphate (pNPP), 0.02 M Na_2CO_3, 0.03 M $NaHCO_3$, 1mM $MgCl_2$

All samples and solutions were equilibrated to a room temperature. A microtitre plate (F16 MaxiSorp NUNC-IMMUNO MODULE) was coated with a solution of antigen. The antigen solution was prepared by the dilution of antigen to a concentration 4 μg/ml in immobilization buffer. Into each well 100 μl of the antigen solution was added and the plate was incubated overnight at 4°C.

The next day, all unbound antigen was washed off by a washing buffer (4×200 μl). After that, 150 μl of a blocking solution was added into each well and the plate was incubated at 37°C for 1 hour. After the incubation, the microtitre plate was washed by a washing buffer (4×200 μl) and the primary antibody was added at following concentrations: 3.3; 10.0; 30.0 and 90.0 μg/ml in PBS and incubated at 37°C for 2 hours. Subsequently, the microtitre plate was washed again and 100 μl of alkaline phosphatase-conjugated rabbit anti-chicken antibody in PBS was added to each well (diluted in ratio 1:1 400). This step was followed by 1 hour incubation at 37°C and washing. At the end, 100 μl of substrate solution with pNPP was added and the reaction was stopped by addition of 100 μl of 3 M NaOH after 10 minutes. The absorbance at 405 nm was measured immediately (Microplate absorbance reader Sunrise) and data were evaluated.

3.3.6 Affinity Purification of Anti-human CYP2W1 Antibody

Because the reactivity of anti-peptide antibodies is often not specific and they may display cross-reactivity with other antigens, affinity purification was performed. In this process, we took advantage of the terminal cysteine in the peptide sequence. Sulfhydryl-containing peptide was immobilized on SulfoLink® Coupling Resin by its reaction with iodoacetyl groups resulting in thioether bond (Fig. 3.2). After the peptide was immobilized, the gel was incubated with the blocking buffer. The affinity column was then filled with a stationary phase and incubated with the target antibody solution. Finally, the fraction of specific antibodies was released and used for immunoassay.

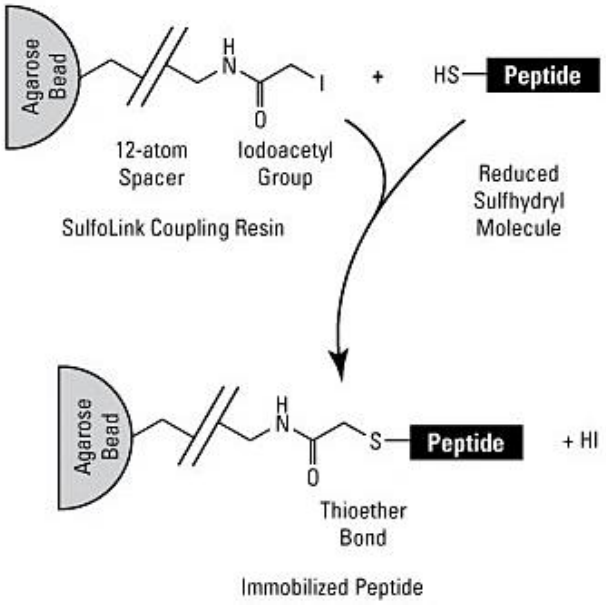

Figure 3.2: Scheme of peptide immobilization. The figure was adapted from [97].

Reagents:

Coupling buffer: 50 mM Tris/HCl, 5 mM EDTA; pH 8.5

Washing buffer: 1.0 M NaCl

Blocking buffer: 50 mM L-cysteine·HCl, 50 mM Tris, 5 mM EDTA; pH 8.5

Storage buffer: PBS, 0.1% (w/v) NaN_3

Elution buffer: 50 mM diethylamine; pH 11.5

Neutralization buffer: 1 M potassium phosphate, pH 6.7

Potassium phosphate buffer: 0.1 M KH_2PO_4, pH 7.2

The peptide immobilization procedure:

Synthetized peptides were dissolved in 2 ml of coupling buffer to a final concentration of 0.5 mg/ml. Because the peptides contained hydrophobic amino acids and were not easily soluble, 50 µl of DMSO was added prior to the dissolving. The mixture was incubated for 10 minutes at room temperature. Meanwhile, affinity column was filled with 3 ml of the SulfoLink® Coupling Resin, which settled in 1.5 ml of gel. The column was rinsed with 8 ml of coupling buffer and then the gel slurry was mixed with 2 ml of the peptide solution on end-over-end mixer for

15 minutes. This step was followed by 30 minutes incubation upright at room temperature for the gel to settle down. The unbound peptide was removed and the column was then washed with 6 ml of coupling buffer. Free iodoacetyl groups were blocked with 2 ml of blocking buffer and the solution was again mixed on end-over-end mixer for 15 minutes, followed by 30 minutes incubation upright at room temperature. In the next step, the flow through was discarded from the column. The weakly bound antigen was released by: (i) 12 ml of 1 M NaCl; (ii) 6 ml of diethylamine (DEA), pH 11.5; (iii) 6 ml of 0.1 M potassium phosphate buffer, pH 7.2 and (iv) 6 ml of PBS.

Affinity purification of anti-peptide antibodies:

Solution of anti-human CYP2W1 antibody (20 ml ~ 824.6 mg of 2W1c and 10 ml ~ 405.1 mg of 2W1d) was incubated with immobilized peptide on end-over-end mixer overnight at 4°C. Next day, the unbound IgY fraction was collected for the ELISA assay. The column was washed with PBS until the absorbance at 280 nm of the flow through reached the 0.04 value. The weakly bound IgY fraction was eluted with 10 ml of 1 M NaCl in PBS and 1 ml-fractions were collected into Eppendorf tubes. After washing the column with 15 ml of PBS, the specific antibodies were eluted using an elution buffer and 1 ml-fractions were collected into Eppendorf tubes containing 200 µl of neutralization buffer. Fractions with the highest amount of protein were pooled and dialyzed against 10 l of storage buffer overnight at 4°C. The column was carefully washed with the storage buffer and stored at 4°C.

To the affinity purified antibodies, bovine serum albumin (BSA, fraction V) was added to a final concentration of 1 mg/ml to prevent nonspecific adsorbtion of antibodies to glass vials while storage.

3.3.7 Determination of Protein Concentration

Protein concentration in lysate of colorectal adenocarcinoma cell line DLD-1 (cells were kindly provided by RNDr. Alena Hyršlová-Vaculová, Ph.D.; Institute of Biophysics, Academy of Sciences of the Czech Republic) was determined using bicinchonic acid (BCA) protein assay. The principle of this method is based on the ability of certain amino acids (cystine, cysteine, tyrosine, and tryptophan) and the peptide bond to reduce a copper cation Cu^{2+} to a cuprous cation Cu^{+}. In alkaline environment, BCA forms with Cu^{+} a violet complex. This complex exhibits a strong absorbance at 562 nm, which is linear with increasing protein concentrations.

Reagents:

Reagent A: 2% (w/v) $Na_2CO_3.H_2O$, 0.95% (w/v) $NaHCO_3$, 0.4% (w/v) NaOH, 0.16% (w/v) sodium tartarate

Reagent B: 4% (w/v) $CuSO_4.5H_2O$

BCA working reagent: 50 parts of reagent A + 1 part of reagent B + BCA in a final concentration of 1% (w/v)

Compatibility reagent solution: content of one Compatibility reagent tube diluted with 100 µl of Working Reconstitution Buffer

Working Reconstitution Buffer: Reconstitution Buffer diluted 1:1 with ultrapure water

The protein concentration in DLD-1 cell lysate was determined using Thermo Scientific™ Pierce™ BCA protein kit – Reducing Agent Compatible, based on method by Wiechelman et al. [99]. In this method, the BSA was used as the standard.

At first, 60 µl of electrophoresis sample buffer (the composition is described in chapter 3.3.9) was added to an approximate cell amount of 10^6. The mixture was boiled for 5 minutes. After that, 60 µl of ultrapure water was added and a 5 minute centrifugation at 13 500 RPM (Centrifuge 5418, Eppendorf) was performed.

Standards for the calibration curve were prepared by diluting the Albumine Standard Ampule (2 mg/ml) with ultrapure water to concentrations: 1.5; 1.0; 0.75; 0.5; 0.25 and 0.125 mg/ml. The standards were applied in duplicates to reduce the error probability.

The sample was diluted 25×, 50×, 100× and 200× and pipetted in triplicates into 96-well microplate in a volume of 9 µl (blank sample contained 9 µl of ultrapure water). Into each well containing sample, 4 µl of Compatibility Reagent Solution were added and the plate was incubated at 37°C for 15 minutes. After that, 260 µl of BCA working reagent was added to each well and the plate was incubated at 37°C for 30 minutes. After 5 minutes, the absorbance at 562 nm was measured. Data were evaluated by the Kim32 program.

3.3.8 Cell Cultures and Other Biological Material

The biological material that was used for determining the presence of CYP2W1 or CYP2S1 is shown in Tab. 3.1 (page 46).

As a standard the following material was used:

- HEK293T: human CYP2W1 transfected embryonic kidney 293T cells,
- purified CYP2W1 expressed in *Escherichia coli*,
- purified CYP2S1 expressed in *Escherichia coli*.

Table 3.1: Used human cells.

Biological material	Specification
CRCA	human colorectal cancer cells
RPE	transformed human retinal pigment epithelial cell line
U2OS	human bone osteosarcoma epithelial cell line
A549	human lung adenocarcinoma epithelial cell line
A375	human malignant melanoma cell line
HeLa	human cervical cancer cell line
Saos-2	human osteosarcoma cell line
Cal51 MCF7 MDA-MB-231 ZR-75-1 ZR-75-30 BT-474	human breast cancer cell lines
Aspc BxPC3 CAPAN MiaPaCa Panc PaTu	human pancreatic cancer cell lines
A2780	human ovarian carcinoma cell line
HCT116 DLD-1	human colorectal carcinoma cell lines

More detailed information about the used human cells is provided in Appendix (page 89).

3.3.9 SDS-polyacrylamide Gel Electrophoresis

Electrophoresis is an electrochemical technique separating proteins based on their molecular weight and charge. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) uses an anionic detergent sodium dodecyl sulphate (SDS) which denatures proteins in the sample and imparts them a negative charge. Proteins are then separated according to their molecular weight when they pass through the gel.

