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Enzymatic crosslinking of human serum albumin with mouse IgG1 monoclonal antibody

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Abstract

The work carried out in this master thesis was embedded in the EU-NANOFOL project which deals with the development of antibody based nanobiodevices used for targeted drug delivery. One component needed is human serum albumin (HSA) linked to mouse IgG1 monoclonal antibody. Thereby the potential of mushroom tyrosinase to enzymatically cross-link these two protein based molecules was used to replace the conventional method using toxic chemicals.

Enzyme characterization was performed to find the pH and temperature optimum for tyrosinase *Agaricus bisporus*. Model substrates were used to optimize the reaction conditions in order to favor the cross-linking. Denaturation of human serum albumin using chemicals and high temperatures improved accessibility by enzymes due to opening of the structure. Unfolding was demonstrated using GPC, intrinsic fluorescence and dynamic light scattering. Cross-linking was accomplished with different linker molecules according to SDS analysis.

Enzymatic cross-linking of HSA and AB was achieved using low molecular weight phenolic compounds like caffeic acid in a concentration of 2 mM as cross-linkers. Coupling products were identified by using fluorescence scanning of the FITC labeled HSA-AB conjugates and tryptic digestion of the product band followed by Maldi-TOF analysis.

Kurzfassung

Diese Masterarbeit wurde im Rahmen des EU- Projekts NANOFOL durchgeführt, welches sich mit der Entwicklung von Antikörper basierenden Nanobiokonstrukten beschäftigt. Diese Elemente finden Anwendung in der zielgerichteten Pharmakotherapie und setzten sich aus Humanalbumin zusammen, das mit von der Maus abstammenden Antikörpern (AB) verknüpft wird. Sie werden konventionell durch Einsatz toxischer Chemikalien hergestellt. In dieser Arbeit wurde ein neuartiger Ansatz basierend auf enzymatischer Vernetzung entwickelt.

Nach Bestimmung des pH und Temperaturoptimums der Tyrosinase *Agaricus bisporus* wurden Modellsubstrate eingesetzt, um die optimalen Bedingungen für die Vernetzungsreaktion festzustellen. Das Substrat Humanalbumin (HSA) wurde sowohl durch chemische als auch durch thermische Denaturierung erfolgreich für die enzymatische Reaktion zugänglicher gemacht. Der Entfaltungsprozess wurde mittels GPC, intrinsischer Fluoreszenz und der Veränderung des hydrodynamischen Durchmessers verfolgt. Der Einfluss verschiedener Linkermoleküle auf die Bildung von enzymatischen Kopplungsprodukten wurde getestet und mittels engmaschiger SDS Gele analysiert.

Enzymatische HSA-AB Kopplungsprodukte konnten durch den Einsatz von phenolischen Komponenten mit niedrigem Molekulargewicht wie z.B. Kaffeesäure als Quervernetzer erzeugt werden. Dies wurde mit Hilfe von Fluoreszenzaufnahmen des mit FITC markierten HSA-AB Konjugats und eines tryptischen Verdaus der Produktbande gefolgt von einer Maldi-TOF Analyse verifiziert.

Table of contents

Table of Figures

List of Tables

1 Introduction

1.1 Theoretical

1.1.1 The NANOFOL Project

The NANOFOL Project is funded by the European Union and has 13 partnering institutions. The overall aim is to develop a folate/antibody based nanobiodevice enclosing a drug, which can be used for the treatment of chronic inflammatory diseases such as Rheumatoid Arthritis.

The motivation for the search of new treatment in this area is that more than 80 million people worldwide suffer from inflammatory diseases, which cause premature death and economic loss. Existing drugs for rheumatoid arthritis are linked to several challenges and risks such as high costs, complicated administration, allergic reactions and deadly side effects.

NANOFOL will address these issues through the development of a global approach integrating diagnosis and therapy for chronic inflammatory diseases. It aims to explore the tremendous potential of nanomaterials as an effective drug delivery system to develop nanodevices that will directly target effector cells and reduce long term effects of chronic inflammatory disorders [1].

1.1.1.1 Rheumatoid arthritis

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune disorder that is characterized by inflammation of the joints. It affects over 2,9 million people in Europe [2]. The main contribution to the characteristics of the disease which are inflammation and destruction of cartilage and bones comes from synovial macrophages. The bones transform into osteoclasts, which are bone cells that destroy bone tissue [3,4].

Common treatment strategies used in the last decade are on the one hand non-steroidal anti-inflammatory drugs such as salsalate and aspirine. On the other hand cyclooxygenases were used which are enzymes that control production of prostaglandins involved in inflammation, pain and fever [5]. The problem is that those drugs do not sufficiently prevent joint damage or slow down the disease progression.

1.1.1.2 Activated macrophages

Macrophages are white blood cells that are part of the immune system. They are found either in the circulation or stationary in tissues. Their main role is to phagocytise invaders and process them for presentation to the adaptive immune system [6].

The resident tissue macrophages can generally be found in a resting state, but when exposed to some sort of stimuli they get activated. This activation becomes visible through the expression of specific receptors on the surface, of which more than 100 are classified. The activated macrophages

are phagocytes that scavenger live or dead microorganisms, debris and dying cells [7] but also play a key role as mediators for inflammatory diseases like rheumatoid arthritis. Their numbers and level of activation correlate with the extent of joint inflammation and tissue degradation. Approaches to treat the activated macrophages were to either get rid of pro-inflammatory by-products or eliminate the entire population of macrophages. Due to their importance in fighting infectious diseases and promoting tissue repair, eliminating them can be very harmful for the patient.

Therefore, the new plan of action is to only remove the subpopulation of macrophages that promotes the specific inflammatory disorder by delivering therapeutic agents selectively to pathologic cells. Studies have shown that the folate receptor is specifically expressed by activated macrophages [8]. Selective elimination could be achieved by linking either folate or a bi-specific antibody recognizing the folate receptor isoform β and a macrophage-specific marker onto the surface of the nanodevice [1].

1.1.2 Targeted drug delivery

The use and development of biodegradable nanoparticles (NPs) as effective and targeted drug delivery systems became especially interesting over the past few decades. The big advantage of using various polymers for the drug delivery is, that they can effectively deliver the drug to a target site and thus increase the therapeutic benefit, while minimizing side effects [9]. The major goal in the design of such devices is to control particle size, surface properties and the controlled release (CR) of pharmacologically active agents in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen [10,11].

At first liposomes were of high interest due to their unique advantages but problems such as low encapsulation efficiency and rapid leakage of water-soluble drug in the presence of blood components has decreased the developmental work [12]. As a good alternative synthetic polymers like poly lactic acid (PLA), poly D,L lactide and poly cyanoacrylate (PCA) can be used as a material for nanoparticles offering the advantage of increasing the stability of drugs and proteins [10]. Additionally, natural polymers like for example human serum albumin (HSA) are promising materials to generate nanoparticles [13].

The size of nanoparticles varies from 10-1000 nm and the drug is dissolved, entrapped, encapsulated or attached to a NP matrix. The preparation method decides whether nanoparticles, nanospheres or nanocapsules are obtained [11].

1.1.3 Tyrosinase from Agaricus bisporus

Tyrosinases (polyphenoloxidases, ([EC 1.14.18.1]) are copper containing oxidoreductases that play an important role in melanogenesis e.g. the biosynthesis of melanin pigments. In plants and vegetable this process causes the undesired browning that takes place during senescence or damage at the time of postharvest handling [14]. The tyrosine-related melanogenesis in mammals on the other hand is responsible for skin, eye and hair pigmentation [15].

It is a bifunctional enzyme catalyzing the *o*-hydroxylation of monophenols, also referred to as monophenolase- or cresolase activity, and the subsequent oxidation of *o*-diphenols to *o*-quinones, the so called diphenolase- or catecholase activity [EC 1.10.3.1]. For both reactions molecular oxygen is required as an electron acceptor which is then reduced to water [15]. *O*-quinones are a highly reactive species that undergo non-enzymatic reactions with amino acid residues containing amino and sulfhydryl groups such as cysteine or lysine [14,16].

Figure 1 displays the pathway of melanogenesis containing the two enzymatically catalysed reactions:

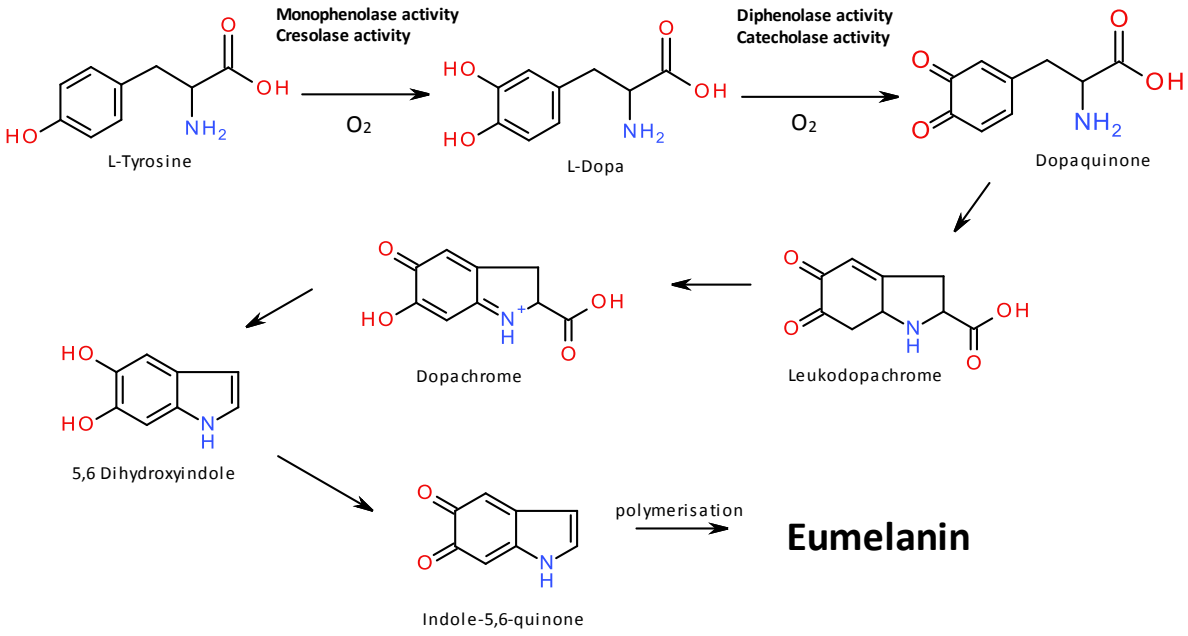


Figure 1: Pathway of melanogenesis [14]

Tyrosinases are common in many fungi, plants and animals but the enzyme originating from the common mushroom (*Agaricus bisporus*) is the most well-known and best studied in this family.

Figure 2: *Agaricus bisporus*-the common mushroom

The active site contains a binuclear type 3 copper centre, that consists of a pair of copper centres each coordinated by three histidine residues [17] and can exist in three main oxidation states depending on the copper-ion valence:

- *oxy*- state: Cu^{II}-O_2-Cu^{II}
- *deoxy*-state: Cu^{I}-Cu^{II}
- *met*-state: Cu^{I}-Cu^{I}

The *met*-form is the resting state of the enzyme and a fraction of 85-90% exists in this form at atmospheric pressure, room temperature and neutral pH. For the catecholase activity the enzyme works both in the *met*-and the *oxy*-form but for the Cresolase activity the *oxy*-form is required as displayed in Figure 3 [16].

