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MASTERTHESIS

Comparison of different Purification Strategies for Anti-Lupine IgY-Antibodies in Sensitivity and Specify

Zur Erlangung des akademischen Grades

Diplomingeneurin (Dipl.Ing.)

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Eingereicht von:

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“Research is what I'm doing when I don't know what I'm doing.”

- *Wernher von Braun*-

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Statement of Authenticity

I certify, that all materials presented here are of my own creation, and that any work adopted from other sources is duly cited and referenced as such.

Vienna, March 21th, 2014

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Signature

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Kurzfassung

Ziel dieser Arbeit ist es Immunglobulin Y (IgY) aus Eidottern zuerst grob mittels unterschiedlichen Fällungsprozessen aufzureinigen und anschließend spezifische IgY Antikörper gegen Lupinienproteine zu finden. Dies wird unter anderem in der Lebensmittelindustrie benötigt, da der Einsatz von Lupinien durch ihre positiven Eigenschaften in immer mehr Lebensmittelprodukten enthalten ist. Durch den vermehrten Einsatz zeigen sich auch immer mehr Personen, die allergisch auf Lupinienproteine reagieren. Um nun nachweisen zu können, ob Spuren von Lupinien in einem Lebensmittel enthalten sind, müssen Schnelltests entwickelt werden, die möglichst schnell, aber auch sehr spezifisch aufzeigen, ob Spuren von Lupinien in einem Lebensmittel enthalten sind oder nicht.

Für diesen Zweck werden im Zuge dieser Arbeit verschiedene Fällungsmethoden und Kombinationen aus diesen getestet, um eine möglichst einfache und schnelle Reinigungsmethode zu finden, die zugleich zu einer sehr hohen Reinheit führt.

Anschließend werden mittels Affinitätschromatographie die Antikörper soweit aufgereinigt, dass spezifische IgY-Antikörper gegen Lupinienproteine vorliegen. Dies ist wichtig, da die Schnelltest natürlich spezifisch gegen bestimmte Proteine, in dieser Arbeit gegen Lupinienproteine, wirken sollen und dadurch falsch positive Ergebnisse zu vermeiden. Zu diesem Zweck wurden unterschiedliche Matrizen ausgetestet, sowie die Bedingungen geändert, wie zum Beispiel die Pufferbedingungen und Eluierungsbedingungen, um auch hierfür eine möglichst große und reine Ausbeute zu erhalten.

Während dieser Arbeit wurden auch ELISA hergestellt, um die technische Anwendbarkeit der aufgereinigten Antikörper zu überprüfen.

Abstract

The aim of the work is to purify Immunoglobulin Y (IgY) from egg yolk as pure and with a as far as practicable high yield. Egg yolk contains between 8 and 20 mg complete IgY per millilitre. In the end, the IgY should be used to produce strip tests or develop ELISA tests to discover traces of lupine. This is necessary, for example, for the food production to prevent allergic reactions. Nowadays there are more and more food products on the market containing lupines. IgY is also important for several subjects like therapeutic, immunodiagnostic and food analysis (toxin detections).

The use of IgY-antibodies became more and more important in the past years because there are a lot of advantages compared to mammalian IgG. Some of them are that it presents no interaction with Fc human serum factor, advances higher antibody production and is compatible with animal protection and ethical laboratory handling regulations. The major problem of the avian antibodies is to purify them or to separate them from other lipids in the egg yolk. It is not possible to bind them with protein G or protein A, like IgG. However it is important to discover a method to produce IgY cheap and as fast as possible while still getting a high purity. It may be that the difficulties in separating the IgY from the high amount of yolk lipids have impeded the development of this source of antibody.

The initial solution was already pre-purified with PEG 8000. In this work different precipitation methods were tried to separate the IgY from the lipids and other proteins in the supernatant, like different precipitation methods and subsequently clean them up specifically for lupine proteins by affinity chromatography using different matrices and chromatographic conditions.

Short Conclusion

To sum up the results of the work, there are several possibilities to purify the IgY from the egg yolk fast and simply but the research has not come to an end. In the end, there is an outcome of specific lupine-IgY but the yield is not as high as expected. The process described in this thesis gained results but there are still possibilities for improvements. At the end of this thesis, some suggestions will be given for future researches and improvements.

Contents

Abbreviations

Ab	Antibody
Ag	Antigen
BCA	Bicinchonic acid
BSA	Bovine serum albumin
EDTA	Ethylenediaminetetraacidic acid
ELISA	Enzyme Linked Immunosorbent Assay
FPLC	Fast protein Liquid Chromatography
HRP	Horse Radish Peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
LL	Lightning Link
MES	N-morpholino-2-ethanesulfonic acid
MWCA	Molecular weight cutoff
NaCl	Sodium Chloride
PBS	Phosphate Buffered Saline
PEG	Polyethylenglycol
SDS	Sodium Dodecyl Sulphate
SDS-Page	Sodium Dodecyl Sulphate-Polyacrylamid gel electrophoresis
TMB	3.3',5.5' Tetramethylbenzidine
UV/VIS	Ultraviolet/visible light

List of tables

List of Figures

1 Introduction

Nowadays there is a wide range of people who are allergic to various food additives or basic food elements. It often happens that food contains traces of elements, which are not listed in the description of ingredients of the final product. The easiest and most effective way to prevent food allergy reactions is to abdicate the offending food. *(Peng Su, Defa Li et al., Food Chemistry, 2009)* It becomes more and more difficult to avoid contamination of food with allergens but antibodies make it possible to find the allergens hidden very easily. The easiest ways to require the hidden allergens are to use polyclonal antibodies from chicken or rabbits because it is ten times more time consuming to produce monoclonal antibodies. The avian antibodies are used more and more often in basic research. Due to the phylogenetic distance between birds and mammals, hens' antibodies in particular have clear advantages over mammalian immunoglobulin. For instance, highly conserved mammal-specific antigens from hens can be recognized as foreign. In part, more epitopes on mammalian antigens can be recognized from hens' antibodies than on those from antiserum from mammals. *(Sigma-Aldrich Co. LLC., 2013)*

In immunological studies it is possible to use an IgY antibody against a mammalian antigen. The reaction is sensible and produces less cross-reactions and background than systems in which the antibody and the antigen in the antibody-antigen complex are both, from mammalian sources. Moreover, the IgY–antibody demonstrates effective affinity to high conserved regions across the mammalian species. *(Gen Way Biotech, 2013)*

Another advantage is that hens' antibodies do not bind with mammalian Fc-surface-receptors. For that reason IgY antibodies can be easily used to detect surface proteins. (*Gen Way Biotech, 2013*) It neither binds to rheumatoid factors nor with proteins A or G. That means that the use of hens' antibodies is rarely likely to produce false positive reactions in certain immunochemical assays. Furthermore, a combination with mammalian antibodies is possible and sensible.

Another major advantage is that the amount of antibodies in the egg yolk is enormous due to the transfer from the plasma through the egg follicle to the egg yolk.

Moreover, the cruelty to animals is minimized because you can replace the use of blood from mammalians. The only unpleasantness for the hens is the immunization.

For all this reasons, the production of antibodies via hens' eggs gets more and more attention. (*Sigma-Aldrich Co. LLC. , 2013)*

1.1 Basic information about IgY antibodies

IgY is the major antibody in birds', reptiles' and lungfishes' blood as well as in the egg yolk. Like other antibodies IgY is a class of glycoprotein that is produced by the immune system in reaction to detect foreign substances with the aim to specifically recognize them.

1.1.1 Hen's immune system:

Hen's immune system, like the mammalian's, is divided into two major components: the non-specific and innate part and the specific and acquired one. Between these parts there are numerous interactions, of critical importance is the event of an immune response. Hen's immune system has also antigen-presenting cells and many functional mechanisms are regulated via interleukins. A disadvantage is that there is less knowledge of the hen's immune system. The focus of scientific research was yet on rat's and mice immune systems. (*Recombinant Protein Purification Handbook-Principles and Methods)*

1.1.2 Hens' vs. Mammalians' Immunoglobulins

Hen's immunoglobulins are similar to mammalians'. They have also a heavy and a light, bridged by disulphide bonds, a variable and a constant part.

Avian immunglobulins are divided into IgM, IgY and IgA. IgM has the same function as mammalian IgM. They eliminate pathogens in the early stages of B cell mediated immunity before there is sufficient IgG. Avian IgA-antibodies are also similar to mammals'. They are also found in the gall bladder and in secretions active in the body. It prevents colonization by pathogens.

IgY is often mislabelled as IgG due to its similar function to mammalian IgG and IgE. *G.A. Leslie and L.W. Clem* have proposed the name immunoglobulin Y in 1969. They proved that the IgY molecule has a different structure and function from IgG.

Further genetic research suggests that the IgY molecule is phylogenetically a progenitor of mammals' IgG and IgE.

IgY, like mammals' IgG, is the immunoglobulin delivering the second response but hens' IgY can also take part in anaphylactic reactions unlike mammalian IgG.

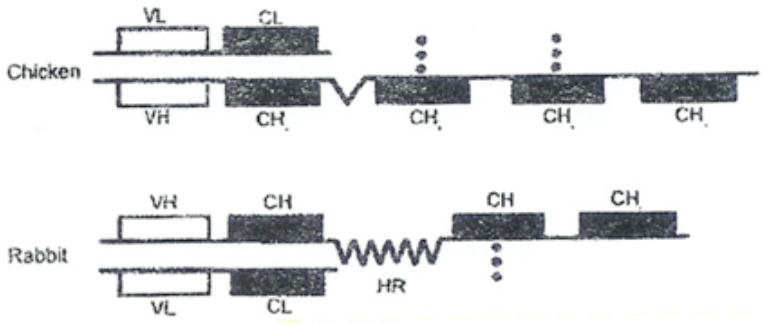

Figure 1 Simplified model of avian (chicken IgY) and mammalian (R. Schade, I. Behn, M. Erhard, A. Hlinak, C. Staak. Chicken Egg Yolk Antiodies, Production and Application - IgY-Technology*,)*

The Figure 1 shows the simplified models of a rabbit IgG and a chicken IgY. The abbreviations stands for: L-light chain, H-heavy chain, V- variable domain, C-constant domain.

There is a clear difference in the structure of avian IgY and mammals' IgG. The heavy chains of hens' IgY have an additional constant domain instead of the hinge region (HR) of mammals IgG. Further differences are the revealed number of the constant domains of the heavy chains (HC) and the carbohydrate chains (black points). *(Guan-Ping et al., 2009)*

Table 1 Main differences of hens' IgY and mammalian IgG structure

	IgY	**IgG**
Molecular Mass	180 kDa	160 kDa
Light chain	25 kDa	23-25 kDa
Heavy chain	68 – 70 kDa	~56 kDa

1.1.3 Biological basis of immunoglobulin-transfer into eggs

There are various ways in which immunglobulins are transferred into hens' eggs. IgA and IgM are transferred together with other proteins of the oviduct into the egg white but only in small amounts. The transfer of IgY into the egg follicle is based on receptors, transferred in large amounts and takes about 5 days (Patterson et al., 1962) while the egg is still in the ovary. The concentration in the egg yolk is comparable to the concentration within the sample (6 to 13 mg/ml). IgY can be isolated from the egg yolk without traces of IgM and IgA. *(Antibodies)*

1.1.4 Immunization of hens

In hens, like in mammals immunization against recombinant antigens is possible. For an antigen to be a good immunogen it is necessary to have an amino acid sequence which is recognized by the hens' immune system as a foreign one. The properties of antigens influencing the development of specific antibodies, means that an immune response is essentially influenced by the quality and the amount of the used antigen. *(25ff)*

1.1.5 Storage of IgY antibodies

Purified IgY fraction is even at room temperature very stable but not recommended. It can be stored at +4 °C with 0.02% sodium azide. IgY can be also stored at -20 °C but freezing and thawing should be avoided.

The IgY preparations were stable over time. No loss of antigen recognition was observed after storage for 3 years at + 4 °C. Affinity purified antibodies are the most fragile ones. The best storage conditions should be tested.

The preparations were subsequently kept at +4 °C and -20 °C and there was no loss of stability captured. However the best conditions should still be tested over time. Some alternatives could be:

-20 °C or -80 °C

+ 4 °C with preservatives like azide (0.02%) or merthiolate

-20 °C with glycerol; Final concentration of glycerol 10 or 50%

-20 °C with BSA at final concentration of 0.05-0.5%

It is recommended to aliquot a big amount of purified IgY to avoid freezing and thawing. *(Agrisera)*

1.2 Lupines

1.2.1 Basic information about lupines

Lupine, lat. *Lupinus*, is a genus of flowering plants and belongs to the legume family, *Fabaceae* or *Legumniosaea*. The genus includes over 200 species, in relation to beans, chickpeas and peanuts that are mostly herbaceous perennial plants and between 0.3 to 1.5 meters tall. The flowers are produced in dense or open whorls on an erect spike where each flower is between 1 and 2 cm long.

The fruit is a pot, which contains several seeds. Lupines are basically grown for their seeds, which can be used as an alternative to soybeans. They contain more protein and less fat. They are gluten free and high in dietary fiber, amino acids and antioxidants. Furthermore, they are considered to be prebiotic. At this time, the market for lupine beans is small but it is already growing and agronomy researchers believe that they have potential not only for the reasons mentioned above.

Lupine allergies

Lupines are also known as allergens. The study "*Cross-allergenicity of peanut and lupine: The risk of lupine allergy in patients allergic to peanuts" by Denise Moneret-Vautrin et al.* shows that 44% of people with peanut allergy had a positive allergy test for lupine and that seven of eight had a positive test experienced a reaction to ingest lupine. For that reason the European Commission requires in 2006 to label food to indicate the presence of "lupine and products thereof" in food. *(Kyprianou M. , 2003) (Denise Moneret-vautin et al., 20136)*

The cross-reactivity between lupine and peanuts could be explained with the similar amino acid sequence, and the structure of the proteins and they belong to the same botanical family, the *Fabaceae* respectively.