Reagents:

Buffer A: 0.375 M Tris/HCl, pH 8.8; 0.1% (w/v) sodium dodecyl-sulphate (SDS)
Polymerization solution A: 30% (w/v) acrylamide; 0.8% (w/v) BIS - in buffer A
Buffer B: 0.125 M Tris/HCl, pH 6.8; 0.1% (w/v) SDS; 0.006% (w/v) bromphenol blue
Polymerization solution B: 30% (w/v) acrylamide; 0.8% (w/v) BIS - in buffer B
Sample buffer: 0.25 M Tris/HCl; 8% (w/v) SDS; 20% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol; 0.004% (w/v) bromphenol blue, pH 6.8
Electrode buffer: 0.192 M glycine; 0.025 M Tris/HCl, pH 8.3; 0.1% (w/v) SDS

For 1 electrophoresis gel:

Resolving gel 8%: 5.5 ml buffer A; 2 ml polymerization solution A; 75 µl (100 mg/ml) ammonium peroxodisulphate (APS); 7.5 µl tetramethylethylendiamine (TEMED)
Stacking gel 3%: 2.7 ml buffer B; 300 µl polymerization solution B; 60 µl (100 mg/ml) APS; 3 µl TEMED

At first, the solution for 8% resolving gel was poured between the glasses (1 mm thickness, 10×8.2 cm), immediately overlaid with distilled water and allowed to polymerize for 30 minutes at room temperature. Next, distilled water was removed and the area above the resolving gel was dried with filter paper. The 3% stacking gel solution was poured on top of the resolving gel, the comb (10 wells) was placed into the gel and the gel was polymerized for 15 minutes at room temperature. After the

polymeration, the glass with gel was placed into electrophoresis rack. The comb was carefully displaced and the rack was filled with electrode buffer.

Cell lysate samples were diluted to the final protein concentration of 1 mg/ml and then were diluted in a ratio 3:1 with the 4× sample buffer. All samples were boiled in 100°C water bath for 5 minutes. PageRuler Prestained Broad Range Protein Ladder – Thermo Scientific/Precision Plus Protein™ standard – Bio-Rad (5 µl) and cell lysate samples (20 µl = 15 µg) were then loaded into the gel wells by a Hamilton syringe.

The electrophoresis chamber was filled with electrode buffer and connected to a power supply. The voltage was set on 200 V and electrophoresis ran until the tracking dye (bromphenol blue) reached the bottom of the gel.

The gel was removed and the separated proteins were visualized either by Coomassies Brilliant Blue staining or using Western blot.

3.3.10 Coomassie Brilliant Blue Staining of Polyacrylamide Gel

Reagents:

Staining bath: 0.25% (w/v) Coomassie Brilliant Blue R-250, 46% (v/v) ethanol; 9.2% (v/v) acetic acid

Distaining bath: 25% (v/v) ethanol, 10% (v/v) acetic acid

Polyacrylamide gel after electrophoresis was transferred into the staining bath for 1 hour at room temperature with a gentle agitation. The gel was then left in distaining bath overnight and scanned.

3.3.11 Western Blot

The Western blot is used for immunodetermination of the relative amounts of proteins present in various samples. After the electrophoresis, separated proteins are transferred on a membrane and then visualized using specific antibodies against desired proteins.

Reagents:

Transfer buffer: 0.025 M Tris; 0.192 M glycine; pH 8.3

PBS Triton X-100: 0.134 M NaCl; 1.8 mM $Na_2HPO_4.10H_2O$; 1mM NaH_2PO_4; pH 7.2; 0.3% (w/v) Triton X-100

Blocking solution: 5% (w/v) non-fat dried milk in PBS Triton X-100

Following the electrophoresis, the gel was equilibrated with the transfer buffer for 30 minutes at room temperature with a gentle agitation. Meanwhile, the nitrocellulose membrane and Whatman papers No. 3 were cut to correspond with the gel dimensions. The membrane was placed into electrode buffer, washed in distilled water for 2 minutes and kept in transfer buffer for 20 minutes.

An anode of the TransBlot® Turbo™ Transfer system was covered with three Whatman papers soaked in transfer buffer. The membrane was placed on the top of the Whatman papers and layered with the gel. The whole assembly was covered with another three Whatman papers soaked in transfer buffer. The blotting sandwich was thoroughly compressed and the cathode was placed on the top of it. The complete blotting cassette was put into Transfer System and ran for 7 minutes at constant voltage 25 V. When the transfer was complete, the membrane was blocked in 5% non-fat dried milk overnight at 4°C with a gentle agitation. Next day, the membrane was incubated with a solution of primary antibody in blocking solution (15 or 30 µg/ml for unpurified antibodies and 0.5; 1; 2 or 5 µg/ml for antibodies purified by affinity chromatography) for 2 hours at room temperature with a gentle agitation. After that, the membrane was washed for 5 minutes in PBS Triton X-100.

For the incubation with a secondary antibody, the Snap i.d. Protein Detection System (Millipore) was used. The membrane was placed on chambers with the protein side down. Air bubbles were gently removed and the blot holder was placed into the apparatus. The membrane was washed three times with 15 ml of PBS Triton X-100 and then incubated with the secondary antibody (rabbit anti-chicken or goat anti-rabbit alkaline phosphatase conjugated) diluted 1:1429 in PBS Triton X-100 for 10 minutes at room temperature.

Finally, the secondary antibody was removed and the membrane was again washed three times with 15 ml of PBS Triton X-100 and then PBS. The membrane was developed with a BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) tablet containing 10 mg substrate for alkaline phosphatase dissolved in 10 ml of distilled water. The membrane was incubated in the developing solution until the bands appeared and then placed into distilled water and dried between filter papers.

3.3.12 "Negative" Affinity Purification

Occasionally, even antigen affinity purified antibodies may suffer from a lack of specificity. The cross-reactivity is traditionally removed by the "negative" affinity purification – a method used to deplete a known contaminant from a protein mixture. The cross-reacting antigen is bound to a column matrix, the cross-reactive antibody binds to it and thus it is removed from the total antibody pool.

Reagents:

Coupling buffer: 0.1 M $NaHCO_3$, 0.5 M NaCl; pH 8.5
Swelling buffer: 1 mM HCl; pH 3.0
Blocking buffer: 0.1 M Tris-HCl; pH 8.0
Washing buffer A: 0.1 M CH_3COOH/CH_3COONa; pH 4.0
Washing buffer B: 0.1 M Tris-HCl, 0.5 M NaCl; pH 8.0
PBS: 13.4 mM NaCl, 1.8 mM Na_2HPO_4, 1 mM NaH_2PO_4; pH 7.2

The antigen immobilization procedure:

At first, the activity of the reactive groups of the CNBr-activated Sepharose 4B was preserved: 15 ml of swelling buffer was added to 0.29 g of CNBr-activated Sepharose 4B and mixed at end-over-end mixer for 30 minutes at room temperature. The swollen Sepharose (approximately 1 ml of gel resin) was then placed into the affinity column and washed with 60 ml of swelling buffer and 5 ml of coupling buffer. A ligand to be coupled (5 nmol of CYP2W1 or CYP2S1) was mixed with 2 ml of coupling buffer and added to the column. The column content was then mixed on end-over-end mixer overnight at 4°C. The unreacted active groups were blocked by incubating the beads for 2 hours at room temperature with 2 ml of blocking buffer. The blocking buffer was removed and the medium was then washed with three cycles of alternating pH (5 ml of washing buffers A and B). Finally, the column was equilibrated with 15 ml of PBS.

The negative affinity purification:

To the gel resin, 2 ml of PBS were added and 1.5 ml of the gel resin was separated into Eppendorf tube containing 1 mg of IgG protein. Incubation was performed at end-over-end mixer for 2 hours at room temperature. After that, the mixture was applied to a respective column with the rest of gel resin. The flow-through was collected and reapplied four times. Protein concentration of the resulting IgG fraction was determined by the BCA protein assay (chapter 3.3.7, page 44).

4 RESULTS

4.1 Peptide Selection

From the primary structure of CYP2W1 (Fig. 4.1) two amino acid sequences were chosen for the immunogen preparation. Both sequences, one from C-terminal and one from N-terminal end of the protein, were selected based on data provided by protein structure prediction software.

Prediction software was used to determine whether the sequence is located on the protein surface (Fig. 4.2, 4.3) and whether it contains any secondary structure (Fig. 4.6). Furthermore, an antigenicity of each peptide was determined (Fig. 4.4, 4.5). Considering these parameters, the following peptide sequences were chosen for the immunogen preparation:

- CYP2W1c: C-^{466}SLDTTPARAFTMRPR^{480}
- CYP2W1d: ^{21}CAQDPSPAARWP^{32}

C- indicates cysteine which was added to the original protein sequence for further conjugation with the carrier protein (KLH)

```
        10         20         30         40         50
MALLLLLFLG LLGLWGLLCA CAQDPSPAAR WPPGPRPLPL VGNLHLLRLS
        60         70         80         90        100
QQDRSLMELS ERYGPVFTVH LGRQKTVVLT GFEAVKEALA GPGQELADRP
       110        120        130        140        150
PIAIFQLIQR GGGIFFSSGA RWRAARQFTV RALHSLGVGR EPVADKILQE
       160        170        180        190        200
LKCLSGQLDG YRGRPFPLAL LGWAPSNITF ALLFGRRFDY RDPVFVSLLG
       210        220        230        240        250
LIDEVMVLLG SPGLQLFNVY PWLGALLQLH RPVLRKIEEV RAILRTLLEA
       260        270        280        290        300
RRPHVCPGDP VCSYVDALIQ QGQGDDPEGL FAEANAVACT LDMVMAGTET
       310        320        330        340        350
TSATLQWAAL LMGRHPDVQG RVQEELDRVL GPGRTPRLED QQALPYTSAV
       360        370        380        390        400
LHEVQRFITL LPHVPRCTAA DTQLGGFLLP KGTPVIPLLT SVLLDETQWQ
       410        420        430        440        450
TPGQFNPGHF LDANGHFVKR EAFLPFSAGR RVCVGERLAR TELFLLFAGL
       460        470        480        490
LQRYRLLPPP GVSPASLDTT PARAFTMRPR AQALCAVPRP
```

Figure 4.1: Primary sequence of CYP2W1. The figure was adapted from [100]. Chosen peptides are marked by red colour.

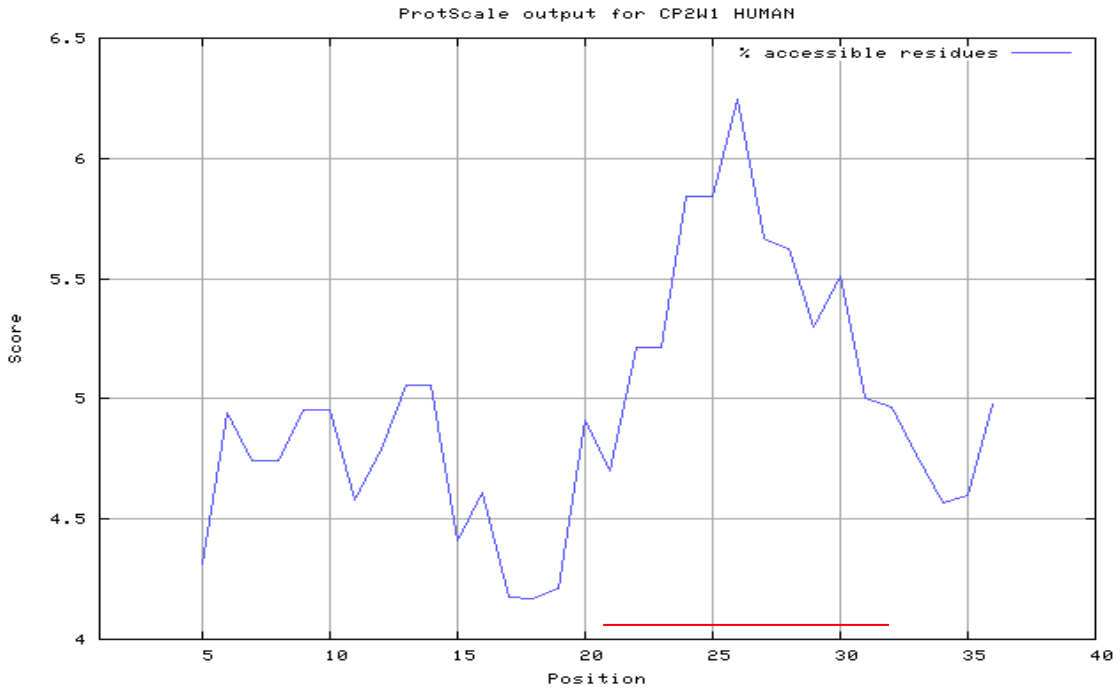

Figure 4.2: Prediction of accessibility of the N-terminal end of CYP2W1. The figure was adapted from [101]. Chosen peptide sequence is located at positions 21-32.