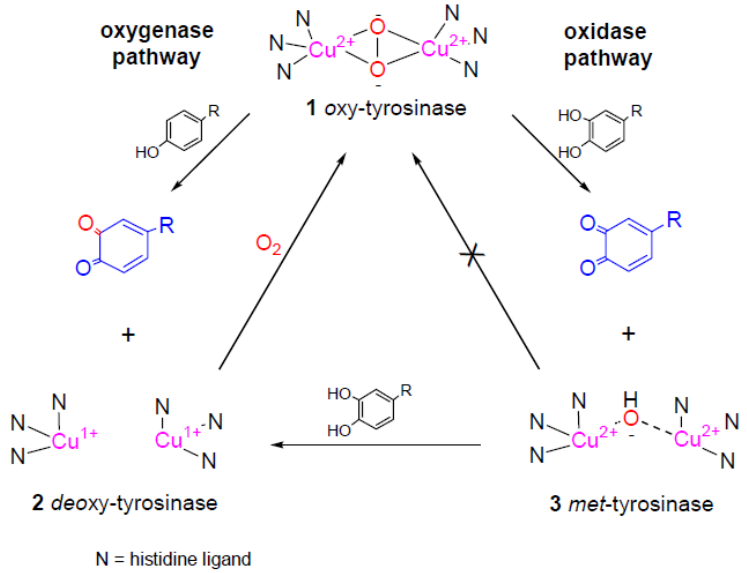


Figure 3: Transformation between *deoxy*-, *oxy*- and *met*-tyrosinase [18]

This fact causes the so called *lag*-period of the enzyme occurring when only a monophenol such as tyrosine is present in solution. Throughout this phase no catalytic activity can be observed because the enzyme needs to be transformed from the *met*- into the *oxy*- state. For this activation DOPA is needed, which is generated by the small amount of *oxy*- form present. The *lag*- period is over when all the enzyme has been transformed into the *oxy*-state [18]. This is the reason for the mono-phenolase activity being the initial rate determining step throughout the catalysed reaction [15].

The applications of mushroom tyrosinase are diverse and reach from food processing over being a potential tool to make novel coatings and composite materials all the way to the functionalization of wool and silk fibres [19].

Specific examples are the crosslinking of whey and wheat proteins [20], the oxidation of wool fibres, and the coupling of functional molecules onto wool fibres [21].

1.1.4 Human serum albumin

The most abundant protein in the human blood plasma is the serum albumin. It acts as carrier for a variety of endogenous substances such as fatty acids, hormones and numerous small ligands. The protein is composed of 585 amino acid residues (18 tyrosine residues) resulting in a molecular weight of approximately 67 kDa. Three homologous domains assemble to form a heart shaped structure with each domain subdivided into two identical subdomains A and B [22,23].

The figure below displays the 3-D structure of human serum albumin in complex with the ligand N-[5-(5-{[(2,4-dimethyl-1,3-thiazol-5-yl)sulfonyl]amino}-6-fluoropyridin-3-yl)-4-methyl-1,3-thiazol-2-yl] acetamide with all tyrosine residues highlighted in dark blue [24].

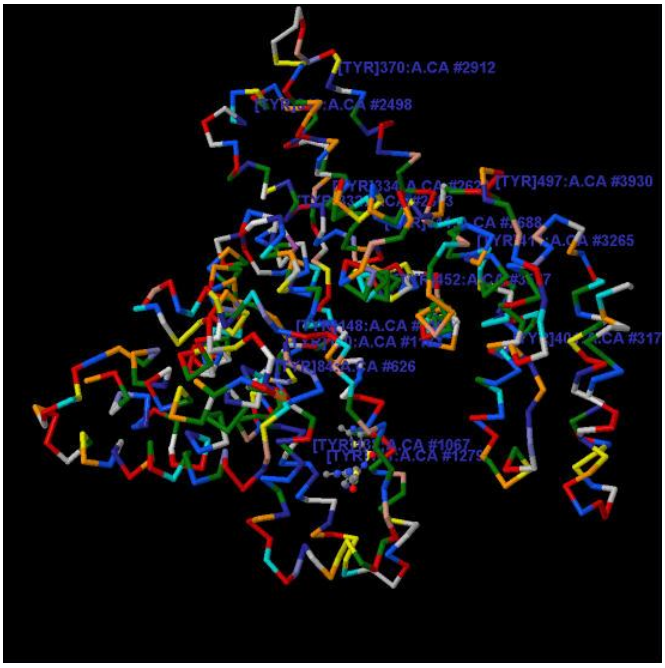


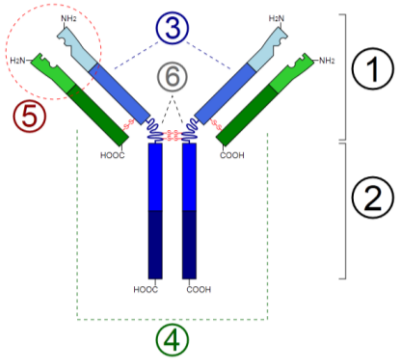
Figure 4: 3D structure of human serum albumin in complex with a ligand [24]

Human serum albumin contains one free thiol group, namely Cys34, which can cause dimerization during the isolation due to oxidation. Depending on age and quality of the product the dimerised albumin content can reach up to 20 %. Those dimers or even higher aggregates may influence the preparation process of nanoparticles, therefore the quality of the product is of major importance [13].

Due to HSA being a major fatty acid transporter with seven fatty acid binding sites on the surface it carries around between 0,1 – 2 mole of fatty acid per mole protein. This is an important factor to take into consideration when using HSA in reactions or unfolding processes, because the bound fatty acids could cause problems [25,26].

1.1.5 Mouse IgG1 monoclonal antibodies

There are five isotypes of antibodies: IgA, IgD, IgE, IgG and IgM. Immunoglobulin G constitutes 75% of serum immunoglobulins in humans and they are synthesized and secreted by plasma B cells [27]. They are composed of amino acid chains and in Figure 5 a schematic diagram of the basic unit of immunoglobulin is shown:



1 Fab: antigen binding fragments
2 Fc: constant region
3 heavy chain
4 light chain
5 antigen binding site
6 hinge regions: flexible region

Figure 5: Basic unit of immunoglobulin [28]

The antibody´s function is to recognise an antigen and with that the initiation of an immunological response.

Monoclonal antibodies (mAbs) are monospecific and are obtained from a single clone of a parent immune cell [29]. Because primary and metastatic tumors generally overexpress certain antigens on their surface that are recognised by this type of antibody they provide a good platform for drug targeting [30].

1.1.6 Denaturation of proteins

The denaturation or the unfolding of proteins and therefore the loss of the function and activity is mostly an undesired process taking place due to harsh reaction conditions. If completely denatured proteins precipitate due to the loss of solubility.

In some cases, such as enzymatic crosslinking, the reversible and partly unfolding of proteins is very interesting because it opens up the structure and can give way to higher reactivity due to better accessibility for enzymes.

There are several ways of denaturing proteins using chemical compounds, higher temperatures, pH-shifts or higher salt concentrations but only the first two procedures will be explained in more detail.

The most commonly used chaotropic agents for chemical denaturation guanidine hydrochloride and urea are able to disrupt the three dimensional structure of proteins because they increase the solubility of nonpolar substances in water. Consequently hydrophobic interactions in proteins are disrupted which destabilizes the overall structure.

Figure 6: Structures of guanidine hydrochloride and urea

The increase of temperature, which is the second method commonly used, causes the weakening of a number of bonds in the protein molecule which consequently leads to a more flexible structure and more groups being exposed to solvent. Due to the more open structure water can interact more easily with the protein chains, followed by the incorporation and the increase of the hydrodynamic diameter of the overall protein [31,32].

The unfolding process of human serum albumin is quite complex due to its three domains that can all unfold separately and mechanism is not completely elucidated yet. But it has been reported that guanidine hydrochloride, urea and higher temperature lead to significant changes in the overall protein structure due to partly unfolding of the three domains [23,31,33,34].

Fatty acids which are frequently bound to the surface of human serum albumin are said to stabilize the protein and to interfere with the denaturation process, so they need to be removed before starting the reaction [26].

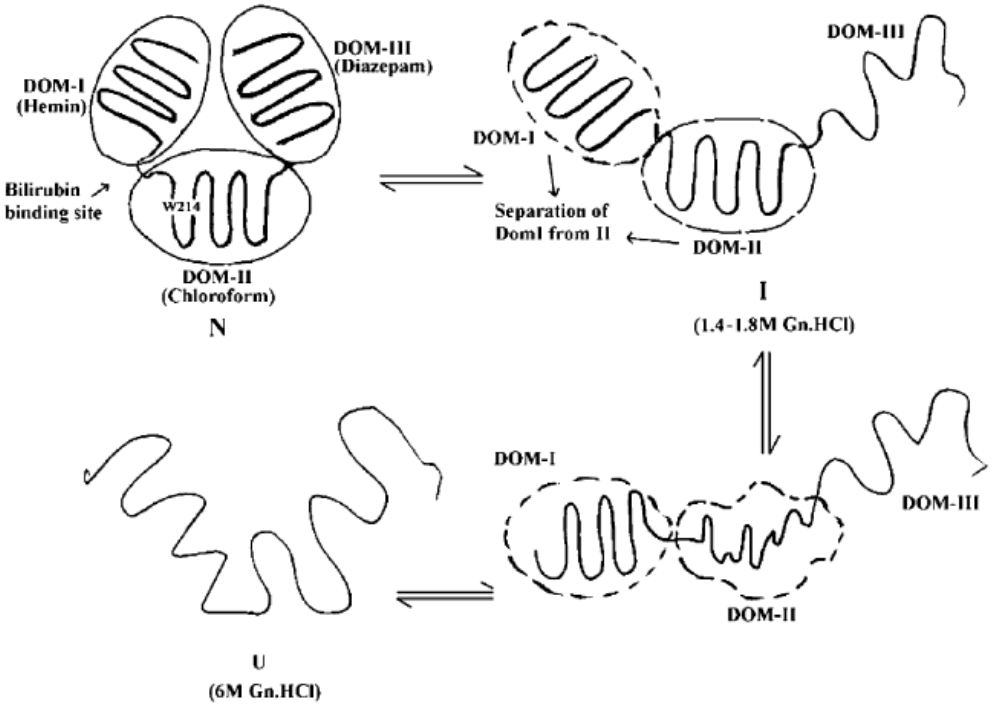


Figure 7: Possible unfolding pathway of HSA domains using guanidine hydrochloride [34]

A way of determining the changes in the structure of a protein is intrinsic fluorescence spectroscopy. This method is a technique both simple and robust that can produce important complementary information.

The aromatic residues tryptophan, tyrosine and phenylalanine, shown in Figure 8, are potent fluorophores with tryptophan being the dominant one due to its indole moiety and its large extinction coefficient.

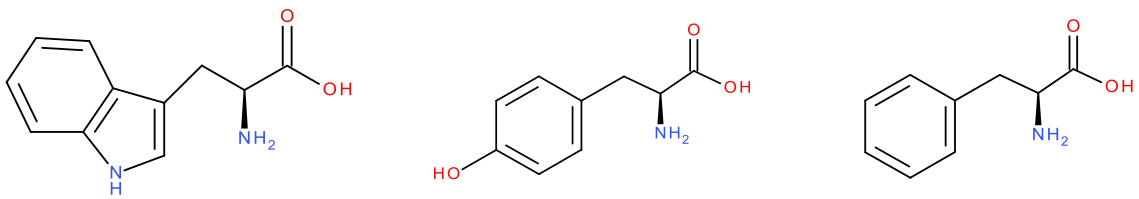


Figure 8: Structures of tryptophan, tyrosine and phenylalanine

Changes in the microenvironment of the tryptophan residue, such as exposure to water, affect the fluorescence maximum (λ_m) and the quantum yield (*q)* [35].

Measuring changes in intrinsic fluorescence intensity is especially suitable for the study of early stages of unfolding due to the often buried position of tryptophan within the protein´s structure [36]. This method can also be applied for unfolding studies of human serum albumin because there is a single intrinsic tryptophan residue present in position 214 [37].

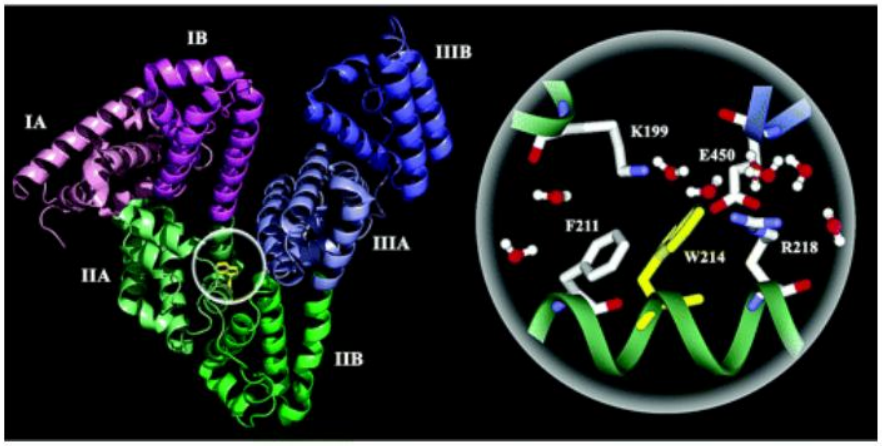


Figure 9: The heart shaped structure of HSA with a single tryptophan residue in position 214 of subdomain IIA [37]

1.1.7 Enzymatic crosslinking

Mushroom tyrosinase is capable of oxidizing tyrosine residues in protein backbones to o-quinone which can undergo a spontaneous Michael like addition (Figure 10) with amino- and sulfhydryl groups of other amino acid residues forming inter- and intermolecular crosslinks [15,20]. The degree of cross-linking highly depends on the accessibility of target amino acids in the substrate protein [19].