The allergens of the lupine are the storage proteins α-, β-, γ-, δ- Conglutin. The symptoms are like most allergic reactions asthma, OAS, hives, rhinitis and even anaphylactic shock.

2 Material and Solutions

MATERIAL:

Table 2 List of used chemicals and their main characteristics

IUPAC Name	Systematic Name	Molecular formula	Molar mass [g/mol]	Density [g/m^3]
Sodium acetate	Sodium ethanolate	$C_2H_3NaO_2$	82.03	1.528
Acetate	Acetate	$CH_3CO_2^-$	56.00	
Sodium hydrogen phosphate	Disodium hydrogen orthophosphate	Na_2HPO_4	141.96	1.7
Sodium dihydrogen phosphate	Monosodium phosphate	NaH_2PO_4	119.98	
Sodium chloride	Saline	NaCl	58.44	58.44
2-Amino-2-hydroxymethyl-propane-1,3-diol	TRIS	$C_4H_{11}NO_3$	121.14	1.328
Sodium carbonate monohydrate		$Na_2CO_3*H_2O$	124.00	2.25
Sodium bicarbonate	Baking soda			
2-(4-Carboxyquinolin-2-yl)quinoline-4-carboxylic acid	bicinchoninic acid	$C_{20}H_{12}N_2O_4$	344.32	
Disodium (2*R*,3*R*)-2.3-dihydroxybutanedioate	sodium tartrate	$C_4H_4Na_2O_6$	194.051	1.545
Sodium hydroxide	Sodium oxidanide	NaOH	39.997	2.13
Copper(II) Sulphate	Cupric Sulphate	$CuSO_4$	159.62	3.603
Bovine serum albumine	BSA		66.5 kDa	
Sodium azide				

2-(*N*-morpholino) ethanesulfonic acid	MES	$C_6H_{13}NO_4S$	195.2	
Sodium lauryl Sulphate	Sodium dodecyl Sulphate - SDS	$NaC_{12}H_{25}SO_4$	288.372	1.01
Ethylenediaminetetraacetic acid - EDTA	2-({2-[Bis(carboxymethyl)amino] ethyl}(carboxymethyl)amino)acetic acid	$C_{10}H_{16}N_2O_8$	292.24	860 mg/ml
Hydrogen chloride	Hydrochloride	HCl	36.46	1.490
Isopropyl alcohol	2-Propanol	C_3H_8O	60.10	0.786
Ethanol		C_2H_6O	46.07	
Citric acid	2-hydroxypropane-1.2.3-ticarboxyl acid	$C_6H_8O_7$	192.124	

Table 3 List of used materials

Name	Type
Antibody solution	Anti-Lupine IgY
Lupine foul	CDL-Allergene, gel. 10.11.08, nicht entfettet
Detection Ab	Anti-Lupine-Mono 260110SP^{2}C12-A11-C10-C8 (13.2.12 BG)
Electrophoresis gel	Gel BT 12% (#0021776-1036)
Matrix for affinity chromatography	CNBr-activated Sepharose
Matrix for affinity chromatography	NHS-activated Sepharose
ELISA plates	96-well plates high binding
BCA Kit	BCA Kit (Solutions and Standard)
BCA plates	96-well plates non-binding
Coupling of the Ab	Lightning Link Kit
FPLC	FPLC Pharmacia – FPLC-System
Software for FPLC	
ELISA reader	Sunrise remote - TECAN
Software ELISA	Magellan 5
Standard gelelectrophoresis	Seeblue Plus 2 Prestained (#1089578)
Loading buffer for electrophoresis	Invitrogen Tricine SDS-Sample Buffer
Roth Roti Spin Midi	Roti Spin Midi - 100
Freeze-dryer	
PD 10	GE Healthcare
IgY Buffer	Na_2HPO_4 – 32.22 g NaH_2PO_4 – 2.62 g NaCl – 116.90 g NaN_3 – 0.5 g → dissolved in 1000 ml RO – H_2O
TMB-Stock Solution	0.375 g Tetramethylbenzidine 5 ml Dimethylsulfoxide 25 ml MeOH

Table 4 Used methods

Used Methods
Precipitation: 3.3% Caprylic acid precipitation, 45% Ammonium Sulphate precipitation
Affinity chromatography
BCA
ELISA – Enzymed Linked Immunosorbent Assay
SDS-Page electrophoresis
Desalting: PD 10, dialysis
IgY-HRP coupling: LL-Kit, Periodate Method
Concentration procedures: Roti-Spin Tube, Lyophilisation

Preparation of Solutions:

Precipitation:

- Acetate Buffer: 2.45g sodium acetate + 100 ml RO-H_2O
  2 ml Acetate + 198 ml RO-H_2O
  pH 4.8
- <u>Caprylic acid precipitation</u>
  10x PBS: 32.22 g Na_2HPO_4
  2.62 g NaH_2PO_4
  21.18 g NaCl
  10x PBS + 1 M Tris
  0.05M PBS

BCA reagents:

Working solution: 50 parts reagents A + 1 part reagents B

BCA Standard:

Stock solution: Albumine Standard Ampules: containing BSA (bovine serum albumine), 0.9% saline and 0.05% sodium azide.

Table 5 Diluted solutions of the BCA standard

Vial	Volume of 10xPBS	Volume and Source of BSA	Final BSA concentration
A	0 µl	300 µl of Stock Solution	2000 µg/ml
B	100 µl	300 µl of Stock Solution	1500 µg/ml
C	400 µl	400 µl of Stock Solution	1000 µg/ml
D	350 µl	350 µl of Vial C dilution	500 µg/ml
E	350 µl	350 µl of Vial D dilution	250 µg/ml
F	350 µl	350 µl of Vial E dilution	125 µg/ml
G	400 µl	100 µl of Vial F dilution	25 µg/ml
H	400 µl	0 µl	0 µg/ml

Gelelectrophoresis:

MES-buffer: 9,76 g MES
6.06 g Tris
1 g SDS
0.3 g EDTA
→ filled up to 1000 ml with RO-H_20

Extraction buffer:

Stock solution: 10xPBS

Working solution: 0.2 M PBS
0.5 % Tween 20
1% SDS
5 mM DTT

ELISA

Coating buffer: 1.22 g Na_2CO_3
3.25 g $NaHCO_3$
→ dissolved in 1000 ml RO-water

Washing buffer: 0.2 M PBS
0.1% Tween 20

Blocking buffer: 1% Ficoll in Coating Buffer

Assay buffer: 100 ml PBS Stock solution 0.2 M
400 µl Tween 20
→ diluted to 400 ml with RO-H_2O

Substrate solution for ELISA (for one plate):
12.5 ml substrate buffer
2.5 µl 30%-H_2O_2
100 µl TMB Stock solution

Stop solution: conc. H_2SO_4

Periodate methode:

Sodiumperiodate solution 0.1 M: 63 mg sodium periodate
→ dissolved in 3 ml RO-water

Bicarbonate-carbonate buffer 1 M: 560 mg $NaHCO_3$
350 g sodium carbonate
→ Dissolved in 10 ml RO-water

Bicarbonate carbonate buffer 0.1 M: Stock: bicarbonate carbonate buffer 1 M
→ Dilution 1:10

Ethylenglycol solution: 1 ml ethylenglycol
→ diluted in 17 ml RO-water

Sodium borhydride solution: 4 mg borhydride
→ dissolved in 1 ml RO-water

Column preparation:

- CNBr-activated sepharose:

$NaHCO_3$ buffer 0.1 M + 0.5 M NaCl: 2.4 g $NaHCO_3$
29,22 g NaCl
→ dissolved in 1000 ml RO-water
pH 8.3

Tris-HCl 0.1 M: 6.0 g Tris
→ with HCl to pH 8.0

Acetic acid/Sodium acetate buffer 0.1 M: pH 4

→ NHS-activated sepharose

Isopropanol

Coating buffer: $NaHCO_3$ buffer 0.2 M
NaCl 0.5 M
pH 8.3

Washing solution: HCl 1mM

Tris/HCl (0.1 M): 6.0 g Tris
→ with HCl to pH 8.0

Acetic acid/Sodium acetate buffer (0.1 M): pH 4

Affinity chromatography:

Binding Buffer: NaH_2PO_4 20 mM
NaCl 0.5 M
pH 7.5

Elution Buffer: Citrate phosphate Buffer 0.1 M
pH 3.2

3 Methods

3.1 Purification Methods

The precipitation from antigens and antibodies describes a reaction, where an antigen or antibody builds a complex with components of the precipitate. There is also the possibility to precipitate the antigens with antibodies.

3.1.1 Caprylic acid precipitation

Caprylic acid precipitation is an octanoic fatty acid with a molecular weight of 144.2 g mol-1.

It is used to induce the removal of the lipoproteins in the egg yolk. It was considered by *Svenson et al. (1995)* as useless for IgY precipitation *(Sumo Brain, 2007-2013)* but as useful to precipitate the bulk of both high and low molecular weight proteins in the raw egg yolk without affecting IgY. *(Guan-Ping et al., 2009)*

That means it is a precipitation method to precipitate the disturbing parts of the initial solution.

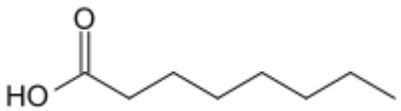

Figure 2 Caprylic acid (Solaray Inc, 2013)

3.1.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation is one of the widely used methods to salt out the proteins by using ammonium sulphate to purify and concentrate enzymes or antibodies. Increases in the ionic strength of the solution cause a reduction in the repulsive effect of charges between identical molecules of a protein. It also reduces the forces holding the salvation shell around the protein molecules. As soon as these forces are sufficiently reduced, the protein will precipitate.

Ammonium sulphate precipitation is efficient and convenient because it is cheap, highly soluble, lacks toxicity to most proteins and has a stabilizing effect. Furthermore the pH concentration and temperature are kept constant.

The needed amount of ammonium sulphate salt depends on the amount of protein or antibody to be precipitated. There is also the possibility to increase the amount of used salt stepwise in order to run a fractionate precipitation.

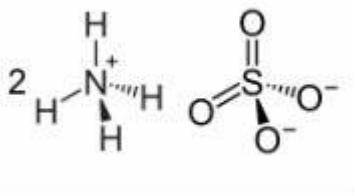

Figure 3 Ammonium Sulphate

3.2 Desalting/buffer exchange techniques

It is important to perform a buffer exchange for the further techniques. Too high salt concentrations could lead to failures in the results.

3.2.1 PD10 desalting column

The PD10 columns are pre-packed columns and ready for use. It is possible to use them to clean up big molecules, like proteins or other biomolecules (>5000MW) and remove the components with low molecular weight. Furthermore, it is an application for desalting or buffer exchange.

Molecules larger than the largest pores in the Sephadex matrix are excluded from the matrix and are eluted first, in or just outside the void volume, which is the column volume outside the Sephadex matrix.

Small molecules, which are smaller than the largest pores in the Sephadex matrix, will enter the pores to a varying extent. They have a larger accessible column volume than the large molecules and therefore they elute after the large molecules just before one total column volume of buffer has passed through the column. *(GE Healthcare)*

The volume of the desalt solution will increase in a ratio of 1:1.4.

3.2.2 Dialysis

Dialysis is a typical technique to remove small, unwanted compounds from macromolecules in solution. Therefore, a semi-permeable membrane is used. The molecular weight cutoff (MWCO) of the membrane is determined by the size of its pores. The sample and a buffer solution (dialysate) are on two opposite sides of the membrane and the sample molecules that are larger than the membrane are retained

on the sample side of the membrane. The small molecules diffuse free through the membrane pores until there is an equilibrium concentration with the entire dialysate volume. It is recommended to exchange the dialysate at least once. In the end the concentration of small molecules in the sample are decreased to acceptable or negligible levels. *(Thermo Scientific, 2013)* (*Wiley Information Services GmbH, 1999-2013)*

3.3 Affinity chromatography

Affinity chromatography is a chromatographic tool to separate biochemical mixtures. It is a highly specific technique, which separates biomolecules according to the differences in their biological function or chemical structure. This feature makes affinity chromatography unique in purification technologies and makes purifications easily possible which would be otherwise time-consuming, difficult or impossible with other techniques. It separates proteins on the basis of reversible interaction between a protein or group of proteins and a specific ligand coupled to a chromatographic matrix. Therefore it can be used to separate active biomolecules from denaturized or functionally different forms, to isolate pure substances present at low concentrations in large volumes of crude samples or just to remove specific contaminants. *(amersham pharmacia biotech)*

Biological interaction between a ligand and a target molecule can be the result of electrostatic or hydrophobic interactions, Van der Waals' forces and/or hydrogen bonding. The target can be eluted specifically by a competitive ligand or non-specifically by changing the pH, ionic strength or polarity.

As mentioned before, affinity chromatography can be an immense time-saving process. It can also handle large volumes of samples. Target molecules can be purified from complex biological mixtures, native forms can be separated from denaturized forms of the same substance and small amounts of biological material can be purified from high levels of contaminating substances. *(amersham pharmacia biotech)*

To achieve a higher degree of purity of the target a multiple-step process must be developed using purification strategy of capture, Intermediate Purification and Polishing (CIPP) because the purification obtained after a single purification step using affinity

chromatography is frequently not sufficient. Sometimes affinity tags may interfere with the post-purification use of the protein and if that happens, a multistep purification will be necessary. *(GE Healthcare)*

With the FPLC either high-performance affinity gels or standard affinity gels can be used.