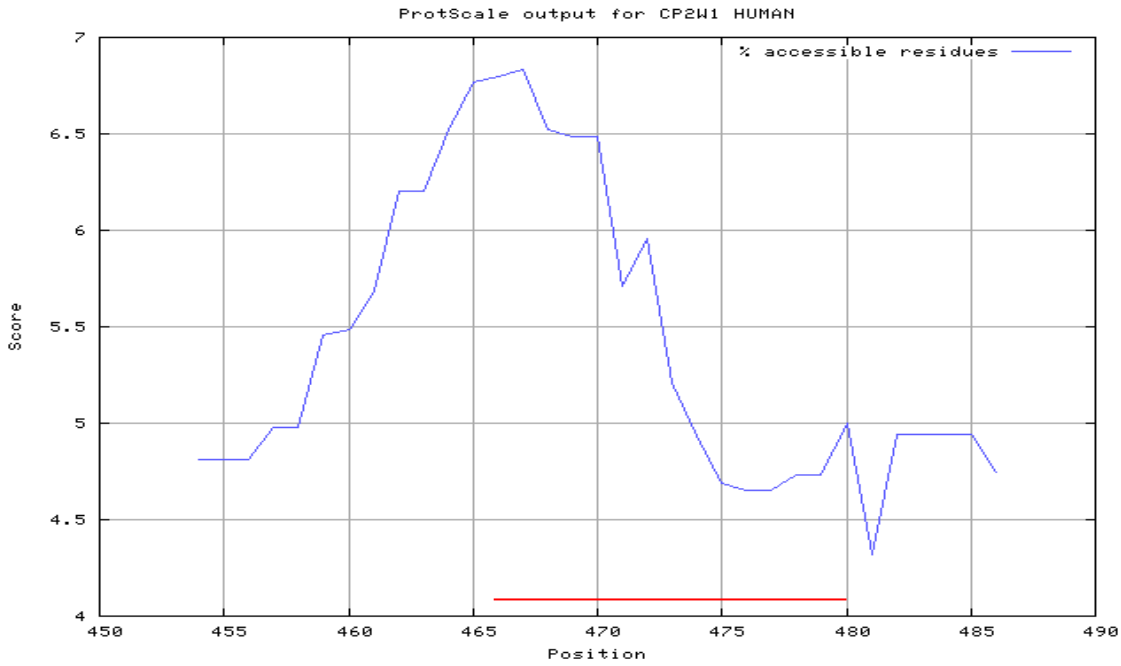

Figure 4.3: Prediction of accessibility of the C-terminal end of CYP2W1. The figure was adapted from [101]. Chosen peptide sequence is located at positions 466-480.

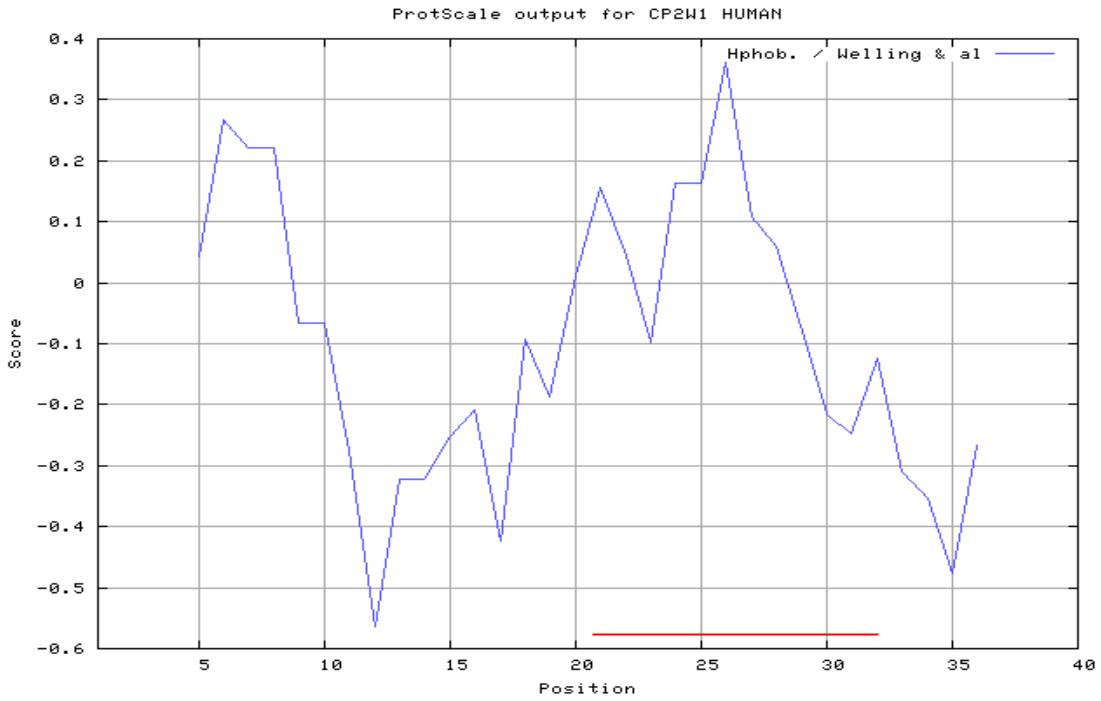

Figure 4.4: Immunogenicity prediction of N-terminal end of CYP2W1. The figure was adapted from [101]. Chosen peptide sequence is located at positions 21-32.

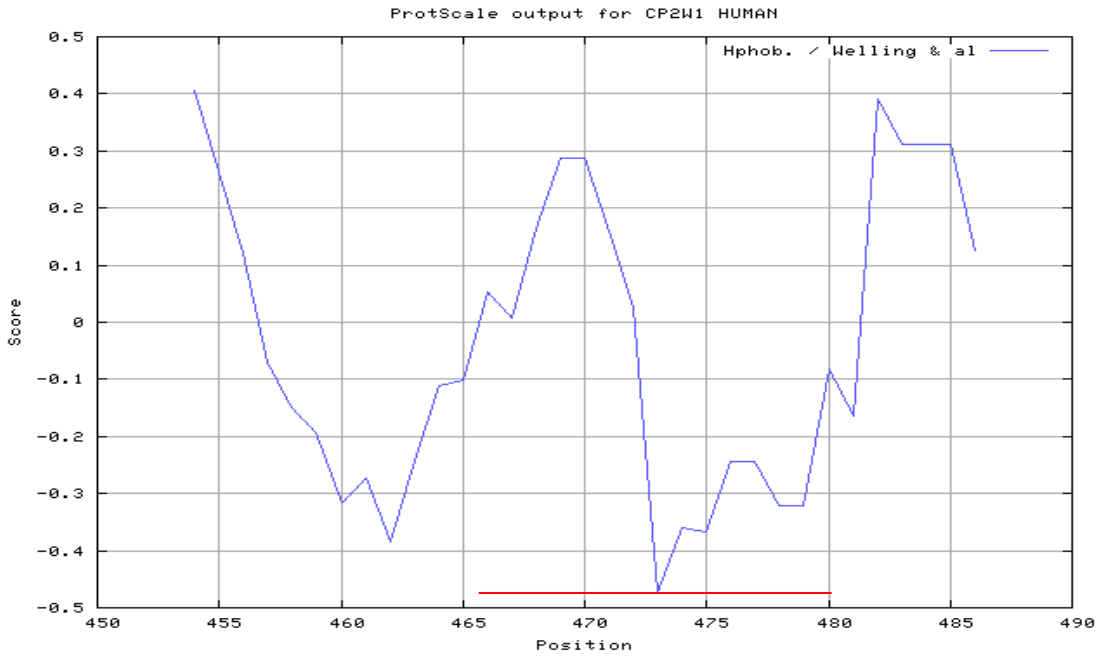

Figure 4.5: Immunogenicity prediction of C-terminal end of CYP2W1. The figure was adapted from [101]. Chosen peptide sequence is located at positions 466-480.