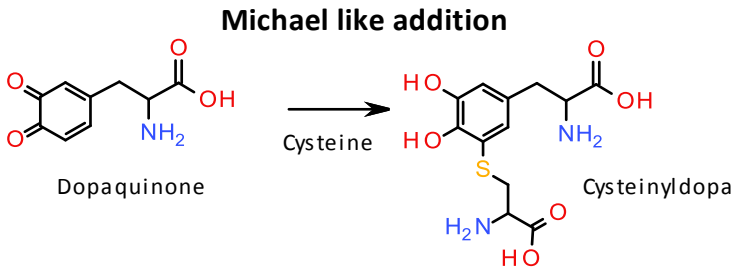


Figure 10: Michael like addition of Dopaquinone to Cysteine

Insulin Chain B oxidized from bovine pancreas is a peptide chain that contains 30 amino acid residues (2 tyrosine residues) with a molecular weight of 3,5 kDa. It is ideal to use as a model substrate to check if the tyrosinase obtained is able to crosslink peptides containing tyrosine residues.

If the substrate is not accepted by the enzyme due to a very tight structure or due to its size, the use of low molecular weight compounds, shown in Figure 11, can enable the enzymatic crosslinking as reported by Thalmann et al. [20].

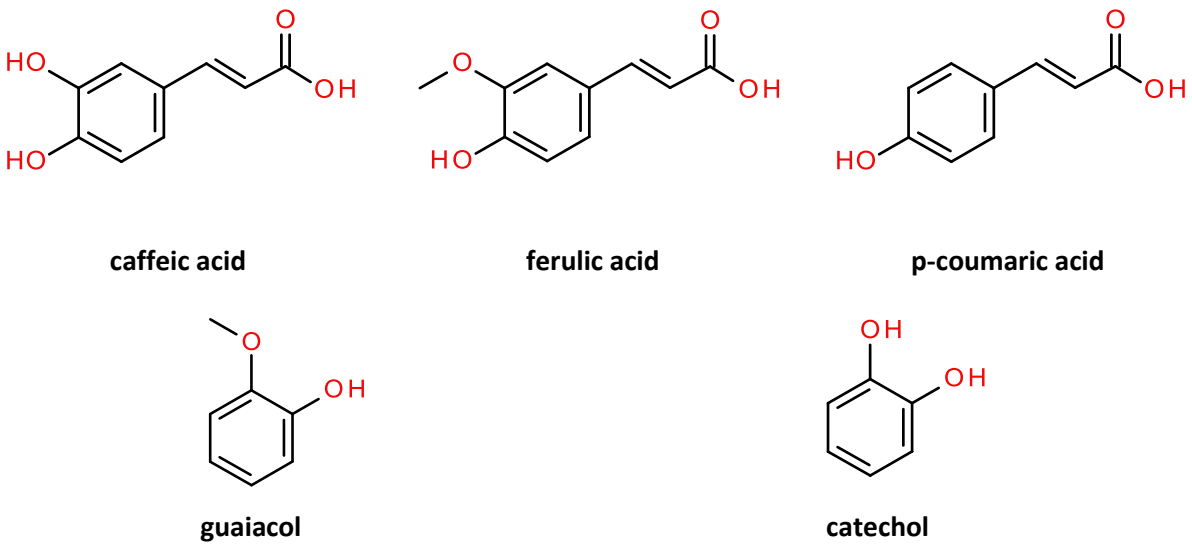


Figure 11: Structures of low molecular weight phenolic compounds

Figure 12 displays the difference between the enzymatic crosslinking with (a) and without (b) phenolic linker molecules. If a small molecular weight phenolic is present in solution it is oxidized very quickly by the enzyme because it is a perfect substrate for mushroom tyrosinase. The oxidized phenolic compounds further react with the amino- and sulfhydryl groups that exist in the protein backbones of larger and less accepted substrates, such as HSA and mouse IgG1 monoclonal antibody, present in solution.

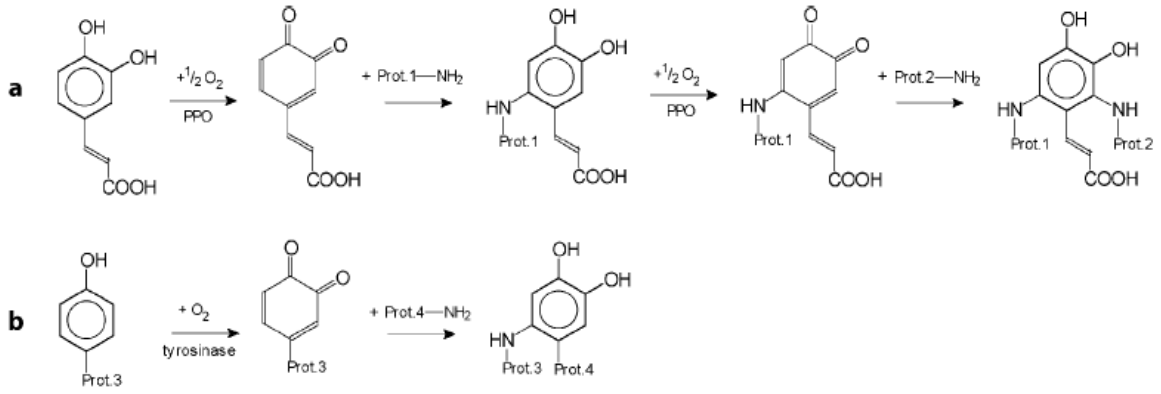


Figure 12: Enzymatic crosslinking of proteins a) with phenolic linker molecules present and b) without phenolic linker molecules present [20]

1.1.8 Aim of this work

The proposed aim of this work is the successful enzymatic crosslinking of human serum albumin with the mouse IgG1 monoclonal antibody using mushroom tyrosinase. A reaction protocol should be set up followed by suitable analysis. The expected advantage of the enzymatic crosslinking is the reduction of the use of toxic chemicals. The conjugate produced following the developed method will be used for the formation of nanospheres which will find application as a targeted drug delivery system.

1.2 Instrumental

1.2.1 Oxygen electrode (Clark cell)

The most commonly used setup to measure the partial oxygen pressure in solution or in the gas phase is the Clark cell. This oxygen electrode consists of two electrodes with the cathode being a central platinum disc working electrode and the anode being the silver ring reference electrode. On the cathode O_2 is reduced to water. With the use of 3 M potassium chloride solution conduction between the electrodes is assured. To seal off the test sample in the incubation chamber but at the same time letting oxygen diffuse through to the electrodes a gas permeable Teflon membrane is used.

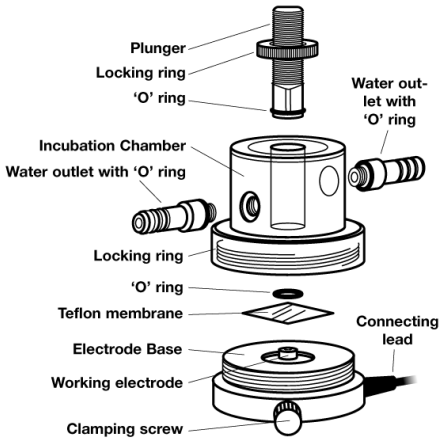


Figure 13: Setup of the oxygen electrode [38]

The **principle of operation** works as follows:

Every oxygen molecule that diffused from the test medium through the membrane and reaches the surface of the platinum electrode, which is polarized at –0,6 V with respect to the silver electrode, is reduced to water through the following reaction.

$O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$ Reduction occurring at the platinum electrode

$4Ag \rightarrow 4Ag^+ + 4\ e^-$

$4Ag^+ + 4Cl^- \rightarrow 4\ AgCl$ Oxidation occurring at the silver electrode

The overall electrochemical process can be written as follows:

$4Ag^+ + O_2 + 2H_2O + 4Cl^- \rightarrow 4AgCl + 4OH^-$

The partial pressure of oxygen is proportional to the resulting current between the two electrodes[39]. It is important to know that the electrode itself is consuming oxygen throughout the measurement, which especially needs to be taken in account when doing long term measurements. To guarantee a proper functioning of the electrode the solution needs to be stirred continuously to assure that the partial pressure of O_2 at the interface between the membrane and the test medium is the same. Due to the electrode being temperature sensitive it needs to be thermostated whenever possible [38].

1.2.2 SDS PAGE

The principle of the sodium dodecyl sulphate polyacrylamide gel electrophoresis is the separation of proteins concerning their molecular weight. Prior to the application on the gel the proteins are incubated in a loading buffer containing the detergent SDS, bromphenol blue, glycerine and buffer. When the mixture is heated to 80°C and incubated for 8 minutes the proteins are partially unfolded and SDS attaches to the surface giving the protein an all over negative charge. This process guarantees that the proteins are only separated by their molecular weight and not by their net charge.

When the samples are applied onto the gel the protein-SDS complex starts to move towards the positive pole in the electrical field and the proteins are separated by the polyacrylamide mesh. The smaller proteins can migrate more easily through the pores and therefore have a higher electrophoretic mobility whereas bigger proteins are held back by smaller pores.

After the run of the gel is finished there are several staining methods available to make the protein bands visible to determine their molecular weight [40].

1.2.3 Size exclusion Chromatography

In size exclusion chromatography particles are separated by their size and not by their molecular weight. It can either be performed under high pressure (high pressure liquid chromatography) or under normal conditions (gel permeation chromatography).

The samples are applied onto a column together with a mobile phase, which is usually buffer when working with proteins. The columns which represent the stationary phase are made of porous materials such as silica. Figure 14 shows the separation principle.

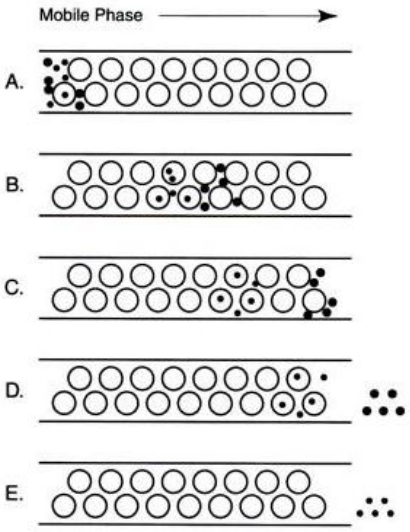


Figure 14: Separation principle of size exclusion chromatography [41]

The separation mechanism is based on the migration of different sized particles through pores of a definite size. The particles that are too large to penetrate the pores elute first whereas smaller

particles diffuse into the pores which holds them back and provokes an extended retention time. The particles should not interact with the column material to guarantee that they are only separated by size and not by chemical properties.

1.2.4 Zetasizer

The Zetasizer Nano Series, produced by Malvern is able to acquire information about the particle size, the zeta potential and the molecular weight of molecules present in the solution to be measured. These properties are assessed with Dynamic Light Scattering (DLS). DLS measures the speed at which the particles are diffusing through a solution due Brownian motion and relates it to the size of the particles. The viscosity of a solvent depends on the temperature, which has to be kept constant throughout the measurement. The values are obtained by measuring the rate at which the intensity of the scattered light fluctuates when detected. Of each sample multiple signals are recorded and compared by a correlating function, giving a correlogram that provides a lot of information about the sample.

From the values obtained the hydrodynamic diameter is calculated, which refers to how a particle diffuses within a fluid. The speed can be influenced by factors such as ionic strength, surface structure and non-spherical particles [42].

2 Materials and Methods

2.1 Enzyme Characterization

Throughout all experiments the tyrosinase from *Agaricus bisporus* (Sigma Aldrich T3824) was used. Before working with it, the yellowish powder was solubilized in 0, 1 M Sodium phosphate buffer (pH 6,8) in the concentration of 1 mg/ml, aliquotted in 100 µl portions and then stored in the -20°C freezer. The first step was to characterise the enzyme in regards to activity, pH/Temperature stability and oxygen uptake.