The affinity medium consists of three components: The matrix, which is important for the ligand attachment, which should be chemically and physically inert. The spacer arm is used to improve binding between the ligand and the target molecule by overcoming any effects of steric hindrance. Last but not least, the ligand is the molecule that binds reversibly to a specific target molecule or a group of target molecules.

The stationary phase for affinity chromatography consists of a matrix to which specific ligands are covalently coupled. The ligand is chosen specifically for the purification of a particular biomolecule or group of molecules due to the natural tendency for the two to bind.

The base-matrix may be activated for the attachment of highly specific ligands or may have ligands already arranged, e.g. Protein A for IgG purification. The choice of the ligand depends on the biomolecule of interest; it can be selective for biological function (e.g. an inhibitor for enzyme purification or an antibody raised for antigen purification) or for chemical structure (e.g. lectins that will bind specific sugar residues for the purification of glycoproteins). As mentioned before IgY cannot be purified with Protein A or G columns but there are other possibilities, which are described below.

There are several activated base-matrices available; each designed to react with a specific attachment group on the ligand.

When a sample is applied to the column, only the biomolecules of interest are specifically adsorbed to the matrix while contaminants are washed through the column. Once the biomolecule of interest is adsorbed and free of contaminants, elution can be effected by several methods. The most common is to lower the pH. *(Pharmacia LKB Biotechnology)*

Description of the matrices for IgY purification

NHS-activated Sepharose 4 Fast Flow

NHS-activated Sepharose 4 Fast Flow is designed for the covalent coupling of ligands containing primary amino groups. The matrix is based on highly cross-linked agarose beads with 10-atoms spacer arms (6-aminohexanoic acid) attached by epichlorohydrin and activated by N-hydroxysuccininimide. Non-specific adsorption of proteins (which can reduce binding capacity of the target protein) is neglible due to the excellent hydrophilic properties of the base matrix. The matrix is stable at high pH to allow stringent washing procedures (subject to the pH stability of the coupled ligand). Ligands containing amino groups couple rapidly and spontaneously by nucleophilic attack at the ester linkage to give a very stable amide linkage. The amide bond is stable up to pH 13 making NHS-activated Sepharose suitable for applications that require conditions at high pH.

CNBr-activated Sepharose

CNBr-activated Sepharose is a pre-activated medium for immobilization of ligands containing primary amines. It offers an option for the coupling of larger ligands, like proteins, peptides, amino acids or nucleic acids. They can be coupled to CNBr-activated Sepharose under mild conditions, via primary amino groups or similar nucleophilic groups.

Cyanogen bromide reacts with hydroxyl groups on sepharose to form reactive cyanate ester groups.

The activated groups react with primary amino groups on the ligand to form isourea linkages. The coupling reaction is spontaneous and requires no special chemicals or equipment. The resulting multipoint attachment ensures that the ligand does not hydrolyse from the matrix. The activation procedure also crosslink sepharose and thus enhances its chemical stability, offering considerable flexibility in the choice of the elution conditions. *(Pharmacia LKB Biotechnology)*

3.4 Electrophoresis

Sodium dodecyl sulphate polacrylamide gel electrophoresis (SDS-Page) is a technique to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility (a function of the length, conformation and charge of the molecule) as well as their molecular weight. It is widely used in biochemistry, forensic, genetics, molecular biology and biotechnology. It allows the characterization of proteins, regarding their composition and their purity. *(Ren C., Tang. L. thang M., Guo S., 2009)* Molecules may be run in their nearly native state, preserving the molecules' higher order structure but unify the charge (e.g. with SDS) or the molecule will be denaturised by adding a chemical denaturant (e.g., DTT or mercaptoethanol) and heat to turn the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. First, the buffer solution is mixed with SDS, an anionic detergent, which denatures secondary and non-disulfide-linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass to charge ratio, as each protein has an isoelectric point and a molecular weight particular to its primary structure (native electrophoresis). A loading dye is added to the protein solution to track the progress of protein solution through the gel during the electrophoretic run.

The detection of the protein bands can be performed with different staining methods but the most common are staining with coomassie blue or silver nitrate. Classical coomassie blue staining can detect a 50 ng protein band and silver nitrate is 50 times more sensitive.

It depends on the molecular weight of the proteins which concentration of acrylamid should be used for the separation. Normally a 12 to 14% of acrylamid is used. *(Universität für Bodenkultur, 2006)*

3.5 Enzyme Linked Immunosorbent Assays – ELISA

Enzyme Linked Immunosorbent Assay (ELISA) is a useful test that uses antibodies and an enzymatic driven colour change to identify a substance or to detect antigens or antibodies in samples. The detection is based on an interaction between antigen and

antibody. The test is performed on high binding polystyrol plates and based on the absorption of polar or non-polar molecules.
There are different types of ELISA, which can be performed. Competitive ELISA is based in competition between marked tracer/antibody and analyte. The signal is proportional to analyte concentration.
For a Non-competitive ELISA a labelled antibody binds to an analyte, which is already bound to another antibody. Therefore, the analyte needs at least two epitops therefore the analyte has to be large enough. It is used, for example, to detect allergens. It is a form of Sandwich-ELISA.
There is also a difference between direct, where the antibody or tracer is directly labelled, and indirect ELISA, where labelled species are used who are specific for the antibody. Another difference is between homogenous (Single step reaction) and heterogeneous (including washing steps and separations) ELISA.
In this work the sandwich format was used to detect the IgY antibodies. The principle is easily described:
First the microtiter plate is coated with specific antibody. Then the sample is added on the plate. After a washing step enzyme labelled second antibody is added into the wells. After a second washing step the substrate (tetramethylbenzide) is further added into each well and after the reaction a stop solution (concentrated H_2SO_4) stops the reaction and the OD can be measured. *(Baumgartner, 2013)*

3.6 HRP Linkage techniques:

It is important to couple the detection antibody to be able to measure a signal and calculate the concentration.

The most important enzymes to couple the antibodies are galactosidase, alkaline phosphatase and the peroxidase, like the horse radish peroxidase. The substrate for the peroxidase is TMB (3,3',5,5'-tetramethylbenzidine). It is a chromogenic substrate and therefore likely used in ELISAs. It forms a pale blue-green liquid in solution with ethyl acetate.

The peroxidase reduces H_2O_2 to 2 OH and further to 2 OH- whiles the oxidation of TMB to 3,3',5,5'-tetramethylbenzidine dimine causes the solution to take on a blue colour.

Using sulphuric acid as a stop reagent turns TMB yellow and can be read on a spectrometer at a wavelength of 450 nm.

3.6.1 Lightning Link Lightning-Link™ HRP Conjugation Kit

The Lightning Link is an innovative technology. It enables direct labelling of proteins, peptides or other biomolecules. A big advantage is that the Lightning Link ™ Kit is a one-step procedure, really easy to use and it allows research to covalently labelled biomolecules in 30 seconds. The conjugate is extremely stable, because the bond between the antibody and the label is covalent.

Another big advantage of the LL-method is that the antibody recovery is 100% because there is no desalting step.

The Lightning Link HRP kit allows conjugations to set up in seconds, simply by adding a solution of the protein to be labelled to a proprietary lyophilized HRP mixture.

By circumventing the dialysis or desalting step, that commonly interrupts traditional protein conjugation procedures, Lightning-Link™ technology can be used to label small quantities of protein with 100% recovery and no excessive dilution of the conjugate.

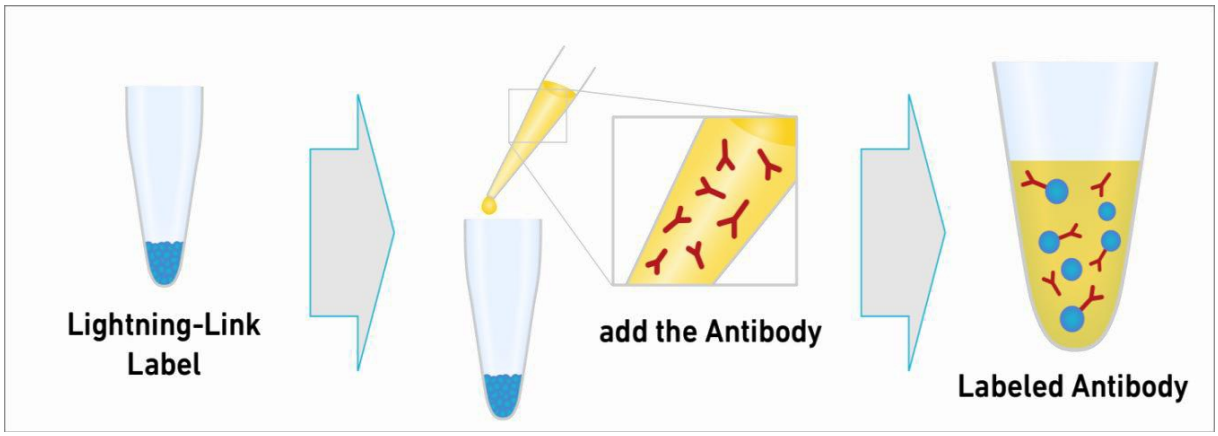

Figure 4 Lightning-Link antibody labelling process

The amount of antibody used for the labelling should correspond to molar ratios between 1:4 and 1:1 antibody to HRP. The volume of the antibody sample ideally should be up to 10 µl (10 µg pack size), up to 1 ml (1 mg pack size) and up to 5 mg (5 mg pack size). The concentration of the Ab in the range 0.5-5.0 mg/ml generally gives optimal results, but concentrations and volumes outside these suggested ranges have also yielded active conjugates.

3.6.2 Coupling of antibodies with the periodate method

Periodate opens the sugar rings of the peroxidase causing them to build aldehyde-residues. These aldehyde-groups can be coupled to the amino groups (-NH2) of the proteins by building peptide bonds. Peroxidase has a large amount of sugars. For that reason the coupling ratio will increase by using the periodate method.

3.7 Concentration methods:

3.7.1 Roti Spin Midi

The Roti Spin is a way to increase the concentration by using ultrafiltration through centrifugal forces. The centrifugal force drives the sample through a membrane and presses all molecules, which are small enough, through the pores. Biomolecules, which are bigger than the MWCO of the membrane stay in the sample reservoir. Liquids and molecules smaller than the MWCO of the membrane are collected on the bottom. It is useful to concentrate the samples before electrophoresis but it can also be used for buffer exchange and some other applications. It is important to choose the right MWCO of the membrane.

3.7.2 Lyophilisation:

Lyophilisation, also known as freeze-drying is a dehydration process, which uses sublimation. Sublimation is the transition of a substance from the solid to the vapour state, without first passing through an intermediate liquid phase. It works by freezing the material and reducing the surrounding pressure by inducing a vacuum. That allows the frozen water in the material to sublimate from the solid to the gas phase. (*Lyophilisation, 2013)*

4 Procedures

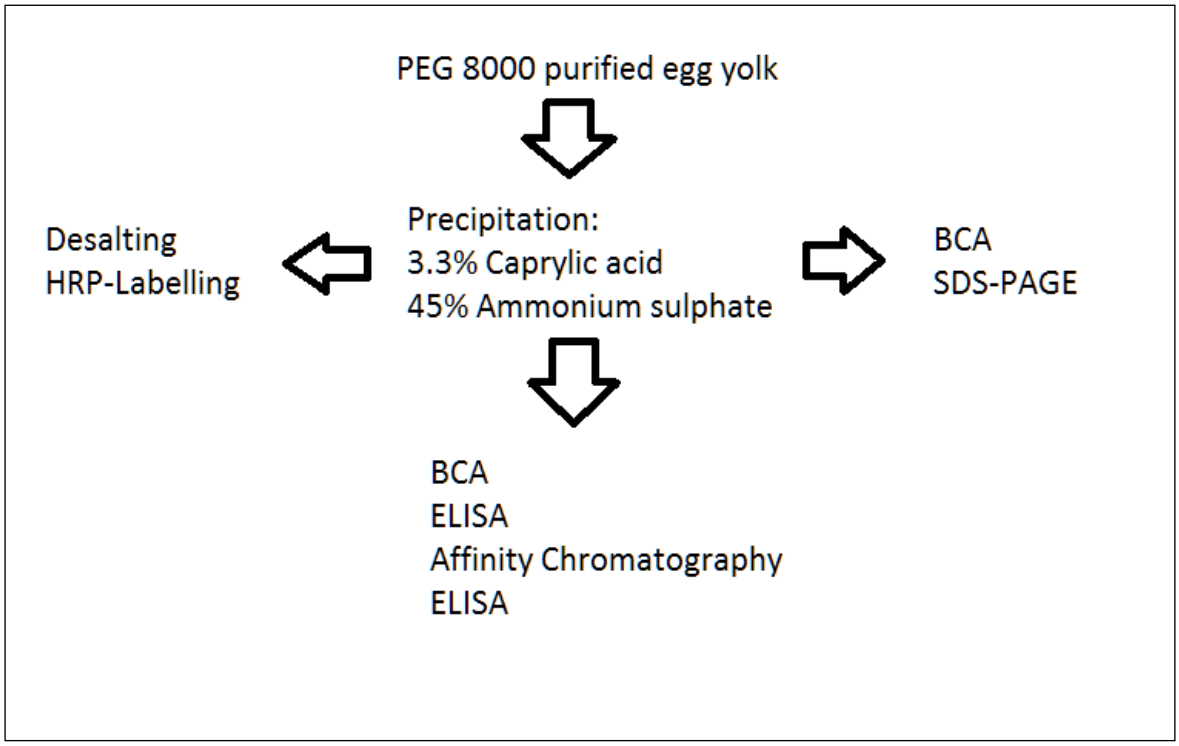

Figure 5 Scheme of the experiment

4.1 Precipitation of IgY from the egg yolk

The egg yolk (lupine) was purified (defatted) with PEG 8000 before the further purification was performed during this thesis. The eggshell was broken and the egg yolk was separated from the egg white. Further the skin of the yolk was removed and the pure egg yolk was collected and the same amount of IgY buffer was added and stirred for 15 minutes. Moreover 3.5% (w/v) PEG 8000 were added, stirred for 15 minutes and shaken for further 15 minutes, centrifuged for 15 minutes at 10 °C with 9500 rpm. The liquid phase, which contains the antibodies, was filtered and after that the extract was stored at -20 °C. This is the raw material for the further purification steps, which were performed for this work. Different precipitation methods were performed before the affinity chromatography.