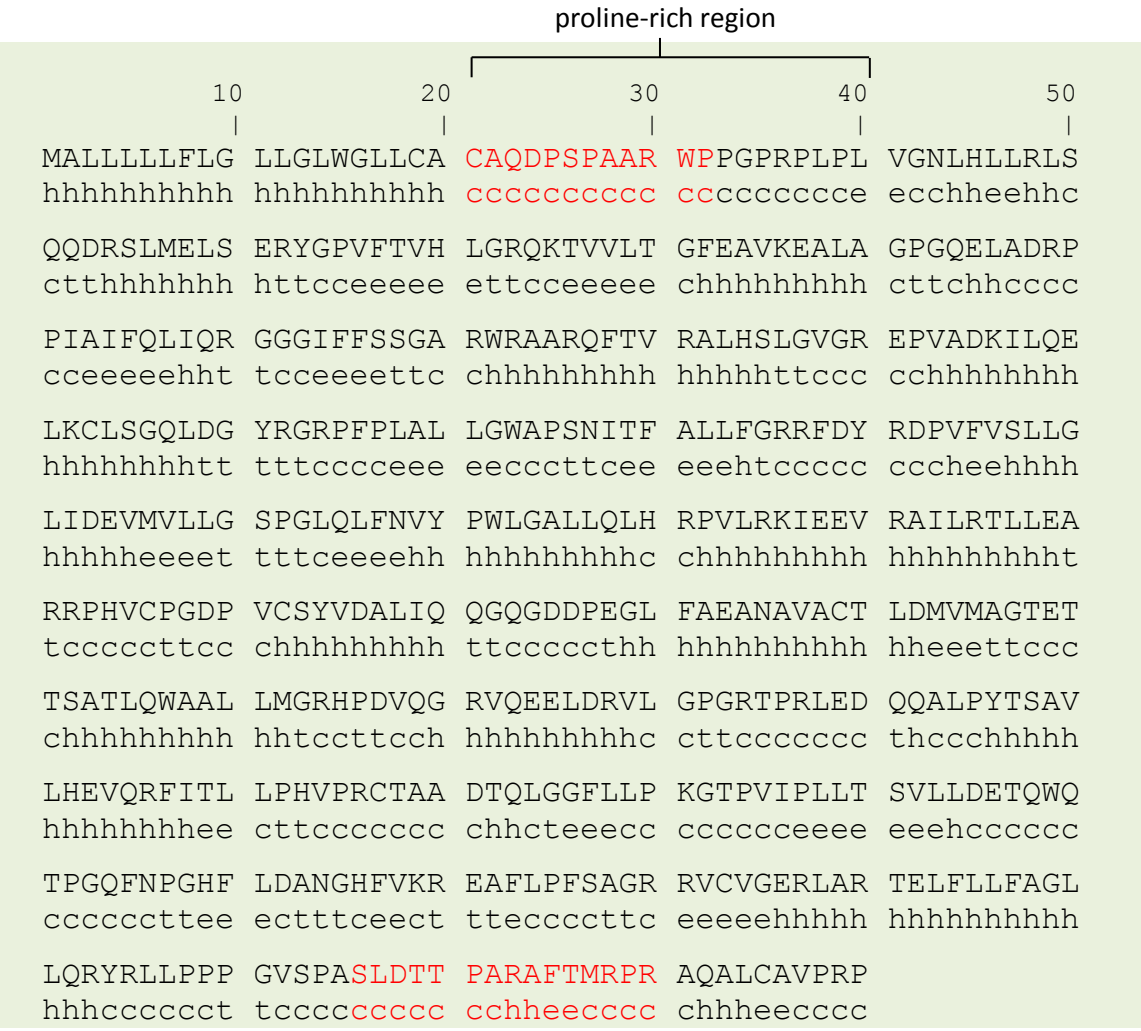

Figure 4.6: Secondary structure prediction of CYP2W1. The figure was adapted from [102]. h = alpha helix, c = random coil, e = extended strand, t = beta turn. Chosen peptides are marked by red colour.

4.2 Immunogen Preparation

Both of the chosen peptide sequences contained terminal cysteine and were highly soluble in conjugation buffer (0.15 M NaCl, 0.1 M NaH_2PO_4, pH 7.2). The peptide solutions were immediately mixed with activated mcKLH and reacted for 2 hours at room temperature.

4.3 Chicken Immunization

Two healthy Leghorn chickens were picked to be vaccinated by prepared immunogens. Five control eggs were collected from each chicken within two weeks before the immunization. Eggs collected after the chicken immunization (7 eggs after the vaccination by CYP2W1c and 4 eggs after the vaccination by CYP2W1d) were used for further antibody isolation.

4.4 Antibody Isolation

The antibody isolation was performed by two-step procedure consisting of extraction of yolks with tap water followed by a specific precipitation of IgY by NaCl at pH 4.0. The final volume of each antibody fraction was measured and the protein concentration was determined based on the absorbance at 280 nm.

The final volumes and IgY concentrations are shown in Tab. 4.1.

Table 4.1: Characterization of IgY fractions.

IgY	Egg yolk [ml]	IgY fraction [ml]	c_{IgY} [mg/ml]	Total IgY [mg]
control 2W1c	100	8	56.9	455.2
2W1c	144	27	41.2	1112.4
control 2W1d	95	8	54.2	433.6
2W1d	76	15	40.5	607.5

Egg yolk – total volume of egg yolk, IgY fraction – total volume of the obtained IgY fraction, c_{IgY} - concentration of the obtained IgY fraction, Total IgY – the total mass of obtained IgY.

4.5 Specificity of Isolated Antibodies

The specificity of isolated antibodies was verified by ELISA. For this assay, all samples were prepared in triplicates with a final IgY dilution of 3.3; 10.0; 30.0 and 90.0 μg/ml. The relevant peptide was bound to microplate surface as an antigen. A rabbit anti-chicken IgY conjugated with alkaline phosphatase was used for the IgY detection. The alkaline phosphatase converted p-nitrophenyl phosphate contained in the developing solution into a yellow product: p-nitrophenol. In order to determine the amount of p-nitrophenol formed during the reaction, the absorbance at 405 nm was measured. The values of measured absorbance corresponding to IgY amount bound to the respective antigen are shown in Fig. 4.7.

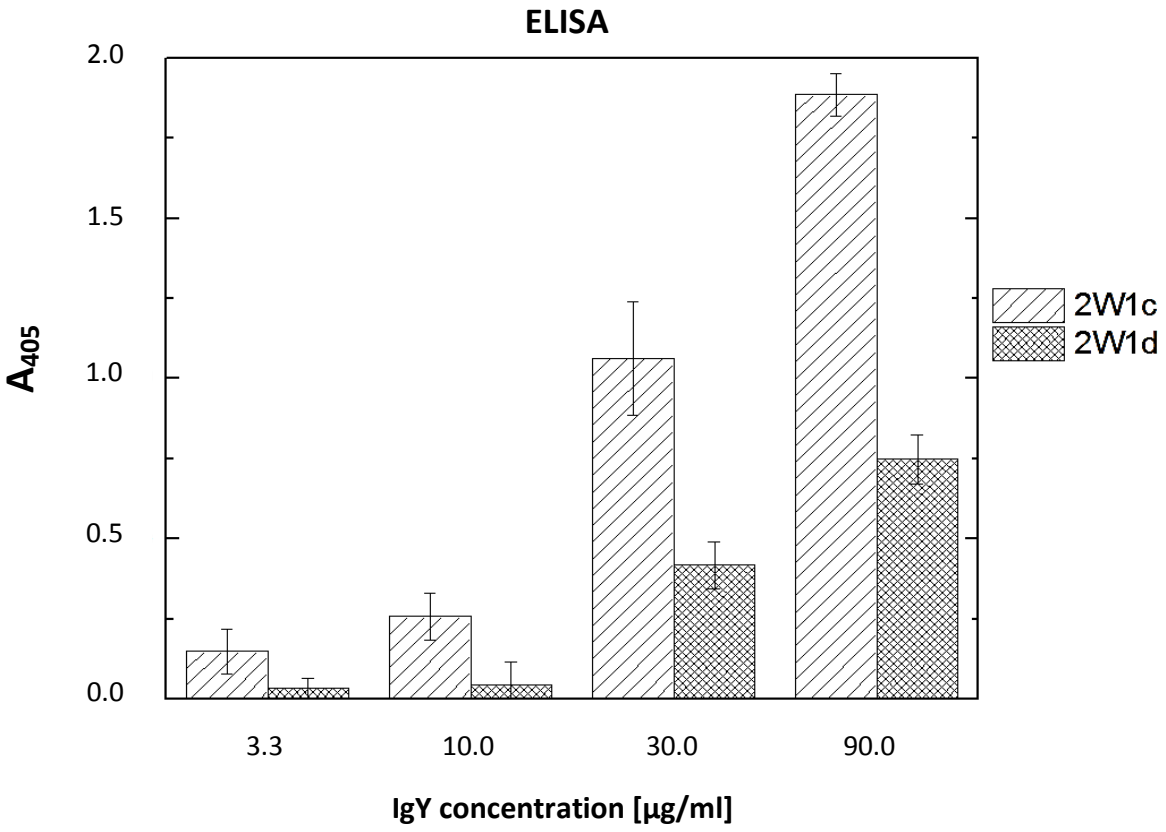

Figure 4.7: Reactivity of IgY fraction with peptide antigens. Graph shows absorbance values at 405 nm of specific IgY fractions after subtraction of control values, at concentrations 3.3; 10.0; 30.0 and 90.0 μg/ml. The data are means of triplicates with plotted standard deviations.

4.6 Affinity Purification of IgY

In order to obtain antigen-specific IgY fraction and thus avoid cross-reactivity, the affinity purification of antibodies was performed. Fractions of IgY (20 ml ~ 824.6 mg of 2W1c or 10 ml ~ 405.1 mg of 2W1d) were used for the purification step. The purification efficiency has been evaluated by an ELISA comparing the absorbance of the original and purified IgY fraction at 405 nm. Figures 4.8 and 4.9 show an increase in absorbance of specific 2W1c and 2W1d antibody fractions compared to the unpurified ones. These results indicate that the procedure of antibody purification has been successful.

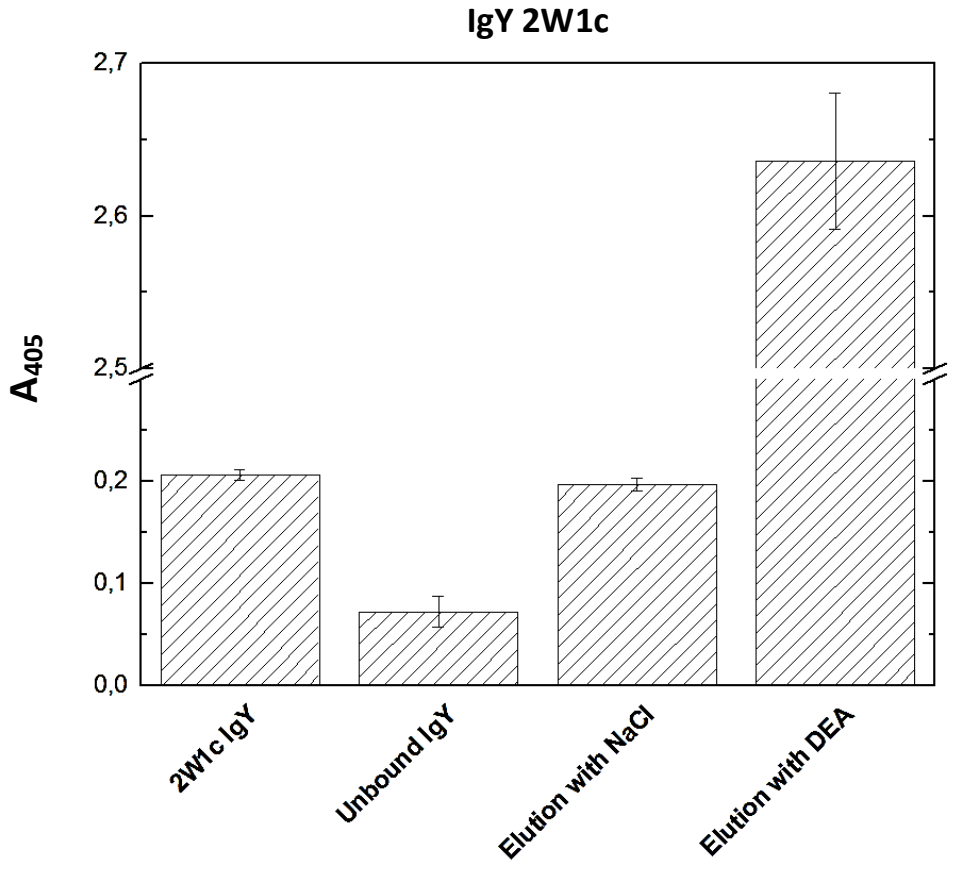

Figure 4.8: Evaluation of 2W1c IgY affinity purification by ELISA. IgY fractions were diluted to concentration 10 μg/ml. Control IgY – fraction isolated from eggs collected before immunization; 2W1c IgY – original (unpurified) fraction isolated from eggs collected after immunization; Unbound IgY – solution collected after overnight incubation of antibody with antigen; Elution with NaCl – fraction of weakly bound antibodies; Elution with DEA – fraction of specific 2W1c IgY. The data are means of triplicates with plotted standard deviations after subtraction of control value.