2.1.1 Enzyme activity assays

There are several ways of determining the activity of mushroom tyrosinase depending on which activity needs to be known. Duration and sensitivity of the assay are also crucial points to consider.

2.1.1.1 Dopachrome activity assay

The dopachrome activity assay makes use of the naturally catalysed reaction of tyrosinases as mentioned in chapter 0 and was adapted from the method of Robb[43]. The compound formation of L-DOPA quinone and dopachrome can be followed easily by measuring the increase in absorption at 305 and 475 nm.

To obtain both the monophenolase and diphenolase activities, L-tyrosine and L-DOPA have to be used as substrates. L-tyrosine is only able to be used in very low concentrations due to its poor solubility in buffer of only 2 mM.

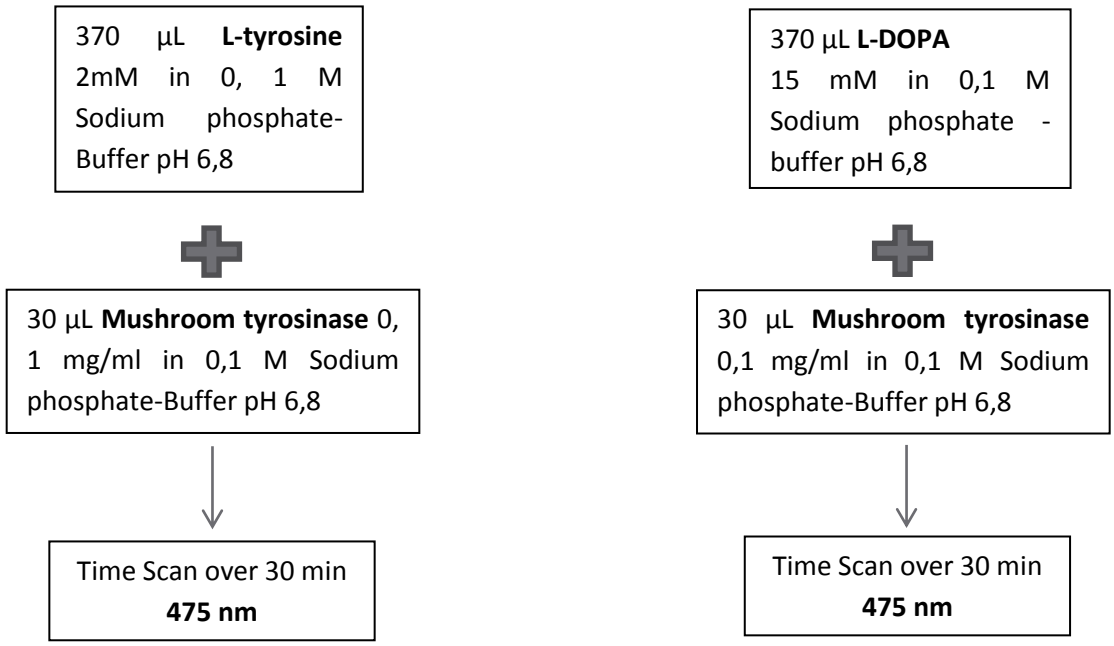


Figure 15: Flowchart of dopachrome activity assay

The assay must be carried out at 25°C and the measurement started immediately after addition of the enzyme. The graphs below display the characteristic activity curves for L-tyrosine and L-DOPA as substrate.

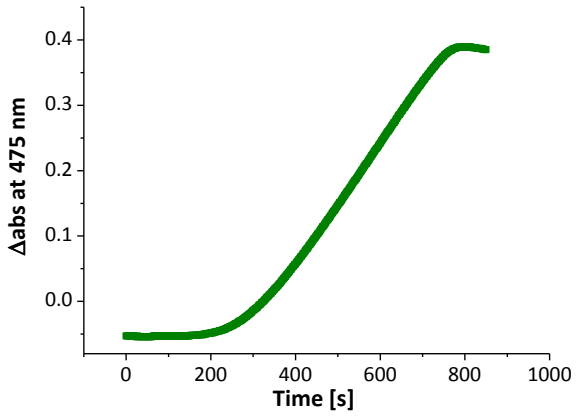


Figure 16: Activity curve of mushroom tyrosinase with L-tyrosine as substrate

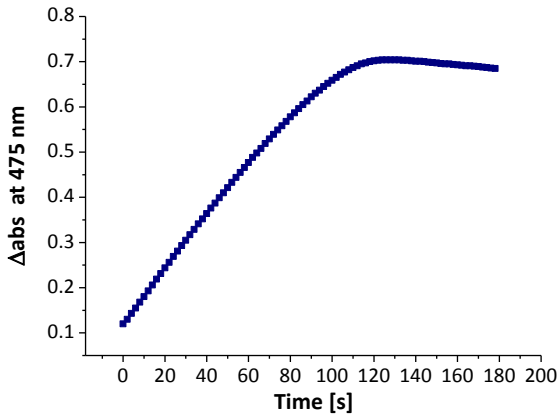


Figure 17: Activity curve of mushroom tyrosinase with L-DOPA as substrate

To obtain reliable values the measurements were done in duplicates.

2.1.1.2 Spectra of enzymatic L-tyrosine conversion

All products of the enzymatic conversion of L-tyrosine by mushroom tyrosinase show a different absorption peak. Therefore it was interesting to investigate the changes in the absorbance scan over a period of 20 minutes. To do this a wavelength scan from 200 to 600 nm was performed on the photometer. The reaction mixture consisting of 370 µl of 2 mM L- tyrosine and 30 µl of 0,1 mg/ml mushroom tyrosinase was measured in a micro quartz cuvette.

2.1.1.3 Spectrophotometric MBTH activity assay

A second method used to determine the activity of Mushroom tyrosinase is the continuous MBTH assay based on the method of Winder [44]. 3-methyl-2-benzothiazoninone hydrazine (MBTH) is a chromogenic and nucleophilic compound that attacks the enzyme generated o-quinone in position six to render a soluble and stable MBTH-quinone adduct. This pink pigment has its absorption peak at λ= 510 nm. This assay is a little more complex than the dopachrome assay but shows several advantages such as higher sensitivity (ϵ_{510}=22 300 l mol^{-1} cm^{-1}), higher stability of the formed compound and a shorter reaction time.

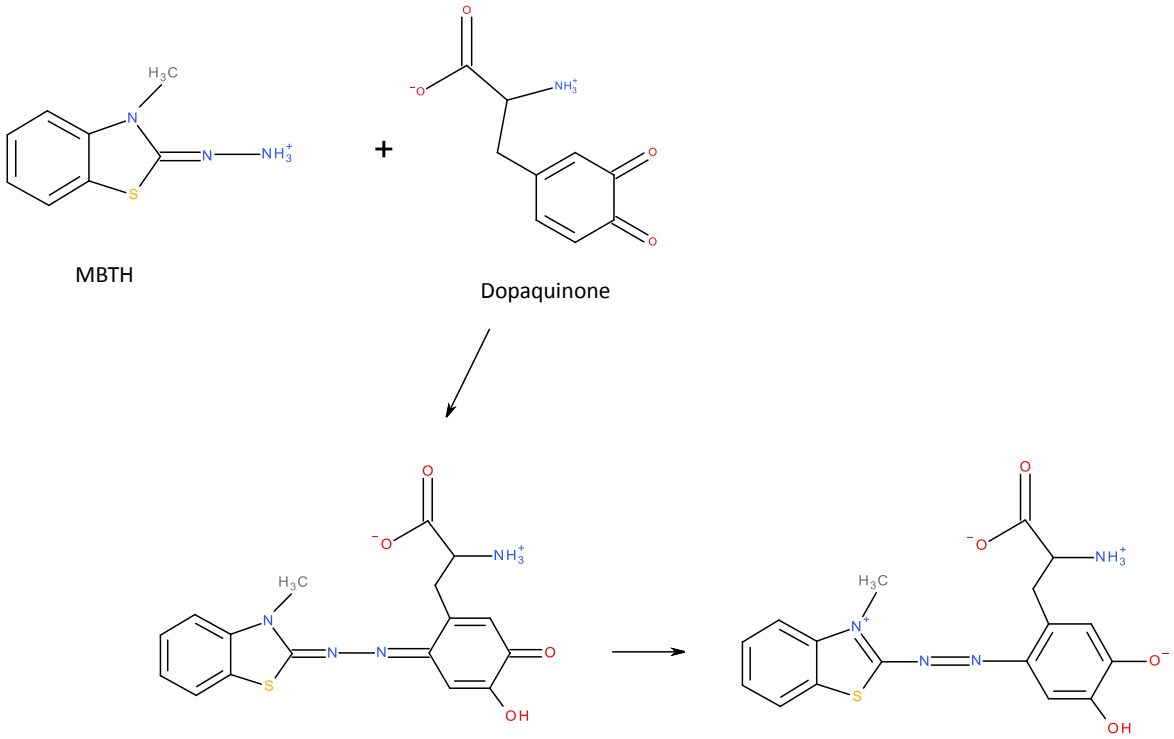


Figure 18: Reaction MBTH activity assay is based on

The method was adapted as follows:

- Assay buffer:
  - 0,1 M Sodium phosphate-Buffer, pH 6,8
  - 4 % (v/v) N,N´-dimethylformamide
  - 0,1 % (v/v) Triton x-100
- L-tyrosine/L-DOPA
  - 2 mM/ 30 mM in 0,1 M Sodium phosphate-Buffer, pH 6,8
- MBTH
  - 5 mM in 0,1 M Sodium phosphate-Buffer, pH 6,8

250 µl of the assay buffer are mixed with 100 µl of substrate and 120 µl of MBTH solution. The solution is then heated to 25° C and after addition of the 30 µl of enzyme (0, 1 mg/ml) the MBTH-Quinone adduct formation is measured at 510 nm over a time period of 20 minutes.

2.1.2 Determination of pH/ temperature stability

To determine the pH/ temperature stability of the enzyme the MBTH activity assay is used with changed reaction conditions. All measurements were done in duplicates.

To investigate the pH stability of mushroom tyrosinase, the assay buffer was prepared with pH values ranging from 4 to 8. The enzyme was then incubated in this solution for 2 hours at 25°C. After the incubation time the missing reagents were added and the measurement was started.

The temperature stability was evaluated by incubating the enzyme in the assay buffer (pH 6,8) at 30, 40, 50 and 60°C for two hours. After this time period the substrate and MBTH were added and the measurement, which was done at the different incubation temperatures, started immediately.

2.1.3 Oxygen uptake

As mentioned in previous chapters mushroom tyrosinase is an enzyme that requires oxygen for its catalysed reaction. The oxygen uptake rate [g O_2/(min g_{enzyme})] is an indicator of how fast a substrate is converted by the enzyme and is therefore an interesting parameter. The Clark electrode which is incorporated in the so called Oximeter is used to measure the oxygen decrease in a solution. The measurements were done in triplicates. 2 mM L-tyrosine and 30 mM L-DOPA were used as substrates. To determine the oxygen uptake of the monophenolase activity 370 µl of 2 mM L-tyrosine solution were pipetted into the reaction chamber and 30 µl of 0,1 mg/ml mushroom tyrosinase were added. For the oxygen uptake of the diphenolase activity 185 µl of 30 mM L-DOPA solution were diluted with 185 µl 0,1 M Sodium phosphate-Buffer (pH 6,8) and then 30 µl of 0,1 mg/ml mushroom tyrosinase were added to the reaction mixture. To guarantee an oxygen saturated solution, it was stirred with the lid open for 15 minutes. The oxygen decrease was monitored for about approximately 15 min (sample recorded every 2 seconds) and the temperature was kept at 25°C with a heated water bath.

2.2 Sample preparation

2.2.1 Denaturation of human serum albumin

Human serum albumin is a very well folded protein and therefore shows a very tight and inaccessible structure. Denaturation leading to a more accessible protein is a very interesting option. There are several ways of achieving the loosening of the structure of HSA using chemical and physical methods.

2.2.1.1 Chemical denaturation

Guanidine hydrochloride and urea are only two of many chaotropic agents that can be used to disrupt the three dimensional structure in proteins. These two compounds were used to make human serum albumin more accessible for mushroom tyrosinase.