A. Precipitation with 3.3% caprylic acid

B. Precipitation with 7.5% caprylic acid

C. Precipitation with 45% ammonium sulphate

D. Precipitation with a combination of 3.3% caprylic acid and 45% ammonium sulphate

A. To 50 ml of the pre-treated egg yolk lupine 100 ml of acetate buffer were added. Then a caprylic acid precipitation was performed by adding 3.3% of caprylic acid drop by drop and 1/10 0.2 M PBS. The solution was stirred for 30 minutes at room temperature and further the following centrifugation was performed at 10.000 g for 15 minutes at 5 °C. The supernatant contains the antibodies so the pellet was removed.

B. To 50 ml of the pre-treated egg yolk lupine 100 ml of acetate buffer were added. Then a caprylic acid precipitation was performed by adding 7.5 % of caprylic acid drop by drop and 1/10 0.2 M PBS. The solution was stirred for 30 minutes at room temperature and further the following centrifugation was performed at 10.000 g for 15 minutes at 5°C. The supernatant contains the antibodies so the pellet was removed.

C. To 50 ml of the pre-treated egg yolk lupine 100 ml of acetate buffer were added. Then a caprylic acid precipitation was performed by adding 45% of ammonium sulphate and 1/10 of 0.2 M PBS. The solution was stirred for 1 hour at 4 °C and further the following centrifugation was performed at 10.000 g for 15 minutes at 5 °C. The pellet contained the antibodies so the supernatant was removed.

D. To 50 ml of the pre-treated egg yolk lupine 100 ml of acetate buffer were added. Then a ammonium sulphate precipitation was performed by adding 3.3% of caprylic acid drop by drop and 1/10 0.2 M PBS. The solution was stirred for 30 minutes at room temperature and further the following centrifugation was performed at 10.000 g for 15 minutes at 5 °C. The supernatant contained the antibodies so the pellet was removed (a small amount was kept for analysing via BCA and SDS-Page). Additionally an ammonium sulphate precipitation was performed. Therefore 45% (w/v) ammonium sulphate was added and incubated for 1 hour at 4 °C stirred. Then again the solution had to be centrifuged at 10 °C with 10.000 g for 15 minutes. This time the precipitate is the part which contained the IgY antibodies. Therefore the supernatant could be removed (a small amount was kept for analysing via BCA and SDS-Page).

Afterwards a buffer exchange to 0.05 M PBS (pH 7.4) was performed.

4.2 Preparation of the lupine extract

To 1 g of lupine flour (CDL-Allergene, gel. 10.11.08, *not-defatted*) 10 ml of extraction buffer were added and well stirred. The incubation is performed for 30 minutes in a 60 °C water bath. During the incubation the mixture was vortexed a few times. A centrifugation was performed by 9500 g for 15 minutes at 22 °C. The supernatant contained the proteins. For that reason the pellet was removed.

4.3 Determination of the amount of protein with BCA test

For concentration determinations, the Thermo Scientific, Pierce BCA Protein Assay Kit was used. The procedure followed the SOP **(Attachment 1)** but the dilutions of the standard. The preparation of the protein standards, as standard protein Bovine Serum Albumin (BSA) and as diluents 0.15 M NaCl-solution were used, performed at first. The working reagents were prepared freshly by mixing one part BCA Reagent A and twenty parts of BCA Reagent B.

Table 6 Preparation of BSA Standards for BCA

Vial	Volume of diluent	Volume and Source of BSA	Final BSA concentration
A	0 µl	300 µl of Stock Solution	2000 µg/ml
B	100 µl	300 µl of Stock Solution	1500 µg/ml
C	400 µl	400 µl of Stock Solution	1000 µg/ml
D	350 µl	350 µl of Vial C dilution	500 µg/ml
E	350 µl	350 µl of Vial D dilution	250 µg/ml
F	350 µl	350 µl of Vial E dilution	125 µg/ml
G	400 µl	100 µl of Vial F dilution	25 µg/ml

20 µl of each standard or sample has been pipetted into a well of the microtiter plate and 200 µl per well working reagent were added and shaken for 30 seconds on a plate shaker. Furthermore the plate was covered and incubated for 30 minutes at 37°C. After cooling the plate to room temperature the absorbance was measured at 562 nm on a plate reader.

4.4 Checking the remaining proteins via SDS-Page

12% Bis/Tris gel was used for the electrophoresis. The concentration should be approximately 5 µg per ml. The required amount of the different samples was calculated from the results after the BCA. The samples contained approximately 5 µg protein and 2.5 µl loading buffer. The rest was filled up with deionised water to a total volume of 10 µl. The electrophoresis chamber was prepared by putting the gel into the equipment and filling up the whole chamber with MES buffer. To denaturise the proteins the samples were heated for 10 minutes at 70 °C, after which the samples were put in the gel chambers. Electrophoresis ran for 45 minutes with 2000 V and 300 mA. Furthermore, the gel was washed three times with deionised water for about 10 minutes and after that it was stained for one hour with coomassie blue and then distained with deionised water over night.

4.4.1 Linkage Lightning Link procedure

The start concentration of the IgY-Ab is 1.66 mg/ml. So in a volume of 10 µl the concentration is 16.6 µg. The ratio between c(Ab) and c(LL-HRP) should be between 1:1 and 1:4.

10 µl…16.6 µg

+1 µl LL-Modifier → c(Ab)=15.09 µg c(LL-HRP)= 100 µg

C(Ab):c(HRP) = 1:6.6

For that reason a start amount of 20 µl is used.

20 µl → 33.2 µg
+ 2 µl LL – Modifier → c(Ab) = 30.18 µg

V=22 µl c(LL-HRP)= 100 µg
c(Ab): c(LL-HRP)= 1:3

The solution of 22 µl containing the antibody and the LL-Modifier was transferred to the HRP-tube containing the LL-HRP powder.
After suspending the powder in the solution the mixture was incubated for three hours at room temperature.
Furthermore 2 µl of quencher was added to the solution and incubated for further 30 minutes. After this step the labelled antibody was prepared and ready to use.

4.4.2 Coupling of antibodies with the periodate method

0.5 ml sodium periodate solution 0.1 M and 2 mg peroxidase per 8 mg antibody, were incubated for 20 minutes at room temperature. Then 30 µl of an ethylene glycol solution (1 M) was added and incubated for five minutes at room temperature. By using a PD10 column a buffer exchange to a bicarbonate buffer 0.1 M (pH 9.5) was performed.

Subsequently, 0.1 ml of a bicarbonate buffer 1 M was added and the solution was incubated for 2 hours at room temperature. Then 0.2 ml sodium borohydride solution was poured into the mixture and incubated for 2 hours at room temperature. In the end, another buffer exchange with a PD10-sephadex column to a 0.2 M PBS buffer was performed.

4.5 Preparation of ELISA-plates after purification

Three plates were coated with different concentration of the IgY-anti lupine antibodies: 2 µg/ml, 1 µg/ml and 0.5 µg/ml. The initial solution had a concentration of 0.5 mg/ml. 100 µl per well were pipetted into each well and incubated at 4 °C over night.

The plates were emptied and 300 µl of Blocking Buffer, containing 1%Ficoll, were added into each plate and incubated for two hours at 37 °C. After spilling out the plates were washed five times with washing buffer.

Furthermore, dilutions of the lupine flour as a standard were prepared and 100 µl per well were pipetted. After incubation for 30 minutes at room temperature, 100 µl of the capture antibody (Lupine-Ak-Mono 260110SP2C12-A11-C10-C8, IgY-HRP, once coupled with Lightning Link Kit and once with the periodate method) were added into each well. The plates were washed three times and 100 µl of the substrate solution (TMB) were pipetted into each well. After incubation for 30 minutes the reaction was stopped with the stop-reagents (concentrated H_2SO_4).

Table 7 Preparation of lupine standards for ELISA

Vial	Dilution	Concentration
A	1:150	100.000 µg/l
B	1:200	75.000 µg/l
C	1:300	50.000 µg/l
D	1:1.500	10.000 µg/l
E	1:3.000	5.000 µg/l
F	1:15.000	1.000 µg/l
G	1:150.000	100 µg/l
H	0	0 µg/l (=Blank)

4.6 Packing the columns

In this experiment two different types of matrices were chosen for the affinity chromatography. The procedures are described below.

4.6.1 Preparing the medium and Packing of the Column with NHS-activated Sepharose

Two different matrices were used. The selection criteria were based on their affinity to the primary amino groups of the lupine proteins, which were used as a ligand and for that reason had to be coupled with the matrix.

4.6.1.1 Preparing the medium for the NHS-activated Sepharose

NHS-activated Sepharose was delivered in 100% Isopropanol. 2 ml gel was washed with approximately 40 ml ice cold 1 mM HCl through a sintered glass filter. The coupling solution (0.2 M $NaHCO_3$ + 0.5 M NaCl, pH 8.3) was prepared and the ligand to be coupled, lupine extract, was dissolved in the coupling buffer in a high volume (ratio: CB: lupine= 1:4). The gel and the coupling solution containing the ligand were mixed and incubated end over end over night at 4 °C. To block the remaining non-reacted groups the medium was kept in blocking solution (0.1 M Tris-HCl, pH 8.5) for two hours at room

temperature. The following washing procedure contained two alternating buffers, one with high pH (0.1 M Tris-HCl, pH 8), and the other one with low pH (0.1 M acetate buffer + 0.5 M NaCl, pH 4). The medium was washed first with the high pH buffer and afterwards with the low pH buffer and this cycle was repeated six times. The medium was then ready for use but in order to prevent the microbial contamination it was transferred to 20% ethanol.

4.6.1.2 Packing the column

At first it was necessary to remove the air from the end-piece of the column and adapter by flushing with binding buffer (20 mM NaH_2PO_4 + 0.5 M NaCl, pH=7.5). The medium was re-suspended and transferred in a single step into the column which was filled up with binding buffer up to the top. After waiting for 30 minutes the column was connected to the FPLC pump and the column was flushed with a low flow rate until the medium was collected at the bottom of the column. The flow rate was increased and left for approximately ten minutes. After stopping the pump the column outlet was closed.

The column was flushed for one hour with binding buffer (flow rate: 0.8 ml/min) before it was ready to use.

4.6.2 Preparation of the medium and packing of the column with CNBr-activated Sepharose

4.6.2.1 Preparing the medium

CNBr-activated Sepharose was supplied as a lyophilized powder in the presence of additives. 2 g of the lyophilized powder was dissolved in 1 mM HCl, pH=8.0 and washed through a filtered glass filter with approximately 400 ml 1 mM HCl for 15 minutes. 10 ml coupling solution (0.1 M $NaHCO_3$ + 0.5 M NaCl, pH= 8.3) and 10 ml lupine protein solution were mixed together and incubated end over end at 4 °C over night. The medium was washed with approximately 100 ml coupling buffer through a sintered glass filter five times. To block the remaining non-reacted groups the medium was kept in blocking solution (0.1 M Tris-HCl, pH=8.5) for 2 hours at room temperature. The following washing procedure contained two alternating buffers, one with high pH (0.1 M Tris-HCl, pH=8) the other one with low pH (0.1 M acetate buffer + 0.5 M NaCl, pH=4). The medium was washed first with the high pH buffer and after with the low pH buffer

and this cycle was repeated six times. Furthermore, the medium was degassed with a vacuum pump.

4.6.2.2 Packing of the column

At first it was necessary to remove the air from the end-piece of the column and adapter by flushing with binding buffer (20 mM NaH_2PO_4 + 0.5 M NaCl, pH=7.5). The medium was re-suspended and transferred in a single step into the column, which was filled up to the top with binding buffer. After waiting for 30 minutes the column was connected to the FPLC pump and the column was flushed with a low flow rate until the medium was collected at the bottom of the column. The flow rate was increased and the flushing procedure was continued for approximately ten minutes. After stopping the pump the column outlet was closed.

The column was flushed for one hour with binding buffer (flow rate: 0.8 ml/min) before it was ready to use.

4.7 Affinity chromatography

Different procedures were tried to discover the best binding buffer and elution buffer for the affinity chromatography.

Procedure 1: Different salt concentrations of binding buffer (20 mM NaH_2PO_4 + 0.5 M NaCl, pH=7.5) and elution buffer (20 mM NaH_2PO_4)

The procedure for NHS-activated Sepharose and CNBr-activated Sepharose is the same. First the column was flushed for one hour with binding buffer. Furthermore different amounts of samples were filtered through the column and the rate of flow was collected. To remove the non-binding elements the column was flushed for 30 minutes with the binding buffer. To elute the IgY-lupine antibodies the column was flushed with elution buffer. But the elution concentration increased to 100% after ten minutes. The rate of flow was collected with the fraction collector in 1ml per tube.

Procedure 2: Different pH values of the binding buffer (20 mM NaH_2PO_4. pH=7.5 + 0.5 M NaCl) and elution buffer (0.1 M citrate-phosphate buffer).