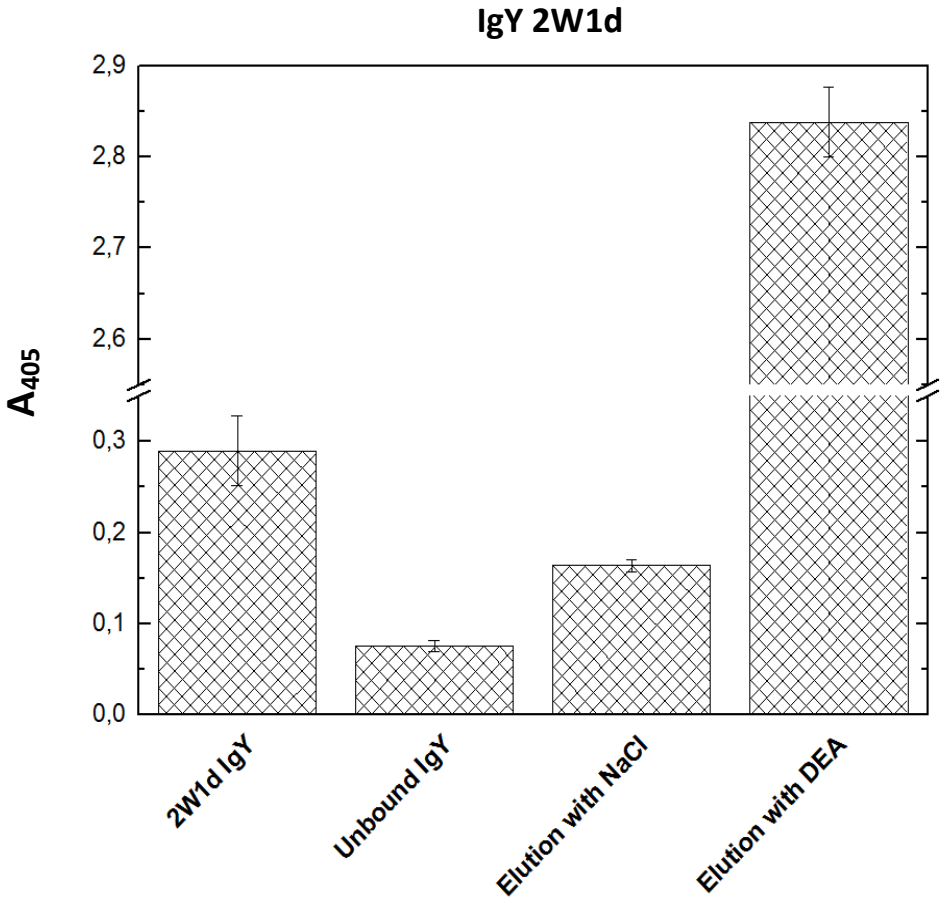

Figure 4.9: Evaluation of 2W1d IgY affinity purification by ELISA. IgY fractions were diluted to concentration 10 μg/ml. Control IgY – fraction isolated from eggs collected before immunization; 2W1d IgY – original (unpurified) fraction isolated from eggs collected after immunization; Unbound IgY – solution collected after overnight incubation of antibody with antigen; Elution with NaCl – fraction of weakly bound antibodies; Elution with DEA – fraction of specific 2W1d IgY. The data are means of triplicates with plotted standard deviations after subtraction of control value.

A protein concentration of each fraction of affinity purification was determined by measuring the absorbance at 280 nm after their dialysis against PBS. The original protein concentration of the isolated IgY fractions listed in the Table 4.1 (page 56) was recalculated as the column with gel resin contained approximately 1 ml of PBS. The results are shown in Tab. 4.2 and 4.3 (page 60).

Table 4.2: Protein concentration and IgY amount of fractions obtained by affinity purification of antibody 2W1c.

IgY fraction	Protein concentration	IgY mass	Yield [%]
2W1c IgY	39.3 mg/ml	824.6 mg	100.00
Unbound IgY	33.9 mg/ml	678.4 mg	82.27
Elution with NaCl	126.9 µg/ml	126.9 µg	0.02
Elution with DEA	171.3 µg/ml	651.0 µg	0.08

Table 4.3: Protein concentration and IgY amount of fractions obtained by affinity purification of antibody 2W1d.

IgY fraction	Protein concentration	IgY mass	Yield [%]
2W1d IgY	36.8 mg/ml	405.1 mg	100.00
Unbound IgY	28.5 mg/ml	284.5 mg	70.23
Elution with NaCl	164.1 µg/ml	164.1 µg	0.04
Elution with DEA	117.8 µg/ml	471.3 µg	0.12

Despite a low amount of specific anti-peptide antibodies in isolated IgY fractions (< 0.15% of IgY), the affinity purification was successful and concentrated fractions of specific IgY were obtained.

4.7 SDS-polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins according to their molecular weight. Separated proteins were then visualized using Coomassie Brilliant Blue (Fig. 4.10 and 4.11).

A protein concentration of the DLD-1 cell lysate was characterized using the BCA protein assay. The protein concentration of the rest of biological material that was used in this diploma thesis was determined by its kind providers: RNDr. Pavel Souček, CSc. and RNDr. Kamila Burdová, Ph.D.

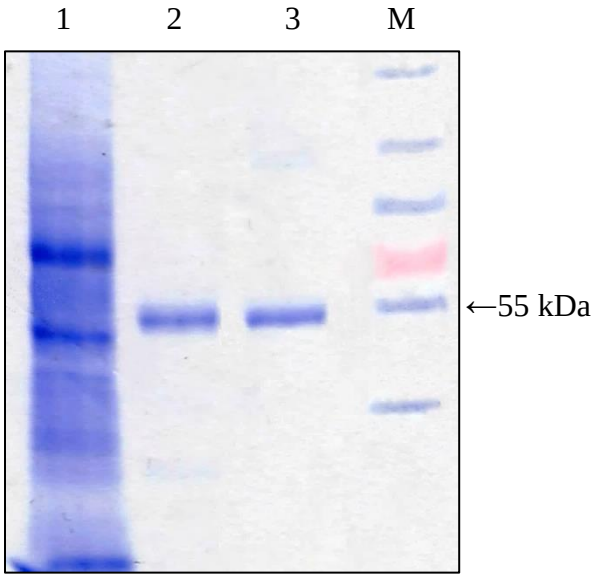

Figure 4.10: SDS-PAGE of CRCA cell lysate, CYP2W1 and CYP2S1 standards.
The following protein amounts were applied into wells – 1: 20 µg protein of CRCA cell lysate; 2: 1 pmol of purified CYP2W1 protein; 3: 1 pmol of purified CYP2S1 protein. Into the line M, 5 µl of PageRuler^{TM} Plus Prestained Protein Ladder (Thermo Scientific) was applied.

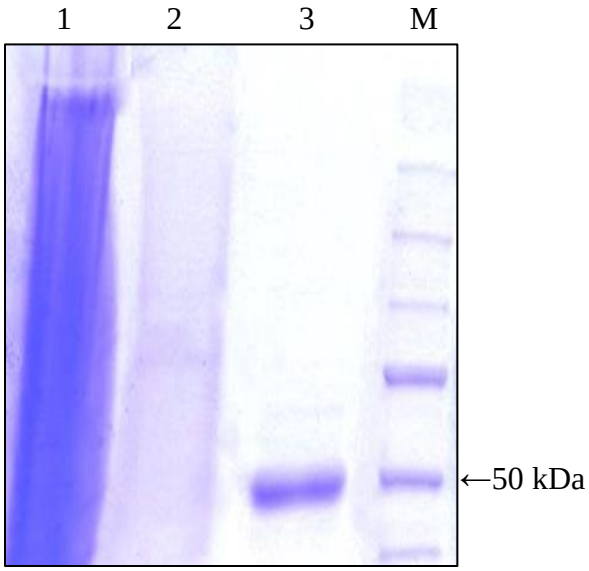

Figure 4.11: SDS-PAGE of DLD-1, HEK 293T cell lysates and CYP2W1 standard. The following protein amounts were applied into wells – 1: 146 µg protein of DLD-1 cell lysate; 2: 5 µg protein of HEK 293T (human CYP2W1 transfected) cell lysate; 3: 10 pmol of purified CYP2W1. Into the line M, 5 µl of Precision Plus Protein™ Unstained Standard (Bio-Rad) was applied.

4.8 Western Blot

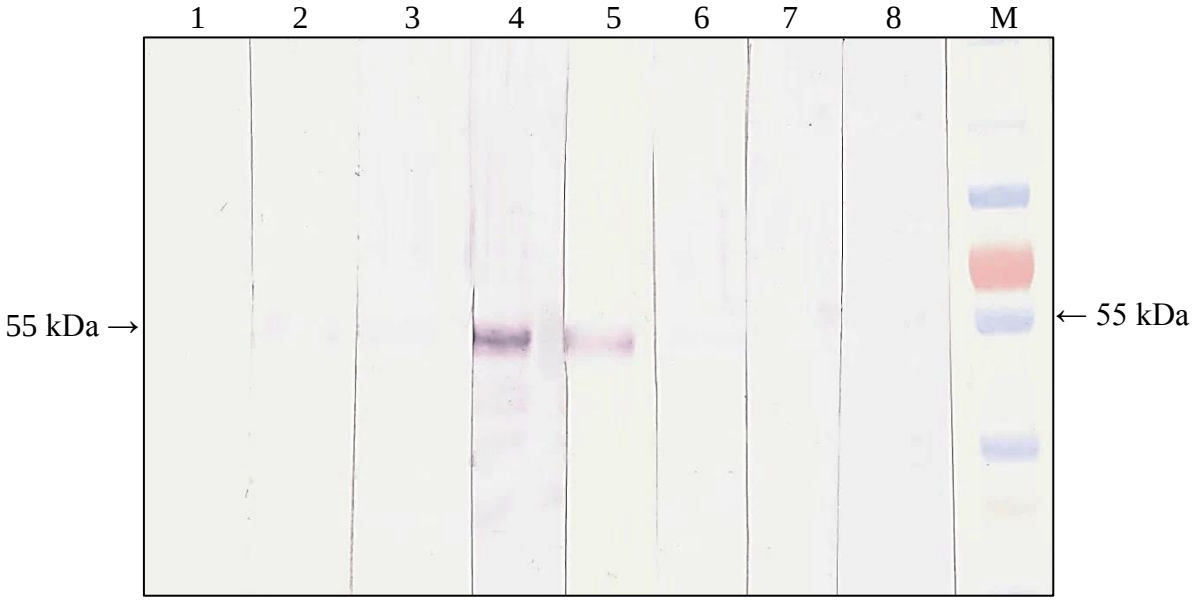

Figure 4.12: Western blot analysis of CYP2W1 standard. Electrophoresed CYP2W1 standard (2 pmol) was transferred to a membrane and probed with:

1: affinity purified IgY 2W1a; c = 1 µg/ml; (isolated by Mácová [103]);

2: affinity purified IgY 2W1b; c = 1 µg/ml; (isolated by Mácová [103]);

3: control IgY 2W1c; c = 15 µg/ml;

4: IgY 2W1c before affinity purification; c = 15 µg/ml;

5: affinity purified IgY 2W1c; c = 0.5 µg/ml;

6: control IgY 2W1d; c = 15 µg/ml;

7: IgY 2W1d before affinity purification; c = 15 µg/ml;

8: affinity purified IgY 2W1d; c = 0.5 µg/ml.