HSA/ HSA essentially fatty acid free with a concentration of 0,6 mg/ml (dissolved in 0,1 M Sodium phosphate-Buffer, pH 6,8) was incubated with GnHCl (final concentration 1,8 M) and urea (final concentration 5 M) in a 96 well plate at room temperature for 12-24 hours and was shaken at 500 rpm. The reaction was monitored by measuring the intrinsic tryptophan 214 fluorescence on the platereader with the excitation wavelength being 280 nm and the emission being scanned from 300-400 nm every 2-3 hours.

Table 1: Platereader Tecan settings of fluorescence measurements

Excitation wavelength	**280 nm**
Emission Scan	300-400 nm
Number of reads per well	20
Manual gain	100

To be able to use the denatured protein for further reactions, it required purification using gel permeation chromatography (AEKTA Purifier).

Table 2: AEKTA purifier settings of chemical denaturation

Column	**5 ml desalting column**
Flow rate	0,7 ml/min
Sample loop	200 µl
Fraction size	200 µl
Mobile phase	0,1 M Sodium phosphate-Buffer, pH 6,8

Through purification the protein was diluted, therefore it was necessary to concentrate the solution via 2 ml Vivaspins 2 (Cut off: 100 kDa) to end up with a volume of around 200 µl.

The protocol had to be adapted numerous times to improve the results. Table 3 shows the protocol variations investigated.

Table 3: Reaction conditions of chemical denaturation

#	Protein	Concentration [M] GnHCl	Concentration [M] urea	Temperature [°C]	Rotation [rpm]	Time [h]
1	HSA	1,8	-	RT	500	24
	HSA	-	5	RT	500	24
2	HSA	1,8	-	RT	500	12
	HSA	-	5	RT	500	12

2.2.1.2 Temperature denaturation

Another way of denaturing proteins is exposing them to high temperatures. Before using this method it was necessary to find out which temperature would result in a measureable change in the structure of human serum albumin. The unfolding was monitored by intrinsic tryptophan 214 fluorescence on the platereader and by the change of the particle size determined by Dynamic Light Scattering using Zetasizer Nanoseries. For the analysis on the platereader 150 µl of HSA and HSA fatty acid free with a concentration of 1 mg/ml in 0,1 M sodium phosphate-Buffer (pH 6,8) were incubated for 3 hours at temperatures ranging from 20 to 90 °C (in 10°C intervals). The intrinsic fluorescence of 100 µl of each sample was then measured on the platereader (Settings see Table 1).

For the analysis on the Zetasizer only HSA fatty acid free was used with a concentration of 1 mg/ml. The solution required filtration through the use of a 0,8 µm syringe driven filter unit (Millex®-AA; Millipore) before the measurement. This was done to remove non-protein particles that could influence the results. A sequence was defined which would heat the sample from 20 to 90°C in 10°C intervals with an equilibration time at each temperature of 20 minutes. The sample was measured in a micro quartz cuvette.

Table 4: Zetasizer settings for temperature denaturation

Material	Polystyrene latex
Dispersant	Water
Equilibration time when t is reached	1200 s
Number of measurements	3
Measurement duration	automatic
Seek for optimum positions	Yes

From this point on, temperature denaturation experiments were carried out at 80°C for 2 hours using HSA essentially fatty acid free unless otherwise stated.

2.2.2 FITC- labelling of human serum albumin essentially fatty acid free

To be able to analyse even small amounts of product, human serum albumin was labelled with fluoresceinisothiocyanate (FITC). 2 ml of human serum albumin essentially fatty acid free (2 mg/ml in 0, 1 M Sodium phosphate-Buffer, pH 6,8) were incubated with 4,74 µl of FITC solution (5 mg/ml in DMSO) at room temperature for 4 hours and 550 rpm. The reactants were used in equimolar amounts to ensure that only one FITC-molecule would bind to one HSA- molecule during the reaction time. Unlinked FITC was removed from the product by using AEKTA purifier equipped with a 2 ml desalting column.

2.3 Enzymatic Crosslinking

The enzymatic crosslinking was the key experiment of this thesis. The basic reaction setup remained the same so that the buffer used was 0, 1 M Sodium phosphate-Buffer (pH 6,8), the reaction temperature 25 °C and the shaking speed 450 rpm. The reactions were always performed in 1,5 ml eppendorf tubes with 3 holes punched into the lid to provide enough oxygen for the reaction over the long incubation time. Stock solutions were used to achieve the concentrations listed in the tables throughout this section.

Table 5: Stock solutions of compounds used for enzymatic crosslinking

Compound	Abbreviation	concentration	Solvent
human serum albumin	HSA	5 mg/ml	0, 1 M Sodium phosphate-Buffer (pH 6,8)
human serum albumin fatty acid free	HSA-FAF	4 mg/ml	0, 1 M Sodium phosphate-Buffer (pH 6,8)
human serum albumin linked to FITC	HSA-FITC	4 mg/ml	0, 1 M Sodium phosphate-Buffer (pH 6,8)
Mouse IgG1 monoclonal antibody	AB	3,6 mg/ml	
Insulin Chain B oxidized from bovine pancreas	Ins	5 mg/ml	0, 1 M Sodium phosphate-Buffer (pH 6,8)
tyrosinase f. *Agaricus bisporus*	AbT	1 mg/ml	0, 1 M Sodium phosphate-Buffer (pH 6,8)
caffeic acid	CA	30 mM	50% EtOH in H_2O_{dest}
Ferulic acid	FA	50 mM	50% EtOH in H_2O_{dest}
guaiacol	G	69,5 mM	50% EtOH in H_2O_{dest}
catechol	C	60 mM	50% EtOH in H_2O_{dest}
p-Coumaric Acid	PCA	50 mM	50% EtOH in H_2O_{dest}

As previously mentioned, multiple changes were performed on the substrates, by denaturing or modifying them. Additionally phenolic non-protein compounds were used as substrates to act as linker molecules.

2.3.1 Using model substrate

To start off Insulin Chain B isolated from bovine pancreas was used as a substrate to see if mushroom tyrosinase could work with peptides which had a certain amount of L-tyrosine residues in the chain. The numbers in brackets refer to the original order of the couplings done in the laboratory.

- **Coupling 1: Using Insulin Chain B oxidized from bovine pancreas as model substrate (2)**

Table 6: Reaction mixtures of coupling 1

	c [mg/ml] Ins	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
Ins 1	1,33	0,01	200	300
Ins 2	0,67	0,02	200	300
Ins 3	0,13	0,001	200	300
Ins 4	0,13	0,001	200	300

The reaction mixtures were incubated for 96 hours and analysis from the samples t= 0, 6, 89 and 96 hours was performed using size exclusion chromatography at the HPLC and a wavelength scan from 230 – 400 nm at the platereader.

- **Coupling 2: Using Insulin Chain B oxidized from bovine pancreas as model substrate (3)**

The only alteration to coupling 1 was the enzyme concentration applied to investigate whether or not the product formation would increase.

Table 7: Reaction mixtures of coupling 2

	c [mg/ml] Ins	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
Ins 1	1,33	0,07	200	300
Ins 2	0,67	0,13	200	300
Ins 3	0,13	0,01	200	300
Ins 4	0,13	0,01	200	300

The reaction mixture was incubated for 48 hours and the samples t= 0, 5, 24 and 48 hours were analysed at the HPLC and the platereader. Additionally the oxygen consumption was monitored on the Oximeter (coupling 8).

2.3.2 Using substrates in native form

The next step was to see, whether mushroom tyrosinase is able to work with larger substrates such as well folded proteins and antibodies.

- **Coupling 3: Using HSA as the only substrate (4)**

Table 8: Reaction mixtures of coupling 3

	c [mg/ml] HSA	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
HSA 1	1,33	0,07	200	300
HSA 2	1,33	0,01	200	300
HSA 3	0,13	0,07	200	300
HSA 4	0,13	0,01	200	300

The reaction mixtures were incubated for 72 hours and then analysed at the HPLC and the platreader after t = 0, 24, 48 and 72 hours.

- **Coupling 4: Using mouse IgG1 monoclonal antibody as the only substrate (8)**

Table 9: Reaction mixture of coupling 4

	c [mg/ml] AB	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
AB 1	0,96	0,1	200	300
AB 2	0,48	0,17	200	300

The reaction mixtures were incubated for 24 hours and analysed at the HPLC. The oxygen decrease was monitored at the Oximeter.

2.3.3 Using denatured/modified substrates and linker molecules

The concept behind using denatured and modified substrates was to increase the possibility of mushroom tyrosinase to interact with human serum albumin.

- **Coupling 5: Using chemically denatured HSA (10b)**

As mentioned in section 1.1.6 guanidine hydrochloride and urea can be used for protein denaturation. For coupling 5 denatured human serum albumin (0,25 mg/ml) from denaturation 1 (See section 2.2.1.1) was used as a substrate.

Table 10: Reaction mixtures of coupling 5

	c [mg/ml] HSA	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
GnHCl	0,11	0,19	100	260
urea	0,13	0,17	100	300

The reaction mixture was incubated for 24 hours and samples from time points t=0, 3 and 24 hours were analysed via HPLC.

- **Coupling 6: Using chemically denatured HSA (11)**

In coupling 6 human serum albumin from denaturation 2 was used as substrate. On this occasion the protein was only denatured for 12 hours, to see if that would change the effect. Due to the low concentration of HSA after denaturation (0,3 mg/ml) no additional buffer was used in this reaction.

Table 11: Reaction mixture of coupling 5

	c [mg/ml] HSA	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
GnHCl	0,24	0,20	-	250
urea	0,23	0,20	-	230

The reaction mixture was incubated for 18 hours and samples from time points t= 0, 15, 18 hours were analysed via HPLC and SDS PAGE 10%.

- **Coupling 7: Using Insulin Chain B as a linker molecule (6)**

Another idea was to use short peptide chains such as Insulin Chain B oxidized from Bovine Pancreas as a linker molecule to see if that would facilitate the crosslinking of human serum albumin.

Table 12: Reaction mixture of coupling 9

	c [mg/ml] HSA	c [mg/ml] Ins	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
HSA 1	0,67	0,07	0,07	200	300
HSA 2	0,07	0,09	0,07	200	300

The reaction mixtures were incubated for 72 hours and analysed on the plate reader and the HPLC after t= 0, 24, 48 and 72 hours.

- **Coupling 8: Using Insulin Chain B as a linker molecule with activated HSA/AB (9b)**

To achieve better results HSA and AB were incubated with AbT for 2, 6 and 24 hours, followed by a drop wise (3µl) addition of a 0,05 mg/ml Insulin Chain B solution (5 times in 15 minute intervals).

Table 13: Reaction mixtures of coupling 9

	c [mg/ml] HSA/ AB	c [mg/ml] Ins	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction	Activation time [h]
HSA 1	0,48	0,0025	0,17	200	300	2
AB 1	0,67	0,0025	0,17	200	300	2
HSA 2	0,48	0,0025	0,17	200	300	6
AB 2	0,67	0,0025	0,17	200	300	6
HSA 3	0,48	0,0025	0,17	200	300	24
AB 3	0,67	0,0025	0,17	200	300	24

The reaction mixtures were incubated for 27 hours and then analysed by HPLC and SDS-PAGE 8%. Additionally HSA 1 and AB 1 were monitored on the Oximeter to measure the oxygen decrease over time.

- **Coupling 9: Using low molecular weight phenolic compounds as linker molecules (15)**

For coupling 9 caffeic acid, ferulic acid, guaiacol, catechol and p-coumaric acid were used as linker molecules in a final concentration in the reaction mixture of 2 mM. AB and HSA were used in equimolar concentration to ensure that each HSA molecule was able to couple with an AB molecule.

Table 14: Reaction mixtures of coupling 9

	c [mg/ml] HSA	c [mg/ml] AB	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
caffeic acid	0,75	1,71	0,2	20	150
ferulic acid	0,75	1,71	0,2	24	150
guaiacol	0,75	1,71	0,2	26	150
catechol	0,75	1,71	0,2	25	150
p-coumaric acid	0,75	1,71	0,2	24	150

All reagents were added at once and the reaction let run for 24 hours. Samples from time points t=0, 18,5 and 24 hours were analysed using HPLC and SDS-PAGE 8%.