The procedure for NHS-activated Sepharose and CNBr-activated Sepharose is the same. First the column was flushed for one hour with binding buffer. Furthermore, different amounts of samples were filtered through the column and the rate of flow was

collected. To remove the non-binding elements the column was flushed for 30 minutes with the binding buffer. To elute the IgY-lupine antibodies the column was flushed with elution buffer. But the elution concentration increased to 100% after ten minutes. The rate of flow was collected with the fraction collector in 1 ml per tube. Before collecting the outcome 0.5 ml of a 0.1 M PBS (pH=10) was transferred into the tubes to avoid the stress for the antibodies because of the low pH of the elution buffer.

The tubes where the proteins were found by showing a peak on the chromatogram were kept and by BCA the protein concentration was discovered and an electrophoresis was done as well.

4.8 Buffer exchange methods

In this experiment two different techniques were used to exchange the buffer or desalt the solutions. Both are described below.

4.8.1 PD10 desalting column

In the beginning the column was washed five times with the 0.2 M PBS buffer. Furthermore 2.5 ml sample were transferred into the column and the outcome which is just buffer can be removed. After that 3.5 ml 0.2 M PBS buffer were added to the column and after approximately ten drops, the outcome had to be collected because it contained the desalted sample.

4.8.2 Dialysis for buffer exchange

The total volume of purified IgY-lupine antibody was approximately 60 ml. A buffer exchange to 0.5 M PBS was conducted. A dialysis membrane was washed under hot water until it became soft. Furthermore, one end was closed and the sample was carefully filled into the membrane. 10 ml of 0.5 M PBS were used as dialysate and put into a bucket. Moreover, the dialysis membrane was put into the dialysate and stirred over night at 4 °C.

The dialysate was changed and the dialysis membrane was stirred for one hour at 4 °C.

4.9 Concentration methods:

4.9.1 Roti Spin Midi

First the sample reservoir was washed with 70 % ethanol and centrifuged at 7500 g for 15 minutes until all the ethanol left the sample reservoir. The filtrate was removed and the sample reservoir was filled with RO-water was centrifuged again for 15 minutes at 7500 x g.

3.5 ml of the sample were pipetted into the Roti-Spin Midi and centrifuged at 2000 x g for 50 minutes. After the desired volume was reached the filtrate was removed and the sample reservoir was closed with the concentrate catcher. Furthermore the tube was put into the centrifuge bottom up and centrifuged for 3 minutes at 2000 rpm.

4.9.2 Lyophilisation:

Another way to increase the concentration is to lyophilize the dialysate. First, 25 ml of the dialysate were filled up in 50 ml falcon tubes and put into the minus 80 °C freezer to pre freeze them. Furthermore, the solution was put into the freeze dryer over night. The newly formed powder was dissolved in 2.5 ml RO-water. In total approximately 200 ml were lyophilised.

4.10 ELISA after affinity chromatography

Two plates were coated with different concentrations of the IgY-anti lupine antibodies: 2.0 µg/ml and 1.0 µg/ml. The initial solution had a concentration of approximately 0.78 mg/ml. 100 µl per well were pipetted into each well and incubated at 4 °C over night.

The plates were spilt out and 300 µl of blocking buffer, containing 1% Ficoll, were added into each plate and incubated for two hours at 37 °C. After spilling out, the plates were washed five times with washing buffer.

Furthermore, dilutions of the lupine flour as a standard were prepared and 100 µl per well were pipette. After incubation for 30 minutes at room temperature 100 µl of the capture antibody (Lupine-Ak-Mono 260110SP2C12-A11-C10-C8 (after whole purification), IgY-HRP (after precipitation steps) (once coupled with Lightning Link Kit and once with the periodate methode) were added into each well. The plate was washed three times and 100 µl of the substrate solution (TMB) were pipetted into each

well. After incubation for 30 minutes the reaction was stopped with the stop-reagents (concentrated H_2SO_4).

Table 8 Preparation of lupine Standards for ELISA

Vial	Dilution	Concentration
A	1:150	100.000 µg/l
B	1:200	75.000 µg/l
C	1:300	50.000 µg/l
D	1:1.500	10.000 µg/l
E	1:3.000	5.000 µg/l
F	1:15.000	1.000 µg/l
G	1:150.000	100 µg/l
H	0	0 µg/l (=Blank)

5 Results

5.1 BCA results

The amount of antibodies in the original solution was measured via BCA, after the precipitations, after the affinity chromatography as well as after the lyophilisation. After the ammonium precipitation the solution was dialysed against 0.5 M PBS and the remaining amount was measured as well.

The tables below show the results of the different steps during the whole purification process: (Dilutions and values which were not used for calculating the average of the final concentration are not listed).

Table 9 Protein concentration of the initial solution

mean Abs	Std. Dev.	found conc.	Dilution	Calculated conc. [mg/ml]	Final conc. [mg/ml]
0.98	0.06	0.97	1:50	48.68	**46.68**
0.51	0.05	0.49	1:100	48.51	
0.06	0.05	0.04	1:1000	42.84	

Table 10 Protein concentration from the initial solution after adding acetate buffer

mean Abs	Std. Dev.	found conc.	Dilution	Calculated conc. [mg/ml]	Final conc. [mg/ml]
4.16	12.92	7.04	1:1	7.04	**6.52**
0.18	0.05	0.15	1:50	7.63	
0.08	0.05	0.05	1:100	5.40	

To 50 ml of the initial solution 100 ml acetate buffer were added. This means that the volume extended in a ratio of 1:3.

Table 11 Protein concentration after the Precipitation with 3.3% caprylic acid

mean Abs	Std. Dev.	found conc.	Dilution	Calculated conc. [mg/ml]	Final conc. [mg/ml]
3.20	0.82	4.07	1:1	4.07	**5.73**
0.15	0.05	0.13	1:50	6.44	
0.07	0.05	0.05	1:100	5.02	

1.65 ml caprylic acid were added. The new volume was 151.65 ml.

Table 12 Protein concentration from the supernatant after ammonium sulphate precipitation

mean Abs	Std. Dev.	found conc.	Dilution	Calculated conc. [mg/ml]	Final conc. [mg/ml]
0.09	0.05	0.07	1:1	0.07	**0.74**
0.05	0.05	0.03	1:50	1.41	
0.02	0.05	0.00	1:100	0.39	

The remaining concentration in the supernatant after ammonium sulphate precipitation is 0.74 mg/ml.

Table 13 Protein concentration of the pellet after ammonium sulphate precipitation

mean Abs	Std. Dev.	found conc.	Dilution	Calculated conc. [mg/ml]	Final conc. [mg/ml]
1.09	0.06	1.10	1:1	1.10	**13.67**
0.41	0.05	0.38	1:50	18.97	
0.24	0.05	0.21	1:100	20.93	

The pellet was collected and re-suspended in 30 ml 0.2 M PBS buffer. The result is that from an initial volume of 50 ml we can precipitate with this method a total amount from 13.67 mg per ml. The solution has to be dialysed to remove the ammonium sulphate. A buffer exchange was performed with 0.5 mM PBS buffer.

Table 14 Protein concentration after dialysing

mean Abs	Std. Dev.	found conc.	Dilution	Calculated conc. [mg/ml]	Final conc. [mg/ml]
3.06	0.64	3.80	1:1	3.80	**4.13**
0.07	0.05	0.04	1:50	2.22	
0.03	0.05	0.01	1:100	0.87	

After the final precipitation we have a total volume of 30 ml. After the dialysis the volume extended to 75 ml, which corresponds to an approximate ratio of 1:4.

The BCA results of the affinity chromatography show the flow-through, the waste while flushing with binding buffer and the collected fractions. The actual outcome of the specific lupine-IgY could not be measured with BCA. For that reason a lyophilisation was performed before the BCA. The following tables show the results of the concentrated solutions.

Table 15 BCA results of the flow-through of the affinity chromatography

mean Abs	Std. Dev.	found conc.	Dilution	Calculated conc. [mg/ml]	Final conc. [mg/ml]
1.78	0.04	1.79	1:1	1.76	**4.54**
1.445	0.04	1.44	1:2	2.89	
1.01	0.04	1.00	1:4	3.98	
0.64	0.04	0.63	1:8	5.01	
0.38	0.04	0.36	1:16	5.73	
0.21	0.04	0.18	1:32	5.80	

Table 16 BCA result of the outcome of the non-binding IgY

mean Abs	Std. Dev.	found conc.	Dilution	Calculated conc. [mg/ml]
2.471	0.05	2.49	1:1	2.49
2.833	0.06	2.86	1:2	5.71
2.303	0.05	2.32	1:4	9.27
1.483	0.04	1.48	1:8	11.85
0.89	0.04	0.88	1:16	14.10
0.505	0.04	0.48	1:32	15.54
0.26	0.04	0.23	1:64	14.86
0.151	0.04	0.13	1:128	16.07

Table 17 BCA result of the specific lupine IgY

mean Abs	Std. Dev.	found conc.	Dilution	Calculated conc. [mg/ml]	Final. Conc.. [mg/ml]
0.47	0.05	0.72	1:1	0,72	**0.78**
0.35	0.06	0.42	1:2	0.84	
0.24	0.05	0,51	1:4	1.01	
0.17	0.04	0.09	1:8	0.73	
0.08	0.04	0.04	1:16	0.60	

5.2 SDS-Page

After calculation of the protein concentration of the different precipitation steps the best amount and the dilution for the performance of an SDS-Page were calculated.

For the calculation the concentration of each dilution was taken and under the assumption that 5 µg of protein were needed, calculated via 5 µl divided by concentration of dilution µg/µl.

$$c(SDS - Page) = \frac{5\mu L}{c(dilution)}$$

Formular 1 Calculation of the needed concentration for the SDS-Page gelelectrophoresis

Sometimes the calculated amount was too small to be pipetted so a greater amount was pipetted. For the supernatant after the ammonium sulphate precipitation the amount was too big. Therefore the maximum amount was pipetted into the well.
For the sample after dialysis the concentration for a 1:5 dilution was calculated and the needed amount was calculated for this dilution.

Table 18 BCA result of the outcome of the non-binding IgY

Sample	Dilution of the sample	Amount calculated [µl]	Amount sample [µl]	Amount dest. H2O [µl]	Amount loading buffer [µl]
initial egg-yolk	1:50	5.14	5.14	2.0	2.5
Egg yolk + acetate buffer	1:1	0.71	3	4.5	2.5
Supernatant after caprylic acid precipitation	1:1	1.23	3	4.5	2.5
Supernatant after ammonium sulphate precipitation	1:1	69,44	7	0.5	2.5
Pellet after ammonium sulphate precipitation	1:1	4.54	4.50	3.0	2.5
Sample after dialysis	1:5	5.52	5.52	1.5	2.5

Initial egg-yolk	1
Egg yolk + acetate buffer	2
Supernatant after caprylic acid precipitation	3
STANDARD	4
Supernatant after ammonium sulphate precipitation	5
Pellet after Ammonium Sulphate precipitation	6
Sample after Dialysis	7

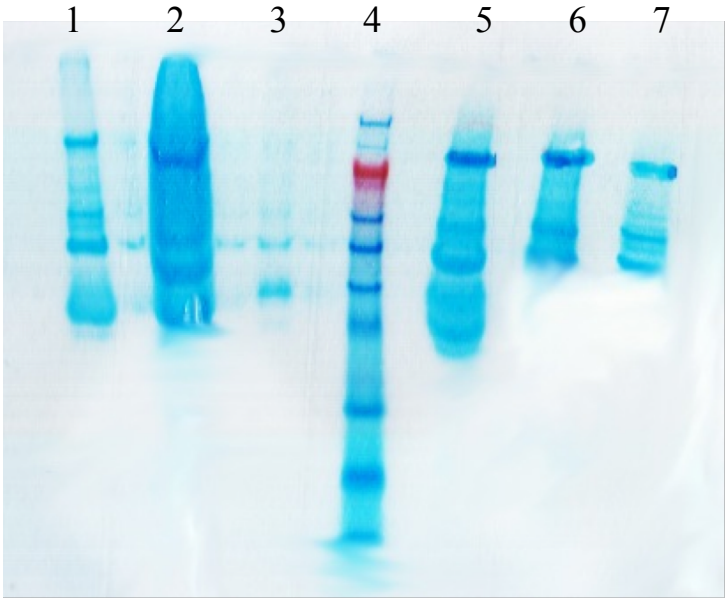

Figure 6 SDS-Page of all precipitation steps

The gel electrophoresis shows all the different steps of the precipitation. In the first lane is the initial egg-yolk. In lane 1 (initial egg-yolk) and 2 (egg yolk plus acetate buffer) equal concentration of protein was loaded. There is a lot protein in the first lane. In the second lane the volume or the dilution was too high so the result is not really clear. But the same proteins are still in the sample solution after adding acetate buffer to the initial egg-yolk solution. This seems like something went wrong with the BCA for the second sample. In the conclusion of the BCA there is too much protein missing.

In the third lane is the supernatant after the caprylic acid precipitation. The bands are lower.

The results of the sample in the third lane compared to the BCA results show, that the missing proteins after the caprylic acid precipitation were still there, but could not be measured in the BCA test. A possible reason could be that the sample was not sufficient vortexed before doing the dilutions for the BCA test. In the end, all the different dilutions showed almost the same result at the BCA test.

In the fourth lane is the standard: Novex-seeblue.

The fifth lane contains the supernatant after the ammonium sulphate precipitation. There are not a lot proteins found. In the next lane the dissolved pellet from the ammonium per-sulphate precipitation, containing the IgY-antibodies, is shown and lane number seven shows the same sample but after the dialysis.