As the secondary antibody, a rabbit anti-chicken antibody conjugated with alkaline phosphatase was used. Into the line M, 5 µl of PageRuler^{TM} Plus Prestained Protein Ladder (Thermo Scientific) was applied and transferred to the membrane.

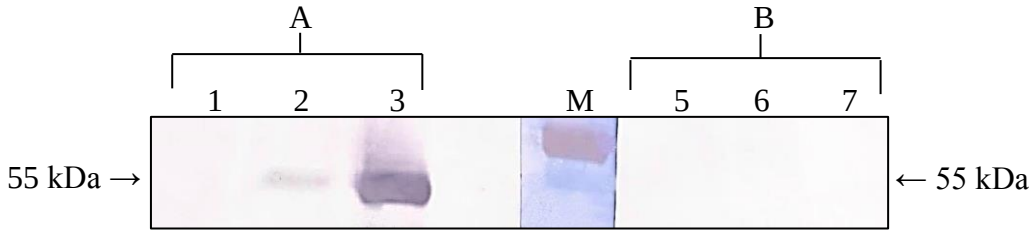

Figure 4.13: Western blot analysis of DLD-1, HEK 293T cell lysates and CYP2W1 standard using chicken antibody. Electrophoresed DLD-1 cell lysate (146 µg protein; lines 1 and 5), HEK293T (human CYP2W1 transfected) cell lysate (5 µg protein; lines 2 and 6) and CYP2W1 standard (10 pmol; lines 3 and 7) were transferred to a membrane and probed with:

A : affinity purified IgY 2W1c; c = 0.5 µg/ml;

B : affinity purified IgY 2W1d; c = 0.5 µg/ml.

As the secondary antibody, a rabbit anti-chicken antibody conjugated with alkaline phosphatase was used. Into the line M, 5 µl of PageRuler^{TM} Plus Prestained Protein Ladder (Thermo Scientific) was applied and transferred to the membrane.

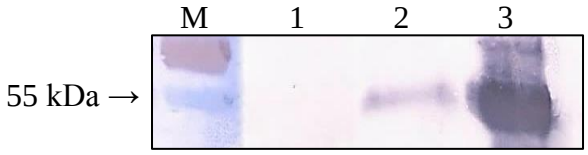

Figure 4.14: Western blot analysis of DLD-1, HEK 293T cell lysates and CYP2W1 standard using rabbit antibody. Electrophoresed DLD-1 cell lysate (146 µg protein; line 1), HEK293T (human CYP2W1 transfected) cell lysate (5 µg protein; line 2) and CYP2W1 standard (10 pmol; line 3) were transferred to a membrane and probed with a rabbit IgG 2W1; c = 5 µg/ml (provided by RNDr. Pavel Souček, CSc., isolated as described in the publication of Wu et al. [47]). As the secondary antibody, a goat anti-rabbit antibody conjugated with alkaline phosphatase was used. Into the line M, 5 µl of PageRuler^{TM} Plus Prestained Protein Ladder (Thermo Scientific) was applied and transferred to the membrane.

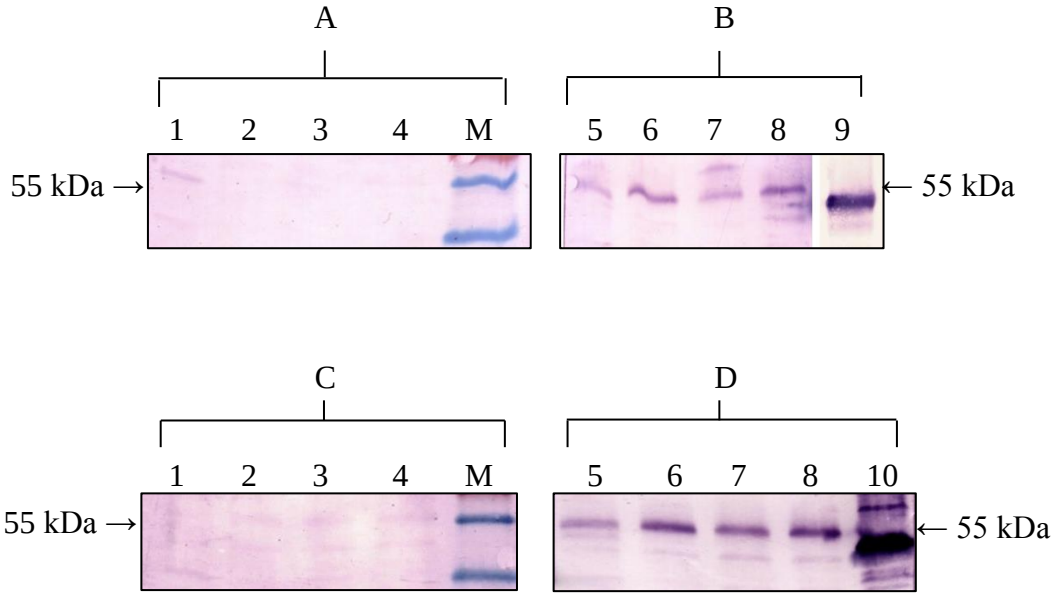

Figure 4.15: Western blot analysis of CRCA cell lysates CYP2S1 and CYP2W1 standards. Electrophoresed 15 µg protein of CRCA cell lysates from four different donors (62 T – lines 1 and 5; 64 T – lines 2 and 6; 120 T – lines 3 and 7; 145 T – lines 4 and 8), CYP2W1 standard (10 pmol; line 9) and CYP2S1 standard (10 pmol; line 10) were transferred to a membrane and probed with:

A: affinity purified IgY 2S1a; c = 2 µg/ml;

B: rabbit IgG 2S1; c = 5 µg/ml (provided by RNDr. Pavel Souček, CSc.);

C: affinity purified IgY 2W1c; c = 2 µg/ml;

D: rabbit IgG 2W1; c = 5 µg/ml (provided by RNDr. Pavel Souček, CSc.).

As the secondary antibody, a rabbit anti-chicken antibody conjugated with alkaline phosphatase (membranes A and C) or goat anti-rabbit antibody conjugated with alkaline phosphatase (membranes B and D) was used. Into the line M, 5 µl of PageRuler^{TM} Plus Prestained Protein Ladder (Thermo Scientific) was applied and transferred to the membrane.

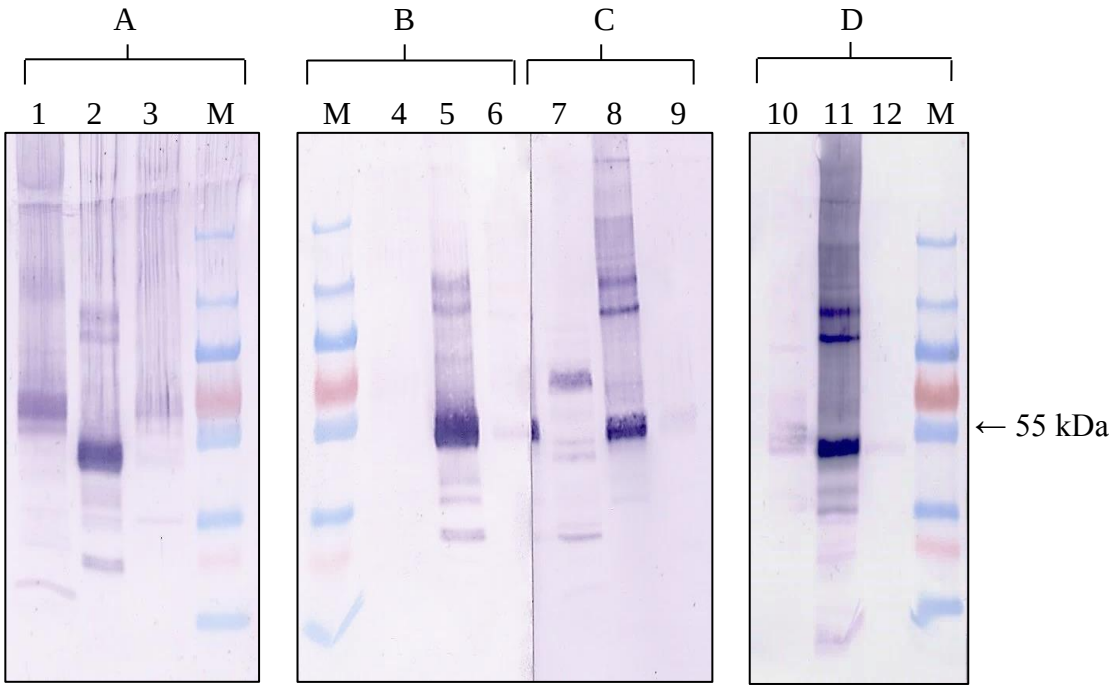

Figure 4.16: Western blot analysis of CRCA cell lysate, CYP2W1 and CYP2S1 standard. Electrophoresed CRCA cell lysate (20 µg protein; lines 1, 4, 7 and 10), CYP2W1 standard (1 pmol; lines 2, 5, 9 and 12) and CYP2S1 standard (1 pmol; lines 3, 6, 8 and 11) were transferred to a membrane and probed with:

A: IgY 2W1c before affinity purification; c = 15 µg/ml;

B: affinity purified IgY 2W1c; c = 5 µg/ml;

C: IgY 2S1a before affinity purification; c = 30 µg/ml (isolated by Mácová [103])

D: affinity purified IgY 2S1a; c = 5 µg/ml (isolated by Mácová [103])

As the secondary antibody, a rabbit anti-chicken antibody conjugated with alkaline phosphatase was used. Into the lines M, 5 µl of PageRuler^{TM} Plus Prestained Protein Ladder (Thermo Scientific) was applied and transferred to the membrane.