- **Coupling 10: Changing concentration of low molecular weight phenolic compounds (16)**

This reaction was performed to determine whether or not a higher concentration of phenolic compounds would lead to a higher product formation. The phenolic compounds caffeic acid, guaiacol and p-coumaric acid were used in final concentration of 2 and 4 mM.

Table 15: Reaction mixtures of coupling 10

	c [mg/ml] HSA FAF	c[mmol/l] phenolic compounds	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
caffeic acid 1	1	2	0,2	28	120
caffeic acid 2	1	4	0,2	20	120
guaiacol 1	1	2	0,2	33	120
guaiacol 2	1	4	0,2	29	120
p-coumaric acid 1	1	2	0,2	31	120
p-coumaric acid 2	1	4	0,2	26	120

The reaction mixtures were incubated for 24 hours followed by HPLC and SDS-PAGE 8,5% analysis of time points t=0 and 24 hours.

- **Coupling 11: Elucidation of compositions of new bands on gel (18)**

The idea behind coupling 11 was to determine which substrate combination leads to which product. The compounds used were HSA FAF, HSA FAF denatured by temperature and AB. caffeic acid, catechol and p-coumaric acid were incubated with HSA FAF (see number 1,5,9)/ AB (see number 2,6,10)/ HSA FAF and AB (see number 3,7,11)/ HSA FAF denatured and AB (see number 4,8,12).

Table 16: Reaction mixture of coupling 11

number		c [mg/ml] HSA-FAF/HSA FAF den	c [mg/ml] AB	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] 0f reaction
1	**HSA FAF CA**	0,75		0,2	35	120
2	**AB CA**		1,71	0,2	23	120
3	**HSA FAF + AB CA 1**	0,75	1,71	0,2	-	120
4	**HSA FAF + AB CA 2**	0,75	1,71	0,2	-	120
5	**HSA FAF PCA**	0,75		0,2	-	120
6	**AB PCA**		1,71	0,2	-	120
7	**HSA FAF + AB PCA 1**	0,75	1,71	0,2	41	120
8	**HSA FAF + AB PCA 2**			0,2	30	120
9	**HSA FAF C**	0,75		0,2	-	120
10	**AB C**		1,71	0,2	-	120
11	**HSA FAF + AB C 1**	0,75	1,71	0,2	47	120
12	**HSA FAF + AB C 2**	0,75	1,71	0,2	35	120

The reaction mixtures were incubated for 24 hours and then analysed via SDS-PAGE 7,5%/6 %.

- **Coupling 12: Using FITC labelled HSA for better analysis (20)**

Using human serum albumin labelled with FITC was another way of revealing the composition of the enzymatic crosslinking products. Due to the high sensitivity of fluorescence even very low concentrations can be detected that are not visible on SDS-gels. The aim of coupling 12 was to compare the crosslinking behaviour of HSA FITC, HSA FAF and HSA FAF denatured for 2,5 hours at 80 °C.

Table 17: Reaction mixture of coupling 12

	Protein used	c [mg/ml]HSA FITC/ FAF den	HSA-FAF/HSA	c [mg/ml] AB	c [mg/ml] enzyme	V [µl] buffer	V [µl] of reaction
1	**HSA FITC**	1,5		1,705	0,2	-	120
2	**HSA FITC**	1,5		1,136	0,2	-	120
3	**HSA FITC**	1,5		0,852	0,2	7	120
4	**HSA FITC**	1,5		0,852	0,2	7	120
5	**AB**			1,705	0,2	23	120
6	**HSA FAF**	1,5		1,136	0,2	-	120
7	**HSA FAF den**	1,5		0,852	0,2	7	120
8	**HSA FAF**	1,5		0,852	0,2	31	120

The reaction mixtures were incubated for 22 hours and samples from time points t=0 and 22 hours analysed by SDS-PAGE (6 % and gradient gel 4%/ 6%) and fluorescent scanning.

- **Coupling 13: Changing the molar ratio of HSA/AB (21)**

To increase product formation, the molar ratio between HSA and AB was changed. The ratio ranged from HSA 2: 1 AB up to HSA 5:1 AB. Another strategy used was the preincubation of HSA and AB with mushroom tyrosinase before adding caffeic acid drop wise (10x 2 µl every 30 minutes) to reach a final concentration of 2 mM. (See reaction number 9-12 in Table 18)

Table 18: Reaction mixtures of coupling 13

	Ratio HSA:AB	c [mg/ml] FAF-FITC	c [mg/ml] AB	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
1	**2:1**	1,2	1,364	0,24	-	150
2	**2:1**	1,2	1,364	0,32	-	150
3	**3:1**	1,2	0,909	0,24	11	150
4	**3:1**	1,2	0,909	0,32	-	150
5	**4:1**	1,2	0,682	0,24	21	150
6	**4:1**	1,2	0,682	0,32	9	150
7	**5:1**	1,2	0,545	0,24	26	150
8	**5:1**	1,2	0,545	0,32	14	150
9	**5:1 CA drops**	1,2	0,909	0,24	11	150
10	**5:1 CA drops**	1,2	0,909	0,32	-	150
11	**5:1 CA drops**	1,2	0,909	0,24	11	150
12	**5:1 CA drops**	1,2	0,909	0,32	-	150
13			0,909	0,24	56	150
14			0,909	0,32	44	150

The reaction mixtures were incubated for 48 hours and samples from time points t=0,24 and 48 hours analysed by SDS-PAGE (6% and gradient gel 5,5%/6%) and fluorescent scanning.

- **Coupling 14: Display all possibilities on one gel (14)**

The last coupling performed included all possible combinations of substrates and strategies that were done using HSA FITC. Additionally, HSA FITC was preincubated with caffeic acid and mushroom tyrosinase before adding the AB (6 x 6µl every hour). This time blanks, meaning substrate mixtures not including mushroom tyrosinase were measured as well, to ensure that the products are only formed with the enzyme present.

Table 19: Reaction mixtures of coupling 14

	Description of sample	c [mg/ml] FAF FITC	c [mg/ml] AB	c [mg/ml] AbT	V [µl] buffer	V_{total} [µl] of reaction
1	**HSA FITC**	1,5		0,24	38	150
2	**HSA FITC Blank**	1,2		0	85	150
3	**AB**		1,364	0,24	37	150
4	**AB Blank**		1,364	0	73	150
5	**HSA FITC + AB 6:1**	1,5	0,568	0,24	14	150
6	**HSA FITC + AB 4:1**	1,5	0,852	0,24	2	150
7	**HSA FITC + AB 8:1**	1,5	0,426	0,24	20	150
8	**HSA FITC + AB Blank**	1,5	0,426	0	56	150
9	**HSA FITC preincubated**	1,5	0,852	0,24	2	150
10	**HSA FITC preincubated**	1,5	0,852	0,24	2	150

The reaction mixtures were incubated for 48 hours and samples from time points t=0, 24 and 48 hours analysed by SDS-PAGE (gradient gel 5,5%/6%) and fluorescent scanning.

2.4 Analysis

Four different methods based on oxygen uptake, size distribution and fluorescence were used to analyse the outcome of the enzymatic crosslinking.

2.4.1 OXIMETER

The Oximeter, as described in the section 1.2.1, can be used to monitor the enzymatic crosslinking reaction by measuring the oxygen decrease in solution caused by the enzyme incorporating oxygen into the substrates. The reaction mixture is pippeted directly into the reaction chamber, kept at 25°C and the oxygen decrease is monitored. The oxygen content was measured for 24 hours with a sample recorded every 10 seconds.

2.4.2 High Pressure Liquid Chromatography (HPLC)

Size exclusion is one of many separation criteria that can be used in the HPLC. Because a successful crosslinking leads to a construct much bigger in size than the original substrates, this is a good method to analyse the reaction products. The samples were either measured undiluted or 10 µl sample were added to 70 µl of water. The vials containing the solution were immediately frozen after sampling to ensure that the enzymatic reaction would no longer take place. They were thawed immediately before being measured. HPLC analysis was performed on a Dionex system equipped with a P680 – HPLC Pump, a variable UV-VIS detector set at 205, 228, 280 and 340 nm, an auto sampler (UltiMate 3000 auto sampler) and a thermostated Column Compartment (TCC 100) using a Zorbax GF 250 size exclusion column.

In Table 20 a summary of the settings of the HPLC can be found:

Table 20: HPLC-settings for the analysis of enzymatic crosslinking

Column	Zorbax GF 250
Mobile Phase	0,1 M Sodium phosphate-Buffer, pH 6,8
Gradient	No
Duration of run	20 min
Flow rate	1 ml/min
Temperature	Room temperature
UV-VIS detection	205 nm
	228 nm
	280 nm
	340 nm
Injection volume	20 µl

2.4.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE is a very simple and efficient way to analyse size changes in proteins. Depending on the expected size of the proteins the gels must be prepared with different acrylamide contents to create smaller or wider mashed grids. Due to dealing with proteins of bigger size, gels ranging from 6 – 12 % were prepared. After successful enzymatic crosslinking the size difference between substrate and product was very large, therefore gradient gels with a separating gel consisting of a 6 % lower and 5,5 % upper part were prepared to guarantee a good separation and resolution of the protein bands.

The composition of the gels the loading and the running buffer are listed in Table 21.

Table 21: Composition of gels, loading and running buffer for SDS PAGE

Separating gel 0,5 mm	**ingredients**	**6% [ml]**	**7,5% [ml]**	**8,5% [ml]**	**10 % [ml]**	**12 % [ml]**
	40% Acrylamide, Bis	1,5	1,9	2,1	2,5	3
	Tris HCl; 1,5M; pH8,8	2,5	2,5	2,5	2,5	2,5
	dd H_2O	5,8	5,4	5,2	4,8	4,3
	10% SDS	0,1	0,1	0,1	0,1	0,1
	10% APS	0,05	0,05	0,05	0,05	0,05
	TEMED	0,012	0,012	0,012	0,012	0,012

Gradient gel 0,75 mm		**5,5 % [ml]**	**6 % [ml]**
Upper part 5,5 % Lower part 6 %	40% Acrylamide, Bis	0,825	0,6
	Tris HCl; 1,5M; pH8,8	1,5	1
	dd H_2O	3,6	2,3
	10% SDS	0,06	0,06
	10% APS	0,03	0,02
	TEMED	0,0072	0,0048

Stacking gel 0,5 mm		**4% [ml]**
	40% Acrylamide, Bis	1,0
	Tris HCl; 0,5M; pH6,8	2,5
	dd H_2O	6,4
	10% SDS	0,1
	10% APS	0,05
	TEMED	0,12

Loading buffer		
2x 20 ml	1x TAE	10 ml
	4 % SDS	0,8 g
	50% Glycerin (w/v)	10 ml
	0,1 ‰ Bromphenol blue	0,002 g

Running buffer		
5x 1L	Tris HCl; 0,125M;pH 3,2	15 g
	SDS	1 g
	glycine	14,4 g

To prepare the samples, 10 µl of the reaction mixture were added to 15 µl of loading buffer. This was then centrifuged for 10 seconds and boiled at 80 °C and 500 rpm for 8 minutes. From the composition of the loading buffer can be seen, that the SDS PAGE was not performed under reducing conditions because agents like DTT or Mercaptoethanol were not added. Subsequently 25 µl were applied onto the gel and let run at 85 V (for gradient gels and 6 -8, 5 % gels) and 100 V (for 10 – 12 % gels) until the bromphenol blue dye was completely run off. (~ 2 hours) After the gels were finished they were washed for 10 minutes in H_2O_{dest} to get rid of all the running buffer components and then stained with either Coomassie Brilliant Blue, Kang staining or silver staining.
The first staining method mentioned works well for protein bands of a sizeable concentration.
In the table below the chemicals for the staining methods are listed.

Table 22: Composition of staining and destaining solution for Coomassie Brilliant Blue and Kang Staining

Coomassie Brilliant Blue	Staining		Destaining	
	0,1 % (w/v) Coomassie blue R-250	1 g	50 % methanol	500 ml
	45 % ethanol	450 ml	10 % acetic acid	100 ml
	10% acetic acid	100 ml	H_2O_{dest}	400 ml
	45% H_2O_{des}	450 ml		
KANG staining	0,02 % (w/v) Coomassie Blau CBB G-250	0,2 g	10 % ethanol (96%)	100 ml
	5 % aaluminum sulfate 18 hydrate	50 g	2 % ortho-phosphoric acid (100 %)	20 g
	10 % ethanol (96%)	100 ml	90% H_2O_{dest}	900 ml
	2 % ortho-phosphoric acid (100 %)	20 g		
	90% H_2O_{des}	900 ml		

After being washing the gels were stained overnight for approximately 12 hours so even the lowest concentrated bands could be seen. The staining was followed by a washing step in H_2O_{dest} to remove the staining solution. To finish the protocol, the gels were incubated in the destaining solution for 3-6 hours until the bands could be seen with high contrast.