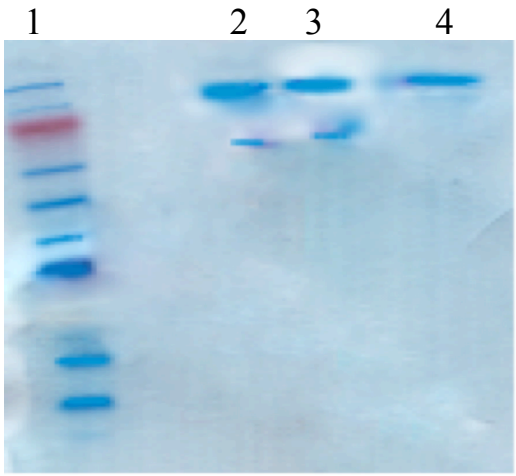

Figure 7: SDS-Page after lyophilisation

1	Standard
2	Flow-through after after affinity chromatograhy
3	Outcome after flushing with binding buffer
4	Specific lupine IgY

After the lyophilisation of the purified IgY another SDS-Page was performed. Figure 7 shows the flow-through, the outcome of the unspecific IgY and the specific lupine-IgY. The first lane contains the standard. The second lane shows the flow-through after the affinity chromatography. There are two bands visible. The third lane contains the outcome while flushing with binding buffer. There are two bands clear visible as well. Last but not least, the fourth lane contains the sample after flushing with elution buffer. It contains the specific-lupine-IgY. There is one band visible at the expected size/place.

5.3 Sandwich ELISA results

5.3.1 Sandwich ELISA with LL-HRP – IgY detection Ab after precipitation

Table 19 Absorbance at a concentration of the coupling Ab of 0.5 µg and LL-HRP detection Ab

c	*1:10*		*1:100*		*1:1000*		*1:10000*		*1:100000*		*1:500000*	
0	2.64	2.63	0.28	1.54	1.47	0.95	0.95	0.25	0.30	0.18	0.08	0.07
100	2.46	2.34	0.40	1.07	1.07	0.71	0.68	0.20	0.22	0.14	0.07	0.07
1.000	1.77	1.83	0.78	0.78	0.70	0.53	0.51	0.12	0.15	0.09	0.06	0.06
5.000	1.54	1.57	0.82	0.63	1.57	0.39	0.44	0.16	0.14	0.09	0.06	0.06
50.000	1.10	0.982	1.52	0.42	0.38	0.32	0.33	0.09	0.11	0.07	0.06	0.06
75.000	0.75	0.858	1.69	0.35	0.31	0.31	0.28	0.10	0.11	0.07	0.06	0.06
10.000	0.60	0.654	1.20	0.21	0.19	0.14	0.16	0.07	0.09	0.06	0.06	0.07
100.000	0.81	0.882	0.32	0.32	0.27	0.15	0.18	0.07	0.10	0.07	0.06	0.06

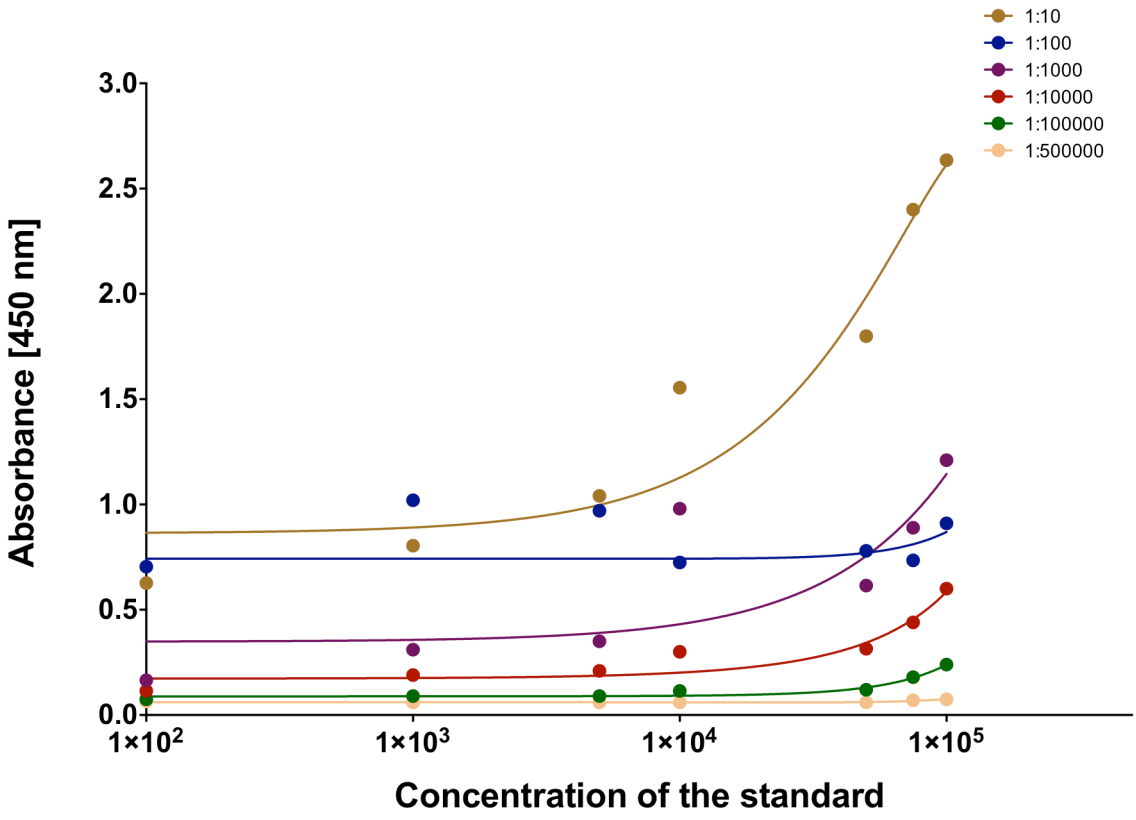

Figure 8: Absorbance dependence on concentration; Coupling Ab concentration: 0.5 µg; Detection Ab: IgY-HRP labelled with LL-kit

Table 20 Absorbance at a concentration of the coupling Ab of 2 µg and LL-HRP detection Ab

c	1:10		1:100		1:1000		1:10000		1:100000		1:500000	
100.000	2.53	2.64	2.10	2.12	1.30	1.30	0.34	0.37	0.25	0.24	0.09	0.07
75.000	2.54	2.482	1.69	1.65	1.04	1.01	0.25	0.29	0.19	0.19	0.10	0.09
50.000	2.03	2.26	1.52	1.46	0.85	0.85	0.19	0.21	0.15	0.15	0.09	0.10
10.000	2.25	2.24	1.20	1.27	0.77	0.78	0.16	0.20	0.14	0.13	0.07	0.12
5.000	1.98	2.12	0.82	0.82	0.61	0.58	0.11	0.14	0.11	0.11	0.06	0.13
1.000	2.11	2.07	0.78	0.85	0.56	0.58	0.11	0.15	0.12	0.10	0.07	0.12
100	1.39	1.19	0.40	0.38	0.29	0.28	0.09	0.11	0.09	0.14	0.08	0.06
0	1.03	1.00	0.28	0.32	0.19	0.18	0.14	0.11	0.09	0.08	0.08	0.06

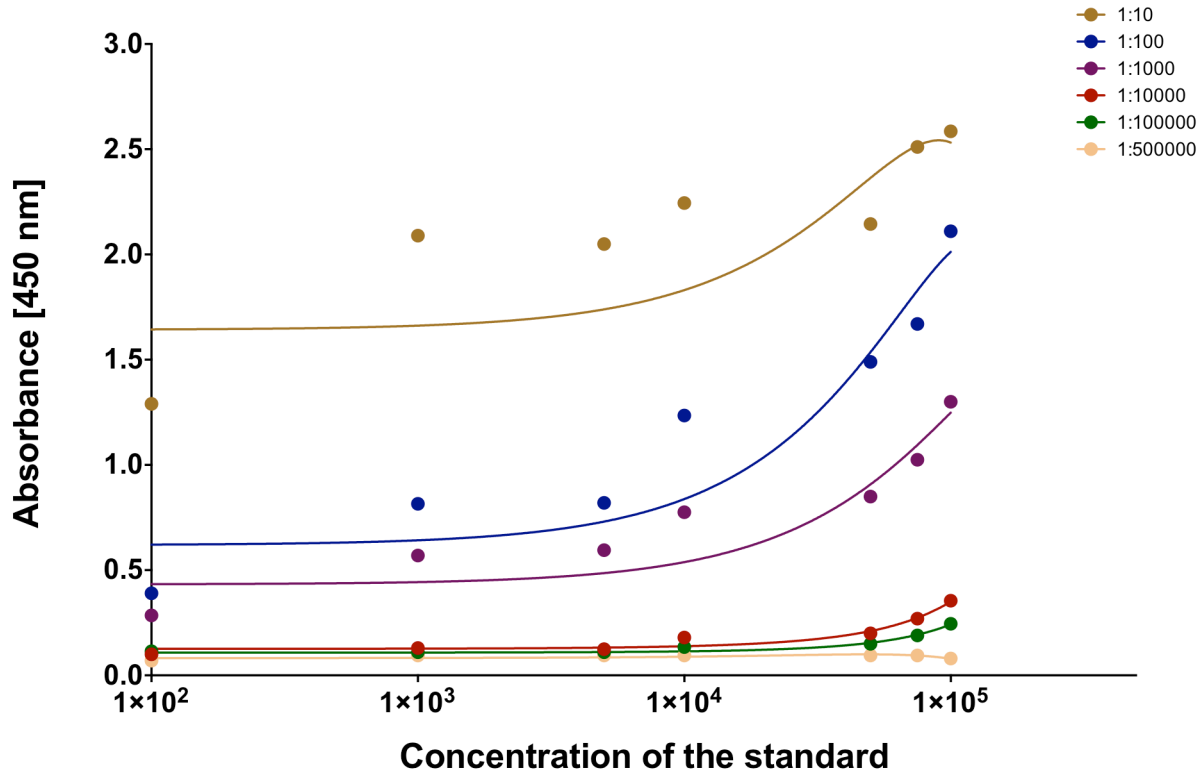

Figure 9: Absorbance dependence on concentration; Coupling Ab concentration: 2 µg; Detection Ab: IgY-HRP labelled with LL-kit

5.3.2 Sandwich ELISA with periodate method coupled IgY-HRP detection Ab after precipitation

Table 21 Absorbance at a concentration of the coupling Ab of 0.5 µg and Periodate-HRP detection Ab

c	1:10		1:100		1:1000		1:10000		1:100000		1:500000	
100.000	0.36	0.27	0.10	0.09	0.07	0.08	0.04	0.07	0.06	0.06	0.06	0.06
75.000	0.32	0.26	0.10	0.09	0.07	0.08	0.04	0.07	0.06	0.06	0.07	0.07
50.000	0.32	0.25	0.09	0.09	0.08	0.08	0.04	0.07	0.06	0.07	0.07	0.06
10.000	0.24	0.24	0.13	0.10	0.08	0.08	0.04	0.07	0.07	0.07	0.07	0.07
5.000	0.22	0.12	0.08	0.08	0.07	0.07	0.04	0.07	0.06	0.07	0.08	0.09
1.000	0.13	0.12	0.08	0.09	0.08	0.09	0.04	0.07	0.08	0.07	0.10	0.09
100	0.12	0.11	0.09	0.09	0.09	0.09	0.06	0.09	0.08	0.08	0.09	0.10
0	0.12	0.13	0.09	0.10	0.08	0.08	0.05	0.07	0.06	0.06	0.07	0.08

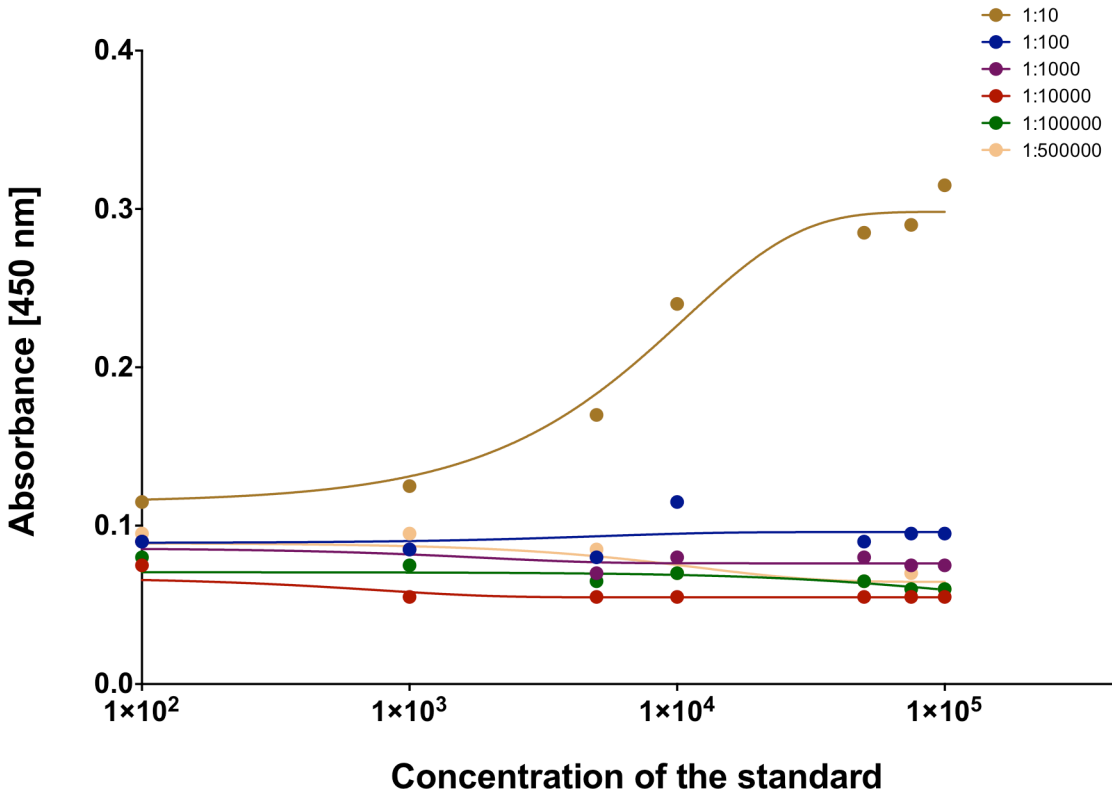

Figure 10: Absorbance dependence on concentration; Coupling Ab concentration: 0.5 µg; Detection Ab: IgY-HRP labelled with Periodate Method