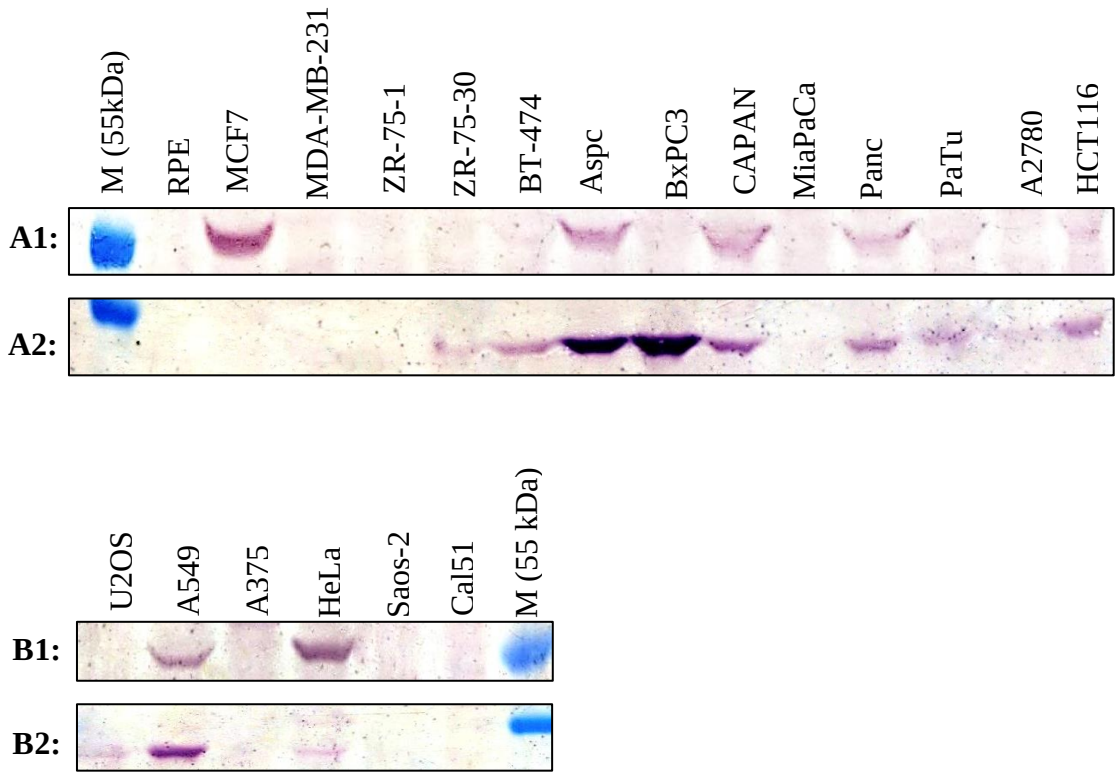

Figure 4.17: Western blot analysis of tumour whole cell lysates. Electrophoresed tumour cell lysates (30 µg protein) were transferred to a membrane and probed with:

A1, B1: affinity purified IgY 2W1c; c = 5 µg/ml;

A2, B2: affinity purified IgY 2S1a; c = 5 µg/ml (isolated by Mácová [103]).

As the secondary antibody, a rabbit anti-chicken antibody conjugated with alkaline phosphatase was used. Into the lines M, 5 µl of PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific) was applied and transferred to the membrane. The protein transfer was kindly performed by RNDr. Kamila Burdová, Ph.D.

4.9 "Negative" Affinity Purification

A "negative" antibody purification was used to deplete the cross-reactive antibody from the total IgG pool. Protein concentration of the resulting IgG fraction was determined by the BCA protein assay. The results are shown in Tab. 4.4 and Fig. 4.18.

Table 4.4: Protein concentration and IgG yield obtained by "negative" affinity purification.

IgG	Amount of applied IgG [mg]	Concentration of purified IgG [µg/ml]	Volume of purified IgG [ml]	Yield [%]
2S1	1	330.8	1.6	52.93
2W1	1	215.9	1.7	36.70

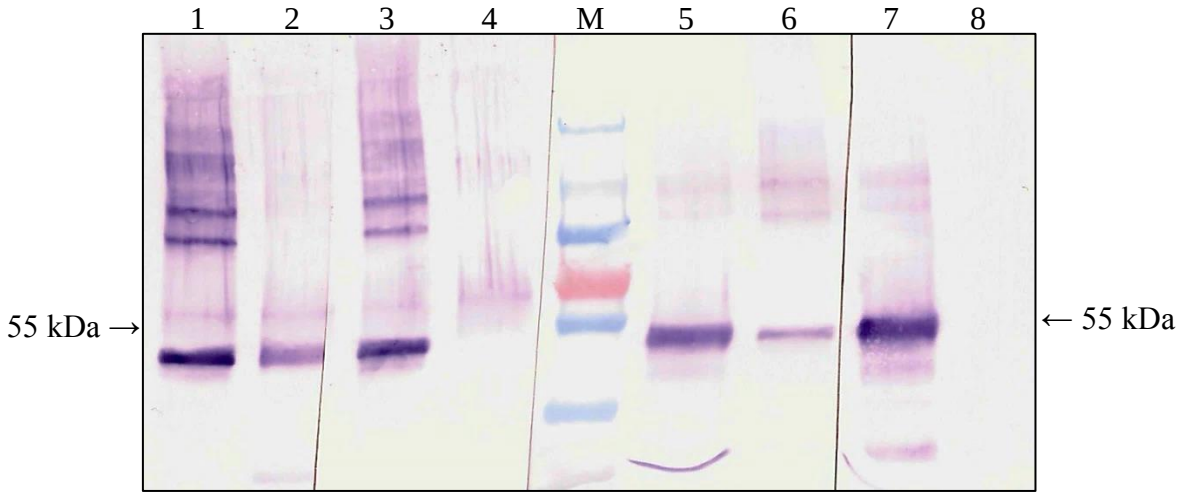

Figure 4.18: Western blot analysis of CYP2W1 and CYP2S1 standards. Electrophoresed CYP2S1 standard (1 pmol; lines 1, 3, 6 and 8) and CYP2W1standard (1 pmol; lines 2, 4, 5 and 7) were transferred to a membrane and probed with:

A: IgG 2S1 before "negative" affinity purification; c = 5 µg/ml;

B: IgG 2S1 after "negative" affinity purification; c = 5 µg/ml;

C: IgG 2W1 before "negative" affinity purification; c = 5 µg/ml;

D: IgG 2W1 after "negative" affinity purification; c = 5 µg/ml.

As the secondary antibody, a goat anti-rabbit antibody conjugated with alkaline phosphatase was used. Into the line M, 5 µl of PageRuler^{TM} Plus Prestained Protein Ladder (Thermo Scientific) was applied and transferred to the membrane.

5 DISCUSSION

Cytochrome P450 is a large family of monooxygenases responsible for the biotransformation of several drugs [104], steroids [3], eicosanoids [6] and many other chemicals. As the P450s can activate many chemical precarcinogens to their active forms (and thus cause the cancer development), the mechanism of their action is necessary to be explored [105]. To date, 57 human P450s are known, of which 13 remain "orphans". The "orphans" are so called, because their function and regulation are still largely unknown [37].

This diploma thesis is focused on two of the orphan P450s - CYP2W1 and CYP2S1. These isozymes are overexpressed in many tumour tissues. Their localization together with the ability to activate some anticancer prodrugs (e.g. AQ4N [48] or duocarmycin analogues [49]) suggests that they may be used for the localized activation of anticancer prodrugs. The expression of CYP2W1 and CYP2S1 can be determined by immunochemical methods. For this purpose, the chicken anti-peptide antibodies recognizing CYP2W1 were isolated. The chicken anti-peptide antibody against the CYP2S1 (2S1a) was isolated by Mácová [103] and the rabbit IgG 2W1 and 2S1 prepared against recombinant proteins expressed in *Escherichia coli* were kindly provided by RNDr. Pavel Souček, CSc.

At first, the immunogens for the chicken immunization were prepared. From the primary structure of CYP2W1 two amino acid sequences were chosen based on data provided by the prediction programme [101]: ^{466}C-SLDTTPARAFTMRPR^{480} from the C-end of protein (2W1c) and ^{21}CAQDPSPAARWP^{32} from the N-end of protein (2W1d). The following parameters were considered while the sequence choosing: (i) the sequences are located on the protein surface, (ii) the sequences do not contain any ordered secondary structure and (iii) the sequences are sufficiently antigenic. The 2W1c sequence is located on the protein surface (Fig. 4.3, page 53) and it is easily accessible. Furthermore, the sequence consists mainly of random coils (Fig. 4.6, page 55) and its immunogenicity is high enough: the value varies between -0.48 and 0.29 (Fig. 4.5, page 54). The 2W1d sequence is also located on the protein surface (Fig. 4.2, page 53), consists only of random coils

(Fig. 4.6, page 55) and its immunogenicity is high: the value varies between -0.25 and 0.36 (Fig. 4.4, page 54).

The chosen peptide sequences were synthetized and conjugated with the carrier protein KLH. The immunization of chickens was performed intramuscularly in three weekly doses of 100 µl of KLH conjugate with peptide (100 µg of peptide per dose).

From the eggs collected one week after the last immunization, the IgY fractions were isolated. A two-step isolation, which uses a tap water extraction (dilution of egg yolks with tap water in ratio 1:7, pH adjustment, freezing and filtration) and a precipitation with sodium chloride, was used. This isolation method is fast, efficient and inexpensive [98]. The isolated IgY yield was 1112 mg from 7 eggs (respectively 608 mg from 4 eggs), which is approximately 152 – 159 mg of IgY per one egg yolk. An average egg yolk contains 100 ± 150 mg antibodies [106]. The used isolation method was, therefore, effective and a high amount of variously specific antibodies was obtained.

Further, the production of antigen-specific antibodies by chickens during the time of immunization was examined by ELISA. As the antigen, the peptide sequence chosen from the primary CYP2W1 structure was used. The primary antibody was applied in a set of serial dilutions (Fig. 4.7, page 57). As the concentration of applied IgY was 3.3; 10.0; 30.0 and 90.0 µg/ml, a threefold increase in absorbance values was expected. At both of the antibodies (2W1c and 2W1d) the absorbance is increasing with the increasing concentration, but the regular threefold increase is not evident. This can be caused by the fact that the absorbance is not directly proportional to the antibody concentration in the whole concentration range but just to approximately 1.0. This trend could mean that in the isolated antibody fractions is a high concentration of antibodies specifically recognizing the CYP2W1 or that these antibodies have a higher avidity [107].

Because the isolated IgY are polyclonal and thus polyspecific, a purification of specific immunoglobulin was performed to avoid a possible cross-reactivity. Therefore, the affinity purification was undertaken with the respective peptide bound to gel sorbent. The affinity purification progress was monitored by ELISA (Fig. 4.8, page 58 and Fig. 4.9, page 59). The obtained data indicate that the purification process was successful and most of the specific antibodies were

separated into the purified fraction. Although the specific antibodies represented a low percentage of the total IgY fraction (approximately 0.08% for 2W1c and 0.12% for 2W1d – Tab. 4.2 and 4.3, page 60), the antibodies were successfully concentrated and prepared for the further usage in immunodetection of CYP2W1 in tissue and cell samples. By comparing the absorbance data obtained using the same concentrations of antibodies before and after the affinity purification, it may be estimated, that the ability of antibodies to recognize the antigen was increased approximately 20-30 times by the purification. Therefore, the 15 μg/ml for the antibodies before the affinity purification and the 0.5 μg/ml for the affinity purified antibodies were selected as the default concentrations to be used for the Western blot analysis.