In cases where the protein concentrations are very low a more sensitive staining method such as the silver staining must be used. This type of staining can detect very low concentration of protein because the silver particles are smaller and can therefore incorporate more easily into the proteins. Silver staining requires many different solutions; however, the staining process itself is not time consuming.

Table 23: Composition of solutions for silver staining

Solution	Composition	
A (Fixing solution)	12 % acetic acid	120 ml
	50 % ethanol	500 ml
	0,05 % formaldehyde (37%)	500 µl
	H_2O_{dest}	380 ml
B (washing)	20 % ethanol	200 ml
	80% H_2O_{dest}	800 ml
C (sensitizing solution)	0,02% $Na_2S_2O_3$	200 mg
	H_2O_{dest}	1000 ml
D (Staining)	0,2 % $AgNO_3$	2 g
	0,076 % formaldehyde (37 %)	760 µl
	H_2O_{dest}	1000 ml
E (Developing)	6 % Na_2CO_3	60 g
	0,0004% $Na_2S_2O_3$	4 mg
	0,05 % formaldehyde (37%)	500 µl
	H_2O_{dest}	1000 ml
F (Terminating)	12 % Acetic acid	120 ml
	H_2O_{dest}	880 ml

After washing the gels in H_2O_{dest} for 10 minutes the staining protocol must proceed as follows:

- 2h to overnight: fixing solution A
- 20 minutes washing B with changing the solution 3 times
- 2 times 1 minute washing with H_2O_{dest}
- 20 minutes cold (4°C) silver staining D
- 2 times rinse gel with large volume of H_2O_{dest} (20-60 seconds)
- 2 to 5 min developing E
- 10 minutes terminating F until bubbling of the solution is over

2.4.3.1 Fluorescence Scanning

Fluorescence scanning of SDS gels is a useful way of detecting proteins with a very low concentration due to the high sensitivity of fluorescent compounds. This method can be used for gels if they are stained with a fluorescent dye or if single proteins are present that were previously linked to a fluorescent compound, in this case fatty acid free human serum albumin linked to FITC. With this, running a SDS gel not only provides information about the molecular weight of a protein but also, if a mixture of several proteins is applied onto the gel, which protein is present in a specific band.

To scan the SDS gel on a fluorescent imager no further preparation is required. The gel only needs to be washed for 10 minutes in H_2O_{dest} before scanning. It is very important that the bromphenol blue dye front completely runs off before the measurement because the colour will interfere with the fluorescence. Normally the gels are incubated in an acidic fixing solution for an hour to guarantee that the protein bands stay sharp and in place. But due to the fact that FITC is very sensitive to pH shifts the scan was done without previous fixing.

The Molecular imager from BIORAD equipped with Laser Scanner FXPro and driven by QuantitiyOne was used to create the images. The excitation laser was set to 488 nm and the emission filter to 530 nm. The 4.5.1 Software package was used to modify the images.

2.4.3.2 Tryptic digestion followed by MALDI-TOF analysis

To ensure that human serum albumin coupled with mouse IgG1 monoclonal antibody, a tryptic digestion followed by MALDI-TOF analysis was performed. The product band was cut out of the SDS-gel and put into a 10% EtOH solution for storage. After digestion the resulting peptide fragments were analysed with MALDI-TOF on a LTQ-FT Ultra by Thermo Scientific. A Spectrum Mill search with the following parameters was then performed with the peptide masses obtained:

- MH+: 200-8000 Da
- NCBInr Mammals
- Max. missed cleavages: 2
- Modifications: Carbamidomethylation (Cys) fixed, Oxidized methionine variable
- Precursor mass tolerance +/- 0,05 Da
- Product mass tolerance +/- 0,7 Da
- Validation: ion score > 20

The whole analysis was performed by the Team CF Mass Spectrometry/Proteomics at the Center for Medical Research (ZMF) of the Medical University of Graz.

3 Results and Discussion

3.1 Enzyme Characterisation

3.1.1 Enzyme activity

The enzymatic activity was calculated using the dopachrome and the MBTH-assay giving different results.

3.1.1.1 Dopachrome assay

To calculate the enzymatic activity of mushroom tyrosinase using the dopachrome assay, the absorption values obtained at 475 nm were plotted vs. time. A linear fit was performed and the slope of the linear equation used for further calculations. As the measurements were done in duplicates, the two resulting slopes are displayed in Figure 19 and Figure 20.

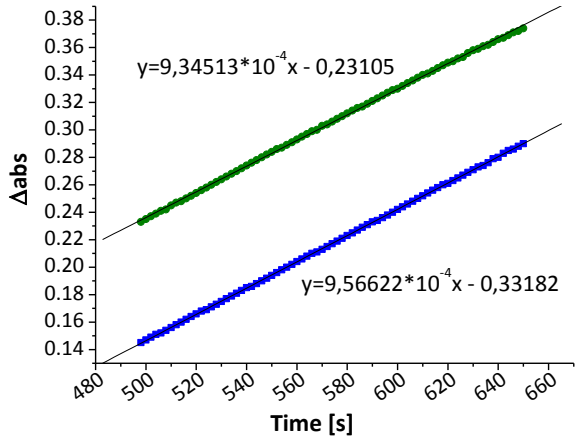


Figure 19: Dopachrome assay with L-tyrosine

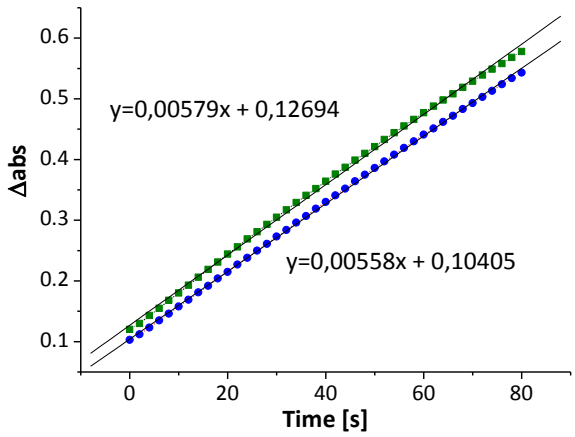


Figure 20: Dopachrome assay with L-DOPA

Equation 1 was used to calculate the activity of mushroom tyrosinase with the Unit U/ml.

$$\frac{\Delta c}{\Delta t} = \frac{\Delta abs}{\Delta t} * \frac{V_{sample}}{V_{ges} * \varepsilon * d}$$

Equation 1

The average values of the slopes for each substrate need to be inserted for Δabs/Δt, the molar extinction coefficient of dopachrome is ε_{475}=3400 l mol^{-1} cm^{-1} and d= 1 cm.

These calculations give an enzyme activity towards L-tyrosine of **0,22 U/ml** and towards L-DOPA of **1,34 U/ml**. The specific activities are **2,23** and **13,38 U/mg** dry enzyme respectively.

3.1.1.2 MBTH assay

The calculation of the enzymatic activity using the MBTH assay is equal to the process in the dopachrome assay. Only the value of the extinction coefficient needs to be changed because it is ε_{510}= 22 300 l mol^{-1} cm^{-1}. In Figure 21 and Figure 22 the slopes of the measurements are displayed.

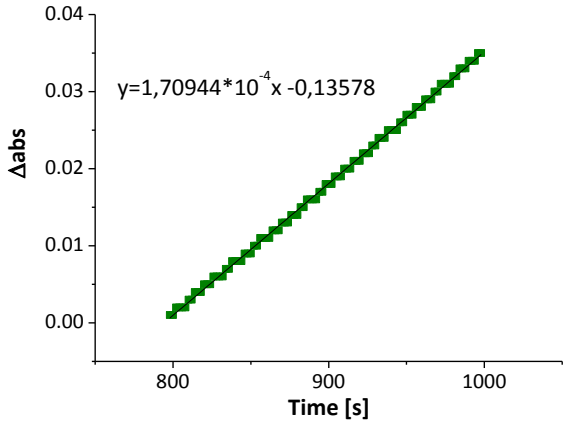


Figure 21: MBTH assay with L-tyrosine

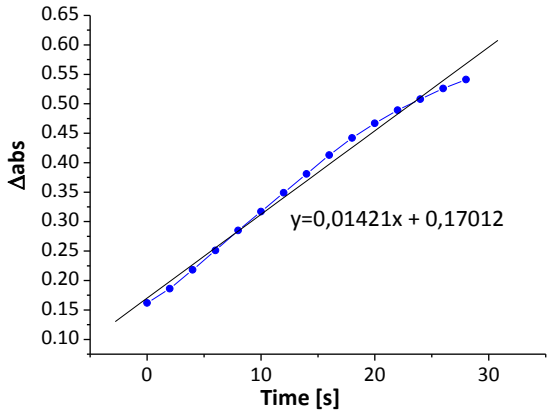


Figure 22: MBTH assay with L-DOPA

Taking the values of the slope and inserting them into Equation 1 gives an enzyme activity towards L-tyrosine of **0,08 U/ml** and towards L-DOPA of **0,63 U/ml**. The specific activities are **0,77**and **6,33 U/mg** dry enzyme respectively.

3.1.1.3 Spectra of enzymatic tyrosine conversion

The spectra that were recorded over 20 minutes show, that during the short lag phase only L-tyrosine (blue arrow) and L-DOPA (green arrow) are present. After this time period L-DOPA quinone (red arrow) is formed with the absorption peak being 305 nm. The following rearrangement to dopachrome (black arrow), with its absorption peak at 475 nm, is a very quick non-enzymatic reaction that causes the red colouring of the solution.

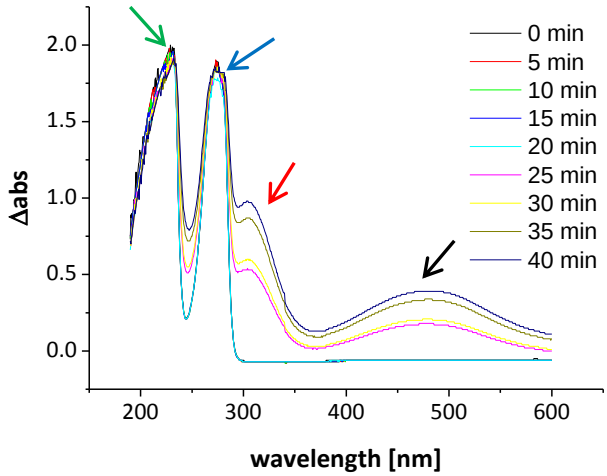


Figure 23: Spectrum of L-tyrosine conversion

3.1.2 Determination of pH/Temperature Stability

The determination of pH and temperature stability brought interesting results which are that mushroom tyrosinase is very sensitive to temperature but not at all to the change of pH.

Up to 40° C the activity only decreases slightly but between 40 and 60 °C the enzyme is nearly inactivated. It doesn´t regain its activity when cooling back down to 25 °C, which proposes that irreversible damages to the proper folding of the enzyme and the active site occur throughout the increase of temperature.

The change of pH from 4 – 8 on the other hand doesn´t affect the activity at all, which is very interesting because enzymes are known to be very sensitive to pH.

In Figure 24 and Figure 25 the results of the measurements are displayed.