Table 22 Absorbance at a concentration of the coupling Ab of 1 µg and Periodate-HRP detection Ab

c	1:10		1:100		1:1000		1:10000		1:100000		1:500000	
100.000	0.42	0.37	0.15	0.14	0.11	0.11	0.08	0.10	0.13	0.05	0.06	0.05
75.000	0.40	0.42	0.14	0.14	0.12	0.12	0.08	0.11	0.13	0.08	0.05	0.05
50.000	0.29	0.33	0.14	1.13	0.12	0.12	0.08	0.11	0.11	0.10	0.05	0.05
10.000	0.23	0.47	0.14	0.14	0.11	0.11	0.75	0.11	0.14	0.12	0.06	0.08
5.000	0.21	0.28	0.13	0.13	0.12	0.12	0.08	0.11	0.10	0.10	0.09	0.13
1.000	0.22	0.19	0.13	0.13	0.13	0.11	0.07	0.11	0.15	0.06	0.09	0.13
100	0.17	0.25	0.12	0.12	0.11	0.12	0.08	0.11	0.13	0.05	0.10	0.08
0	0.21	0.18	0.13	0.14	0.12	0.12	0.08	0.12	0.16	0.06	0.05	0.05

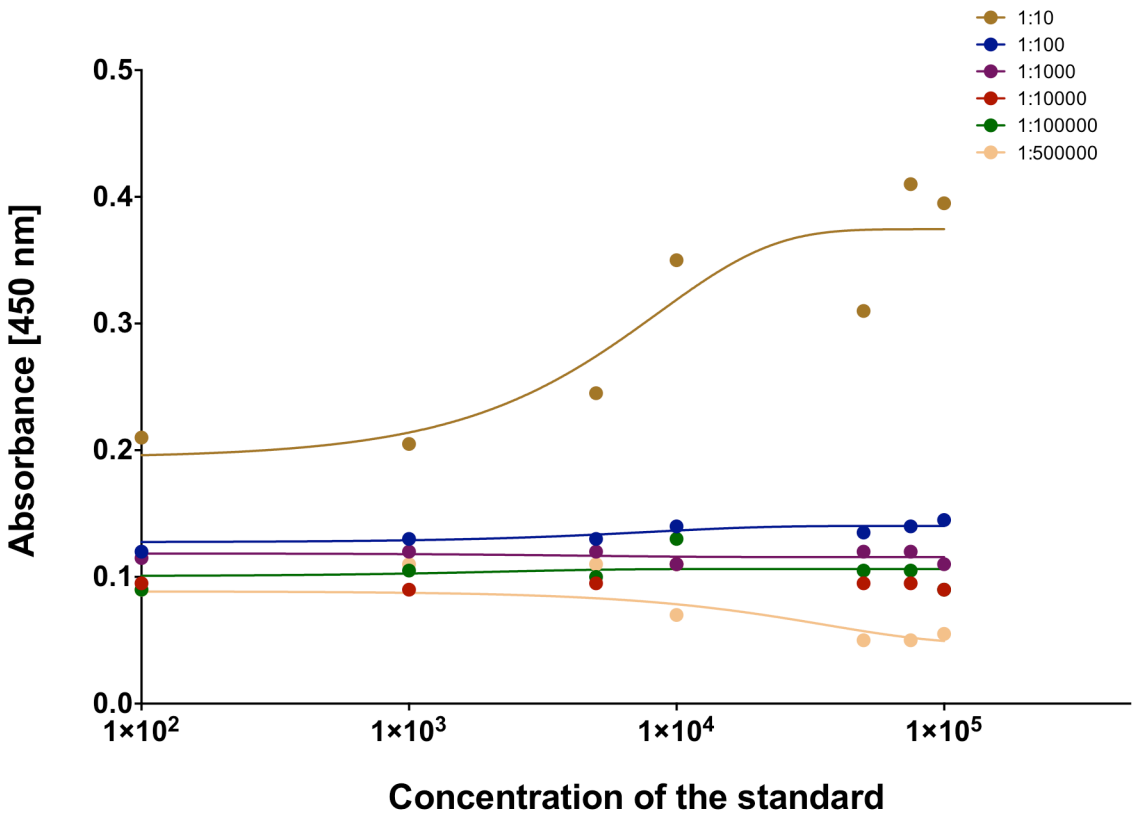

Figure 11: Absorbance dependence on concentration; Coupling Ab concentration: 1 µg; Detection Ab: IgY-HRP labelled with Periodate Method

Table 23 Absorbance at a concentration of the coupling Ab of 2.0 µg and periodate-HRP detection Ab

c	1:10		1:100		1:1000		1:10000		1:100000		1:500000	
100.000	0.43	0.398	0.14	0.13	0.09	0.10	0.05	0.09	0.07	0.08	0.08	0.07
75.000	0.49	0.388	0.14	0.15	0.10	0.09	0.06	0.08	0.08	0.09	0.08	0.09
50.000	0.43	0.335	0.16	0.15	0.11	0.09	0.05	0.09	0.08	0.09	0.11	0.10
10.000	0.28	0.236	0.13	0.12	0.09	0.11	0.07	0.11	0.12	0.11	0.13	0.12
5.000	0.35	0.24	0.14	0.13	0.18	0.13	0.10	0.12	0.13	0.13	0.13	0.13
1.000	0.28	0.21	0.15	0.14	0.12	0.13	0.09	0.12	0.11	0.12	0.13	0.12
100	0.24	0.22	0.14	0.12	0.11	0.13	0.15	0.12	0.11	0.12	0.13	0.14
0	0.29	0.21	0.13	0.11	0.10	0.09	0.08	0.12	0.15	0.13	0.12	0.12

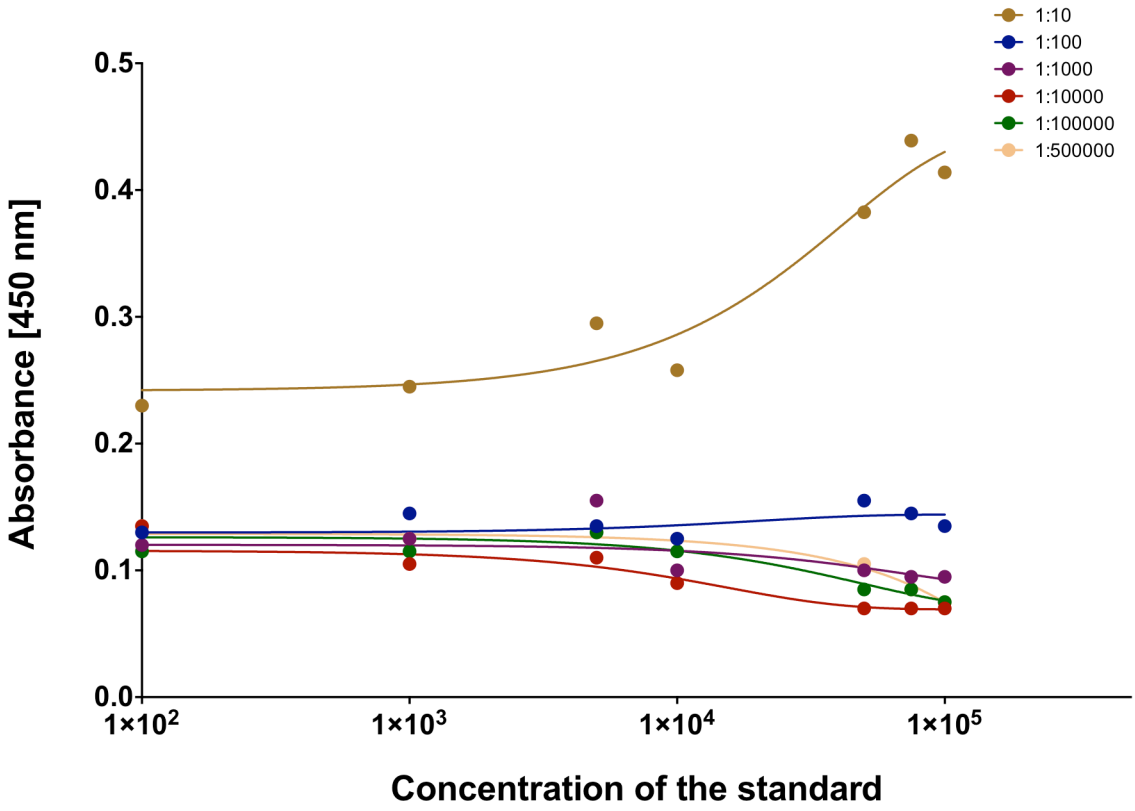

Figure 12: Absorbance dependence on concentration; Coupling Ab concentration: 2.0 µg; Detection Ab: IgY-HRP labelled with Periodate Method

5.3.3 ELISA results after affinity chromatography with NHS-activated Sepharose and lyophilisation

Table 24 ELISA results after purification with NHS-activated Sepharose column on a 0.5 µg coupling Ab plate

c	1:10		1:100		1:1000		1:10000		1:100000		1:500000	
0	0.13	0.14	0.09	0.09	0.07	0.07	0.03	0.06	0.04	0.05	0.05	0.05
100	0.13	0.13	0.09	0.08	0.06	0.06	0.03	0.05	0.04	0.04	0.05	0.05
1.000	0.10	0.10	0.07	0.07	0.06	0.06	0.02	0.05	0.04	0.04	0.05	0.05
5.000	0.10	0.09	0.06	0.06	0.05	0.05	0.02	0.05	0.03	0.04	0.05	0.05
50.000	0.07	0.07	0.05	0.05	0.05	0.05	0.02	0.44	0.03	0.04	0.05	0.05
75.000	0.06	0.06	0.05	0.05	0.05	0.05	0.02	0.44	0.03	0.04	0.05	0.05
10.000	0.06	0.09	0.05	0.06	0.05	0.05	0.02	0.04	0.03	0.04	0.05	0.05
100.000	0.06	0.06	0.05	0.06	0.05	0.05	0.02	0.05	0.04	0.04	0.05	0.04

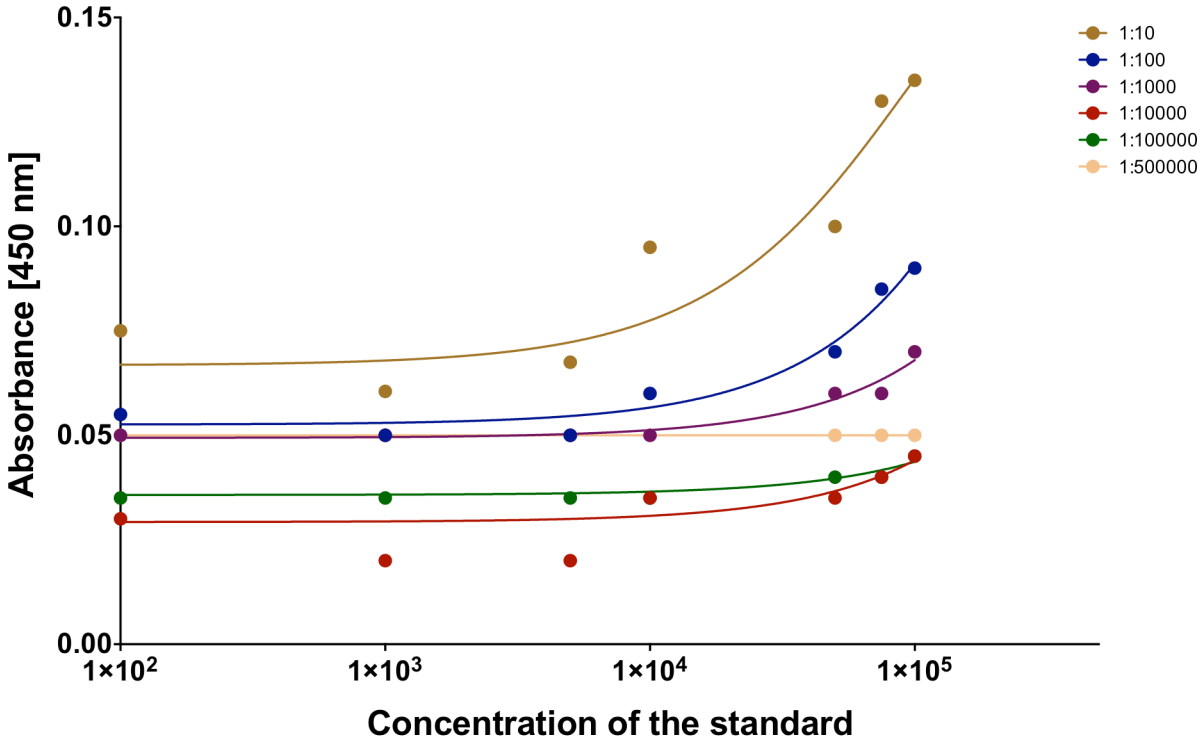

Figure 13: Absorbance dependence on concentration after purification with affinity chromatography (Matrix: NHS-activated Sepharose); Coupling Ab concentration: 0.5 µg, Detection Ab: Anti-Lupine-Mono 260110SP²C12-A11-C10-C8 (13.2.12 BG)