At first, the ability of chicken anti-peptide IgY (2W1a and 2W1b previously prepared by Mácová [103], 2W1c and 2W1d) to recognize the purified CYP2W1 protein was determined (Fig. 4.12, page 63). The IgY 2W1c was as the only IgY able to detect the CYP2W1 presence and therefore it was chosen as the antibody to be used for the further CYP2W1 detection by Western blotting. Figure 4.13 (page 64) shows an immunodetection of CYP2W1 in DLD-1, HEK 293T (human CYP2W1 transfected) cell lysates and purified CYP2W1. Using the IgY 2W1c, low level of CYP2W1 protein expression in HEK 293T cell lysate was detected. This result correlates with the low level of CYP2W1 protein detected in the same cell lysate using a rabbit IgG 2W1 (Fig. 4.14, page 64). Although a presence of CYP2W1 mRNA in DLD-1 cell lysate was established (performed by RNDr. Alena Hyršlová-Vaculová, Ph.D), the expression of CYP2W1 on a protein level was not demonstrated. This result confirms the fact, that the gene's mRNA level does not necessarily predict its protein level [108]. Because the bands obtained by the immunodetection of CYP2W1 standard were not as strong as it was expected, higher concentration of primary IgY (2 μg/ml) was used for the further determination of the CYP protein presence.

Based on the predicted 41% identity between the primary structure of CYP2W1 and CYP2S1 [109], a cross-reactivity of the antibodies was examined. Figure 4.16 (page 66) confirms that the chicken anti-peptide antibodies do not show any cross-reactivity to the non-competent protein standard. On the other hand, a

considerable cross-reactivity between the anti-protein antibodies (IgG 2W1 and 2S1) and the non-competent standards was observed (Fig. 4.15, page 65). On the basis of this result, the "negative" affinity purification of the IgG was necessary to be carried out. Furthermore, it can be seen that the expression of CYP2W1 was determined in all tested CRCA samples by both, IgY 2W1c and IgG 2W1, whereas the detection observed by the IgG 2W1 was significantly more effective. However, it cannot be excluded that the more efficient detection of CYP2W1 by the IgG 2W1 is caused by the antibody cross-reactivity. The expression of CYP2S1 was determined just in 62 T sample by IgY 2S1a, nevertheless the IgG 2S1 determined the CYP2S1 presence in all tested CRCA samples. This result is probably caused by the cross-reactivity of IgG and the bands observed belong to CYP2W1. As the colour of the bands obtained by probing with IgY were still not strong enough and no unspecific bands were detected at the same time, the IgY concentration was increased to 5 μg/ml for the further CYP2W1 and CYP2S1 immunodetection experiments. On the membranes B, C and D, multiple bands are visible. These results are thus consistent with the finding of a possible *N*-glycosylation of CYP2W1 [46]. Besides the refutation of the cross-reactivity of IgY 2W1 and 2S1, Fig. 4.16 (page 66) displays that the affinity purification process was successful. Due to a confirmed presence of CYP2W1 in all CRCA samples and a small amount of the remaining cell lysates, a mixed CRCA cell lysate was prepared (by pooling CRCA 62 T, CRCA 64 T, CRCA 120 T and CRCA 145 T in equal amounts). In so prepared CRCA cell lysate, a presence of CYP2S1 was observed whereas no CYP2W1 presence was established using the chicken anti-peptide IgY 2S1a and 2W1c. Although the CRCA cell lysates were stored at -80 °C, the attempts (shown at Fig. 4.15 and 4.16) were performed 12 days after each other and thus the degradation of CYP2W1 cannot be ruled out. These results also demonstrate that adequate concentrations of IgY were used (resulting bands at the position of standards were strong enough, while no unspecificity of the IgY was observed).

The Fig. 4.17 (page 67) shows results of a Western blot analysis of tumour whole cell lysates. A presence of CYP2W1 was detected in MCF7, Aspc, CAPAN, Panc, A549 and HeLa cell lysates and in a small amount in BT-474, PaTu and HCT116 cell lysates. A band of unknown identity was observed at approximately

62 kDa at the BxPC3 cell line (not shown in the figure). This band may belong to the glycosylated form of the CYP2W1. However, this assumption would have to be supported by further studies. The expression of CYP2S1 protein was determined in Aspc, BxPC3, CAPAN and A549 whole cell lysates and lower CYP2S1 expression was observed in ZR-75-30, BT-474, Panc, PaTu, A2780, HCT116, U2OS and HeLa cell lysates. It is interesting, that the presence of both studied proteins, CYP2W1 and CYP2S1, was determined in 5 out of 6 pancreatic tumour cell lines (except MiaPaCa), cervical cancer cell line (HeLa), colorectal cancer cell line (HCT116) and a lung adenocarcinoma cell line (A549). On the other hand, the presence of CYP2W1 was determined just in one out of six studied breast cancer cell lines (MCF7) and in case of CYP2S1 in two out of six studied breast cancer cell lines (ZR-75-30 and BT-474). These findings are consistent with the study of Hlaváč et al. (2014), who consider the CYP2W1 to be not suitable as an independent biomarker for breast carcinoma, because its expression in breast tumour is not high enough [110]. The cytochrome 2W1 may rather be used as an independent biomarker for stages II and III colorectal carcinoma patients as published by Edler et al. [43; 97]. The cytochrome 2S1 may be suitable as a prognostic marker in both, colorectal and breast cancer, where it is overexpressed [111; 112]. In future studies, it may be useful to examine a suitability of using the CYP2W1 as a biomarker in pancreatic cancer as its expression in pancreatic tumour cells was determined to be high. This finding is consistent with the results of immunohistochemical staining of pancreatic cancer published in The Human Protein Atlas [113]. Furthermore, it may be useful to study an expression of CYP2S1 in pancreatic tumour cells, since its presence in five out of six studied cell lines was found in our experiments. Surprisingly, this result is inconsistent with a study published in The Human Protein Atlas as no CYP2S1 expression was determined by immunohistochemical staining in all eleven studied tissue samples [114].

Although high losses of IgG occurred while the "negative" affinity purification (47.07% of IgG 2S1 and 63.30% of IgG 2W1), the process can be considered successful. As shown in the Fig. 4.18 (page 68), no cross-reactive response of the IgG was observed after the purification.

In this thesis, two types of antibodies were compared: the chicken anti-peptide IgY and the rabbit anti-protein IgG. Based on the results, it may be concluded that the anti-peptide antibodies hold several advantages over the anti-protein antibodies. For example, the peptide synthesis is cost-effective and less time-demanding than the complete protein expression. Furthermore, the anti-peptide antibodies have higher specificity than anti-protein antibodies and sometimes, the affinity purification of these antibodies is not even necessary. Other advantages arise from the host animal which has been used: chicken antibodies have strong avidity [107], broader antigen-binding host range, absence of activation of the mammalian complement cascade [91], bacterial and human Fc receptors [94] or interaction with rheumatoid factors [92]. Moreover, the sampling method is non-invasive and does not cause any stress to the animal [87]. The only disadvantage of the anti-peptide IgY is the possibility that they might not recognize the complete (and often native) conformation of the protein (e.g. IgY 2W1d that successfully recognized the peptide antigen during ELISA, but was not able to detect the protein presence during Western blotting – Fig. 4.12, page 63).

6 SUMMARY

All the objectives of this thesis were fulfilled and the following results were achieved:

- Two chicken anti-peptide CYP2W1 antibodies (IgY 2W1c and 2W1d) were isolated and purified by affinity chromatography.

- Antibody 2W1c was able to recognize the CYP2W1 standard and also detect this CYP in the following biological material and cell cultures: HEK 293T (human CYP2W1 transfected), CRCA, MCF7, Aspc, CAPAN, Panc, A549, HeLa and in a small amount in BT-474, PaTu and HCT116 cell lysates.

- Furthermore, the presence of CYP2S1 in tissue samples and cell cultures was examined by an anti-peptide IgY 2S1a antibody. The presence of CYP2S1 was determined in: CRCA, Aspc, BxPC3, CAPAN, A549 and in a small amount also in ZR-75-30, BT-474, Panc, PaTu, A2780, HCT116, U2OS and HeLa cell lysates.

- Rabbit anti-protein antibodies showed a considerable cross-reactivity with incompetent standards. Thus, a "negative" affinity purification of the IgG was necessary to be carried out.

- Based on the Western blot results, it may be concluded that the rabbit IgG are more effective in recognizing the respective protein at the detriment of the antibody specificity.

- Chicken anti-peptide IgY are in many respects more favourable than the rabbit anti-protein IgG.

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Appendix

Information about the donors of used cell lines and cancer tissues:

CRCA – cells from human colorectal cancer tissue:

- **62 T:** Caucasian male, age: 63 years, stage II, primary tumour (PT)
- **64 T:** Caucasian male, age: 62 years, stage III, PT
- **120 T:** Caucasian female, age: 62 years, stage III, PT
- **145 T:** Caucasian female, age: 79 years, stage III, PT

(Information provided by RNDr. Pavel Souček, CSc.)

Human colorectal carcinoma cell lines:

- **DLD-1:** Caucasian male, age: 45 years, Dukes' stage C, PT
- **HCT116:** Caucasian male, age: 50 years, Dukes' stage D, PT

Transformed human retinal pigment epithelial cell line:

- **RPE:** female, age and ethnicity not known, PT

Human bone osteosarcoma epithelial cell lines:

- **U2OS:** Caucasian female, age:15 years, PT
- **Saos-2:** Caucasian female, age: 11 years, PT

Human lung adenocarcinoma epithelial cell line:

- **A549:** Caucasian male, age: 58 years, PT

Human malignant melanoma cell line:

- **A375:** female, age: 54 years, ethnicity not known, PT

Human cervical cancer cell line:

- **HeLa:** black female, age: 31 years, PT

Human breast cancer cell lines:

- **Cal51:** Caucasian female, age: 45 years, adenocarcinoma, pleural effusion
- **MCF7:** Caucasian female, age: 69 years, adenocarcinoma, pleural effusion
- **MDA-MB-231:** Caucasian female, age: 51 years, adenocarcinoma, pleural effusion
- **ZR-75-1:** Caucasian female, age: 63 years, ductal carcinoma, ascites
- **ZR-75-30:** black female, age: 47 years, ductal carcinoma, ascites
- **BT-474:** Caucasian female, age: 60 years, ductal carcinoma, PT

Human pancreatic cancer cell lines:

- **Aspc:** Caucasian female, age: 62 years, grade 2, ascites
- **BxPC3:** female, age: 61 years, ethnicity not known, grade 2, PT
- **CAPAN:** Caucasian male, age: 56 years, grade 1, liver metastasis
- **MiaPaCa:** Caucasian male, age: 65 years, grade 3, PT
- **Panc:** Caucasian male, age: 56 years, grade 3, PT
- **PaTu:** Caucasian female, age: 64 years, grade 2, PT

Human ovarian carcinoma cell line:

- **A2780:** female, age and ethnicity not known, PT

The information was collected from:

Public Health England: *Culture collections.* URL: <https://www.phe-culturecollections.org.uk/> [cit. 15.4.2015]

LGC Standards: *ATCC®*. URL: <http://www.lgcstandards-atcc.org/> [cit. 15.4.2015]

Sigma-Aldrich: *Sigma-Aldrich Catalog.* URL: <http://www.sigmaaldrich.com/catalog> [cit. 15.4.2015]

Creative Bioarray: *Creative Bioarray Products.* URL: <http://www.creative-bioarray.com/Products.htm> [cit. 15.4.2015]

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