Table 25 ELISA results after purification with NHS-activated Sepharose column on a 1µg coupling Ab plate

c	1:10		1:100		1:1000		1:10000		1:100000		1:500000	
0	**0.16**	**0.14**	0.08	0.08	0.06	0.02	0.05	0.05	0.04	0.04	0.05	0.05
100	0.17	0.16	0.10	0.08	0.06	0.03	0.05	0.05	0.04	0.04	0.05	0.05
1.000	0.14	0.14	0.08	0.08	0.06	0.02	0.05	0.05	0.03	0.04	0.05	0.05
5.000	0.20	0.12	0.08	0.08	0.06	0.06	0.02	0.05	0.04	0.04	0.05	0.05
50.000	0.08	0.08	0.06	0.06	0.05	0.05	0.02	0.04	0.04	0.04	0.05	0.04
75.000	0.07	0.06	0.05	0.05	0.05	0.05	0.02	0.04	0.04	0.04	0.05	0.04
10.000	0.06	0.06	0.05	52.00	0.05	0.05	0.02	0.04	0.03	0.04	0.05	0.05
100.000	**0.09**	0.06	0.05	0.06	0.05	0.05	0.02	0.05	0.04	0.04	0.05	0.05

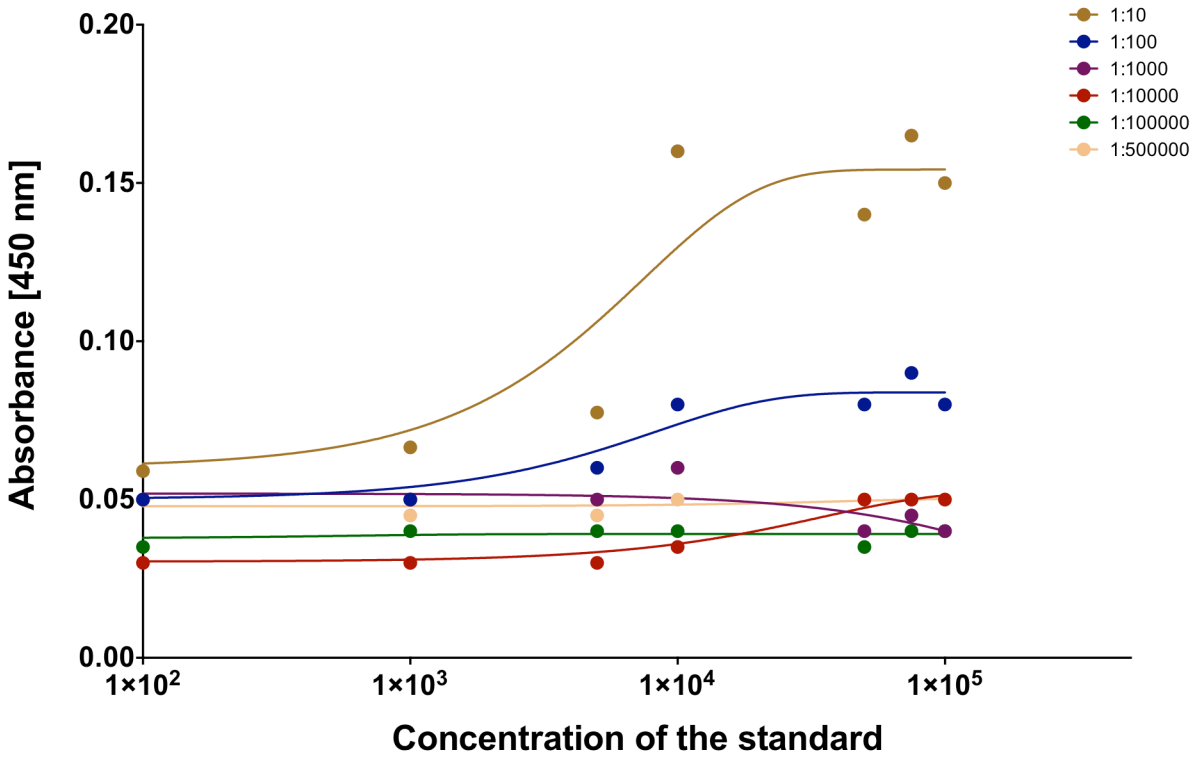

Figure 14: Absorbance dependence on concentration after purification with affinity chromatography (Matrix: NHS-activated Sepharose); Coupling Ab concentration: 1µg, Detection Ab: Anti-Lupine-Mono 260110SP²C12-A11-C10-C8 (13.2.12 BG)

5.3.4 ELISA results after affinity chromatography with CNBr-activated Sepharose

There are no results because there was no specific outcome with this matrix for IgY, neither with BCA nor gel electrophoresis nor ELISA.

6 Discussion

The aim of the precipitation was to purify the anti-lupine IgY proteins as pure and in the highest yield as possible.

The total protein amount decreased after each method. In the end a total amount of 3.01 mg/ml of protein was obtained. This was the initial amount for the affinity chromatography, which should merely contained IgY antibodies.

In the beginning, to 50 ml of the initial solution, 100 ml acetate buffer was added to keep the pH almost constant. This means a dilution ratio of 1:3. The actually concentration should be now approximately 23 mg/ml. There is no explanation why the concentration is approximately one third of the amount it should be.

Furthermore, the amount of total protein decreases again after adding 1.65 ml caprylic acid. The volume extended again to a total of 151.65 ml, which would lead to a protein concentration of 6.45 mg/ml. But the aim of the caprylic acid was to remove unwanted proteins. According to the BCA results it worked or at least some proteins were precipitated.

IgY antibodies should build a complex with the ammonium sulphate and should be found in the pellet and not in the supernatant. The remaining proteins should be the contaminating ones and they have to be removed from the purified IgY solution. Remaining to the BCA 0.74 mg/ml expectantly unwanted protein was removed due to the ammonium sulphate precipitation.

The pellet was collected and re-suspended in 30 ml PBS buffer (0.2 M).

The result is that from an initial pre-precipitated (PEG8000) volume of 50 ml, with this method it is possible to precipitate a total amount from 13.67 mg per ml. The solution has to be dialysed to remove the ammonium sulphate. A buffer exchange was performed with PBS buffer (0.5 mM).

After the final precipitation the total volume was 30 ml. After the dialysis the volume extended to 75 ml what corresponds with an approximately ratio of 1:4. That means that there was no loss of IgY antibodies during dialysis.

First, a buffer exchange with the PD10-desalting column was performed. After proofing if there were still traces of sulphate ions, which should be removed completely, the result was positive and the desalting kind of useless. Secondly, it took a lot of working

time because only 2.5 ml of the sample could be desalted at once and the procedure has to be continuously monitored.
Therefore, buffer exchange and desalting was performed by dialysis. This method should be preferred because it is a time saving and cheap. Time saving in this context is that the method can be performed over night and does not need the presence of an operator. Even it is not possible to remove 100% of the unwanted salt, it is still possible to decrease the amount of the unwanted substance below the measureable value. The extending volume is a disadvantage as well with the PD10 column method and the volume is increasing over the whole process and a lyophilisation has to be performed.

After the precipitation a further purification by affinity chromatography followed. Two possible matrices were used. The first one was the CNBr-activated Sepharose. After coupling the lupine proteins on it, there were still no results measurable. Even after changing the conditions of the chromatography, like different buffers, different flow-rate, and more, there was still no peak measured.

The second matrix, the NHS-activated Sepharose, provided a chromatogram, which showed a peak between the seventh and twelfth fraction.

The volume of the specific lupine IgY was too low to be measured because of all the dilution steps. To concentrate the sample first a Roti-Spin tube was filled with sample and centrifuged to smaller the volume. This method has too many disadvantages. First, even after the concentrating procedure it is not possible to measure the specific IgY by BCA. Secondly, it takes too long to concentrate all the samples because it is only possible to concentrate 3.5 ml at once. The total volume was more than 200 ml. For that reasons the lyophilisation method was used to increase the concentration of the sample. After the lyophilisation process the powder was dissolved in 2.5 ml RO-water. In the end, the total volume of specific Anti-Lupine-IgY is 0.78 mg/ml.

After comparing both methods the lyophilisation was chosen to be the more useful one in this process. There are advantages like that this method can be performed over night and does not need the presence of an operator like the Roti Spin Method, or there is no loss of antibodies.

A major disadvantage is that the salt-concentration increases and that could merge the antibodies or leads to a loss of activity of the antibody or denaturation. A recommendation is to change the buffer during the dialysis process (e.g. RO-water or a 0.005M PBS buffer) to avoid another dialysis step after the lyophilisation. Furthermore, it works better. A disadvantage could be that also a higher salt concentration is present in the end. But performing the dialysis before the lyophilisation needs to be done anyway. This problem can be solved by using a less concentrated PBS buffer for the buffer exchange to keep the salt concentration as low as possible.

After lyophilisation an SDS-Page gel electrophoresis was performed. *Figure 6* shows the results after the whole precipitation steps. In the first lane, which contains initial solution, there are at least four bands, as well as in the second lane, which contains the initial solution plus acetate buffer. The third lane shows only bands lower than the wanted IgY band. It shows, the dissolved pellet after the caprylic acid precipitation and should only contain other proteins than antibodies. There is the acknowledgement that the caprylic acid removes unwanted proteins but does not influence IgY.
In the fifth lane there is the supernatant after the ammonium sulphate precipitation. Actually there should also be only unwanted proteins. Probably the precipitation was not finished or not enough ammonium sulphate was used to precipitate all the IgY from the solution. The sixth and seventh lanes contain the final precipitate, in lane number six before and in lane number 7 after dialysation. A perfect result would be if there would only be one band left at around 180 kDa. Due to the other bands still visible there are still some contaminations in the sample. A suggestion could be to start with the 3.3 % caprylic acid precipitation, increase to 7.5 % caprylic acid precipitation and after precipitate the IgY with 45 % ammonium sulphate but let it incubate for two hours instead of one hour at 4 °C.
Figure 7 shows the results of the SDS-Page after the lyophilisation of the purified specific lupine-IgY. Lane number one contains the standard. Lane number two contains the first flow-through from the affinity chromatography. It shows one band at approximately 180 kDa, which is the size of the IgY-antibodies but also another band below, which contains still contaminations. The second lane contains the outcome while flushing with binding buffer and shows 2 bands and a really small third one. That shows that the unwanted proteins (at least parts) were flushed away and did not bind to the matrix. Last but not least, in the fourth lane containing the specific-lupine-IgY there is

only one band at the expected position, 180 kDa. According to the results the purified solution contains the specific lupine IgY. The reason why there were also bands at the third and second band is, that the IgY has always the same molecular weight and the aim of the work is to remove the non-specific IgY as well. A reason might be that the matrix capacity is not sufficient and therefore the lupine IgYs cannot be entirely captured by the matrix and are found in the flow-through. To exclude this possibility the flow-through was purified again by affinity chromatography. The chromatogram did not provide a peak. This leads to the result that the lupine-specific IgY is completely captured in the matrix and collected in the final solution.

The ELISA tests show different results. After the whole precipitation process ELISA with 0.5 µg, 1.0 µg and 2.0 µg coating antibodies and once with HRP-labelled detection antibodies. As standard the lupine extract in different concentrations is taken to test the technical applicability.

First the detection antibody was labelled with HRP by using the Lightning Link kit. The results are good but the CD_{50} is really high. It still shows a dependency between concentration and absorbance and the lowest CD_{50} value of all ELISA. Also the absorbance is the highest. It is going up to approximately 3.0 nm.

Afterwards, the same test is repeated but the detection antibody is labelled with HRP using the periodate method. This test is not evaluable under the used conditions, because the CD_{50} values are approximately 10^3 too high. Moreover, the absorbance is really low. There is a dependency between concentration and absorbance but this method still has to be improved.

Furthermore, the final sample, containing the specific lupine-IgY, is coated on the plates in two different concentrations 0.5 µg and 2.0 µg. Detection antibody is the anti-lupine-Mono 260110SP^{2}C12-A11-C10-C8 (13.2.12 BG).

It is possible to see the dependency between concentration and absorbance but the CD_{50} values are too high and the absorbance ratio is too low.

To sum up all the results from the ELISA testing, there is a dependency and the purification worked but still it is important to improve the ELISA techniques because the detection limit is not low enough to be used in reality. Furthermore, the background signal is high because the values of the same concentration are showing differences.

7 CONCLUSION

This master thesis focused on the purification of anti-lupine IgY from hens' egg yolk. Different precipitation techniques were compared, as well as different matrices for affinity chromatography and the steps in between were tried to be improved in order to get a high amount and an as pure as possible outcome. Further characterisations were obtained by means of electrophoresis.

Sandwich ELISA techniques with different concentrations of the coupled antibody, different detection antibodies or different labelling were performed and it turned out that sometimes there has to be huge background information or cross-reaction so that many results were not evaluable. For that sake different blocking buffer can be tried out, e.g. containing BSA or fish gelatine.

After lyophilisation the outcome approximately 150 ml of the initial anti-lupine IgY egg yolk, which was pre-precipitated with the PEG 8000 method was 0.7 mg per ml.

A higher outcome was expected. It could be that the egg yolk did not contain a lot of the IgY against lupine proteins. One possibility could be that a lot of this specific IgY got lost during the purification.

To sum up the results the combination of the two precipitation methods is the best way to get a huge amount of IgY-antibodies. Furthermore, the affinity chromatography is specific against lupine and it is the easiest way to get specific anti-lupine IgY. But the outcome should be still higher and the ELISA techniques have to be improved. Some possibilities to reach a higher outcome could be to use more initial solution. Another suggestion would be to incubate the solution after ammonium sulphate precipitation two hours instead of one. Actually, there could not be a increase of IgY antibodies according to several previous test results, which came to the conclusion that ammonium sulphate does not influence the IgY-antibody properties.

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