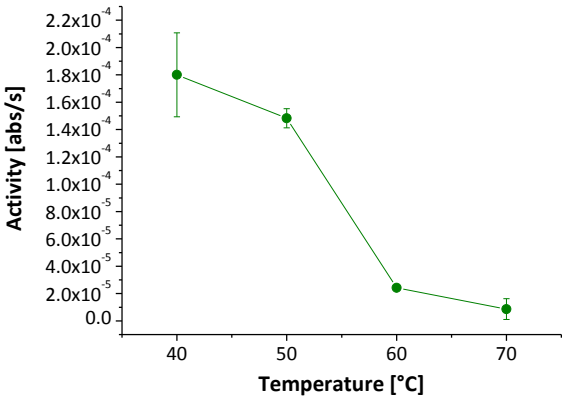


Figure 24: Temperature stability of the tyrosinase from *Agaricus bisporus*

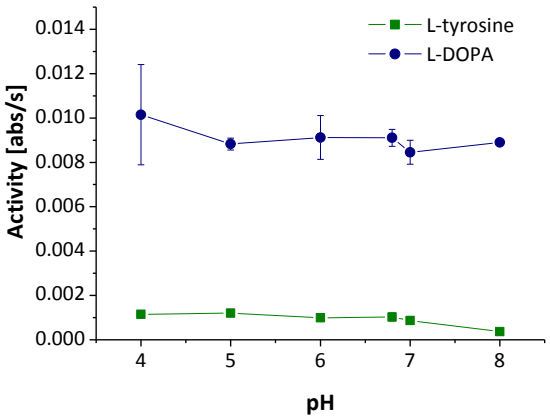


Figure 25: pH stability of the tyrosinase from *Agaricus bisporus*

3.1.3 Oxygen uptake

To calculate the oxygen uptake the oxygen decrease was plotted versus time and the slope of the lines determined. The results are displayed in the figure below.

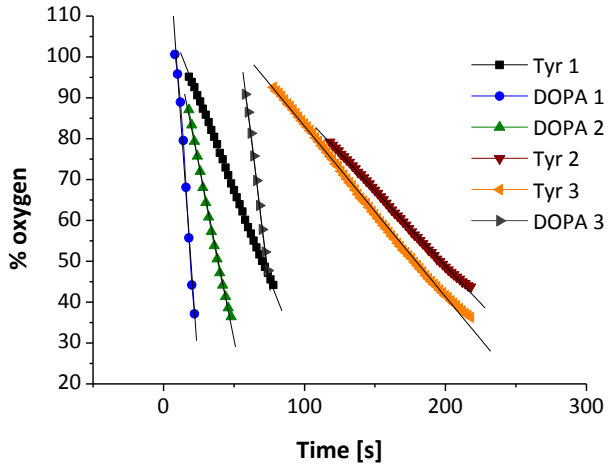


Figure 26: Determination of oxygen consumption in triplicates

The slopes of the lines had the following values:

Table 24: Raw data of oxygen uptake determination

Data	Slope	Average
Tyr 1	-0,9 $\%O_2/s$	-0,5546 ± 0,2814 $\%O_2/s$
Tyr 2	-0,4 $\%O_2/s$	
Tyr 3	-0,4 $\%O_2/s$	
DOPA 1	-4,8 $\%O_2/s$	-3,1168 ± 1,5883 $\%O_2/s$
DOPA 2	-1,7 $\%O_2/s$	
DOPA 3	-2,8 $\%O_2/s$	

Due to the solution being stirred with an open lid for 15 minutes and the oxygen solubility at 25 °C being 8 mg/l, the starting point for the measurement (=100%) can be set at 3,2 ng of oxygen in 400 µl solution. The enzyme concentration in solution is 0,003 g.

Equation 2

$$Oxygen\ consumption\left[mg_{O2}/min * \ g_{enzyme}\right] = \frac{\%\ oxygen\ uptake\ /_{\min} * 0{,}032\ ng\ O_2}{g_{enzyme}}$$

Using Equation 2 the oxygen uptake for L-tyrosine results in being **0,4 ± 0,2 mg O_2/(min*g_{enzyme})** and for L-DOPA **2,0 ± 1,0 mg O_2/(min*g_{enzyme})**.

3.1.4 Discussion

Table 25: Summary of results for enzyme characterization

Substrate	dopachrome Assay U/mg	MBTH Assay U/mg	Oxygen uptake mg O_2/(min*g_{enzyme})
L-tyrosine	2,23	0,77	0,4 ± 0,2
L-DOPA	13,38	6,33	2,0 ± 1,0

Even though Winder et al. [44] report that the MBTH assay is more sensitive than the dopachrome assay, the obtained activities prove otherwise. The results of both methods show, that the monophenolase activity is around 7 times lower than the diphenolase activity as reported in literature [45]. The reason for the lower values obtained with the MBTH-assay could be, that the MBTH-assay is a two-step assay, because not the quinone formed, but the reaction product of the quinone and MBTH is measured. So if MBTH is not present in a sufficient concentration it is the limiting component giving lower values.

The results for the oxygen uptake confirm the results of the activity measurements. About 5 times as much oxygen is consumed per minute when L-DOPA is present as a substrate compared to when L-tyrosine is used.

3.2 Sample preparation

Three types of reactions were performed to prepare the samples for the following enzymatic crosslinking: Chemical denaturation, temperature denaturation and FITC labeling of HSA. The results of the denaturation are presented in this section, but the proof for the successful FITC labeling is given in the enzymatic crosslinking chapter.

3.2.1 Chemical denaturation

Figure 27 and Figure 28 show an example of how the intrinsic fluorescence decreases over time when using GnHCl and urea as denaturing agents over an incubation period of 25 hours. The emission spectra look similar for the two agents used, even the fluorescence intensity is the same. This proves that both GnHCl and urea are equally capable of denaturing HSA.

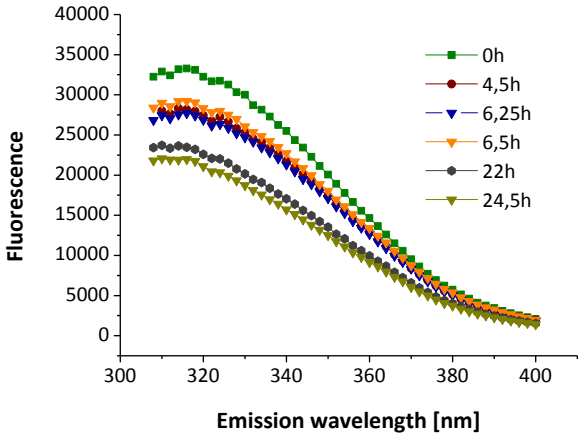


Figure 27: Chemical denaturation with GnHCl

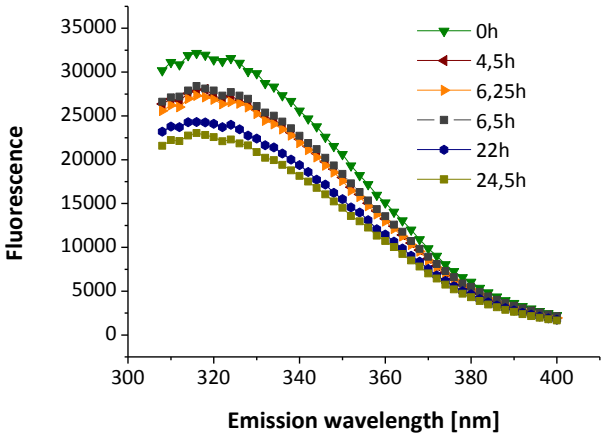


Figure 28: Chemical denaturation with urea

3.2.2 Temperature denaturation

Figure 29 and Figure 30 display the effect of high temperatures on the structure of human serum albumin. To monitor the unfolding process the change of the intrinsic fluorescence was measured. The results don´t show a significant change in the structure, but an overall trend can be observed that the fluorescence activity is decreasing over time with the increase of temperature.

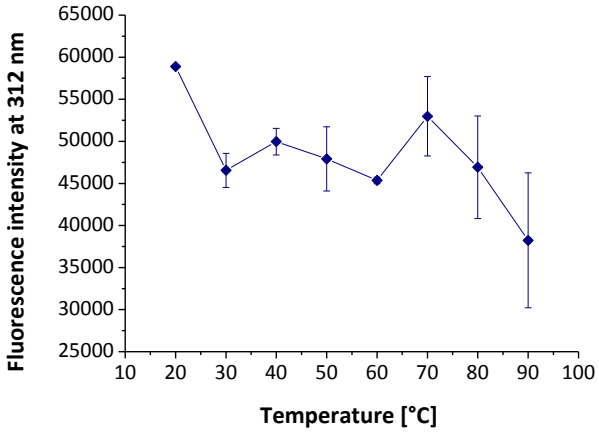


Figure 29: HSA fatty acid free temperature-denaturation profile

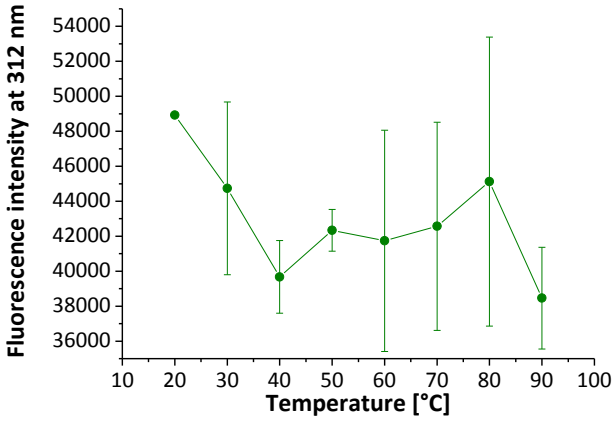


Figure 30: HSA temperature-denaturation profile

Measuring the change of the hydrodynamic diameter of the human serum albumin using dynamic light scattering turned out to be a very good way to monitor the effects of temperature on the structure of the protein. Figure 30 displays the increase of the hydrodynamic diameter with temperature with a significant change between 70 and 90 °C. Cooling the protein solution to 20°C doesn´t affect the hydrodynamic radius, which indicates an irreversible unfolding.

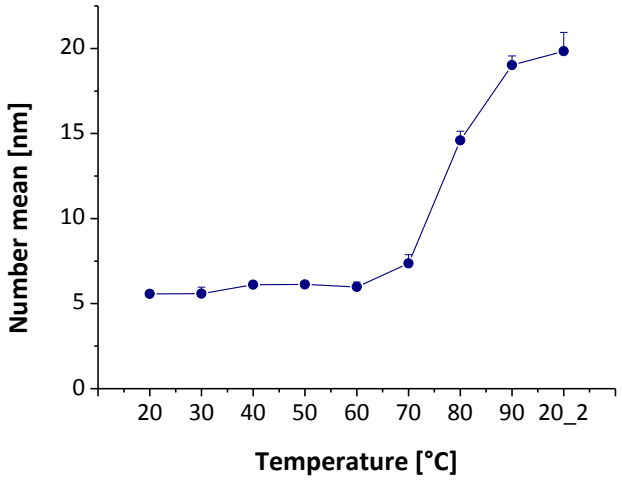


Figure 31: Temperature-denaturation profile of HSA fatty acid free measured on the Zetasizer

3.2.3 Discussion

The chaotropic agents GnHCl and urea are both capable of unfolding HSA at least partially which has also been reported by Ahmad et al. [34]. If the denaturing agents don´t interfere with following reactions where the unfolded HSA is needed, it is a good and easy method to loosen up the structure of HSA. Due to the further use of unfolded HSA for enzymatic crosslinking, the chaotropic agents need to be removed because they would otherwise unfold mushroom tyrosinase. This results in a very time consuming method. There is also no prove that the HSA stays unfolded throughout the purification process.

The temperature denaturation on the other hand needs less incubation time, no possibly interfering compounds and no purification. The results of the Zetasizer also show that HSA stays unfolded even when cooled back down to 20°C. The change of the hydrodynamic diameter is very significant which indicates a big percentage of unfolding which has also been mentioned in literature by Pico et al. [23]. The results obtained indicate that the method of choice to unfold HSA prior to enzymatic crosslinking is the temperature denaturation.

3.3 Enzymatic crosslinking

The results of the enzymatic crosslinking are divided into three parts, starting off with the use of model substrate, followed by using the substrates in native form and closing the chapter with using modified/denatured substrates and linker molecules.

3.3.1 Using model substrate

- **Coupling 1:Using Insulin Chain B oxidized from bovine pancreas as model substrate (2)**

Using Insulin Chain B as a model substrate showed, that mushroom tyrosinase is capable of crosslinking peptides, which indicates that tyrosine residues present in the peptide backbone are accepted as substrates. Noticeable changes can be seen in the wavelength spectra, with new peaks appearing (marked with black arrows) and in the HPLC chromatograms with peaks shifting to higher molecular weights (displayed in Figure 32) Due to low enzyme concentration first changes don´t appear until the time point of 89 hours.

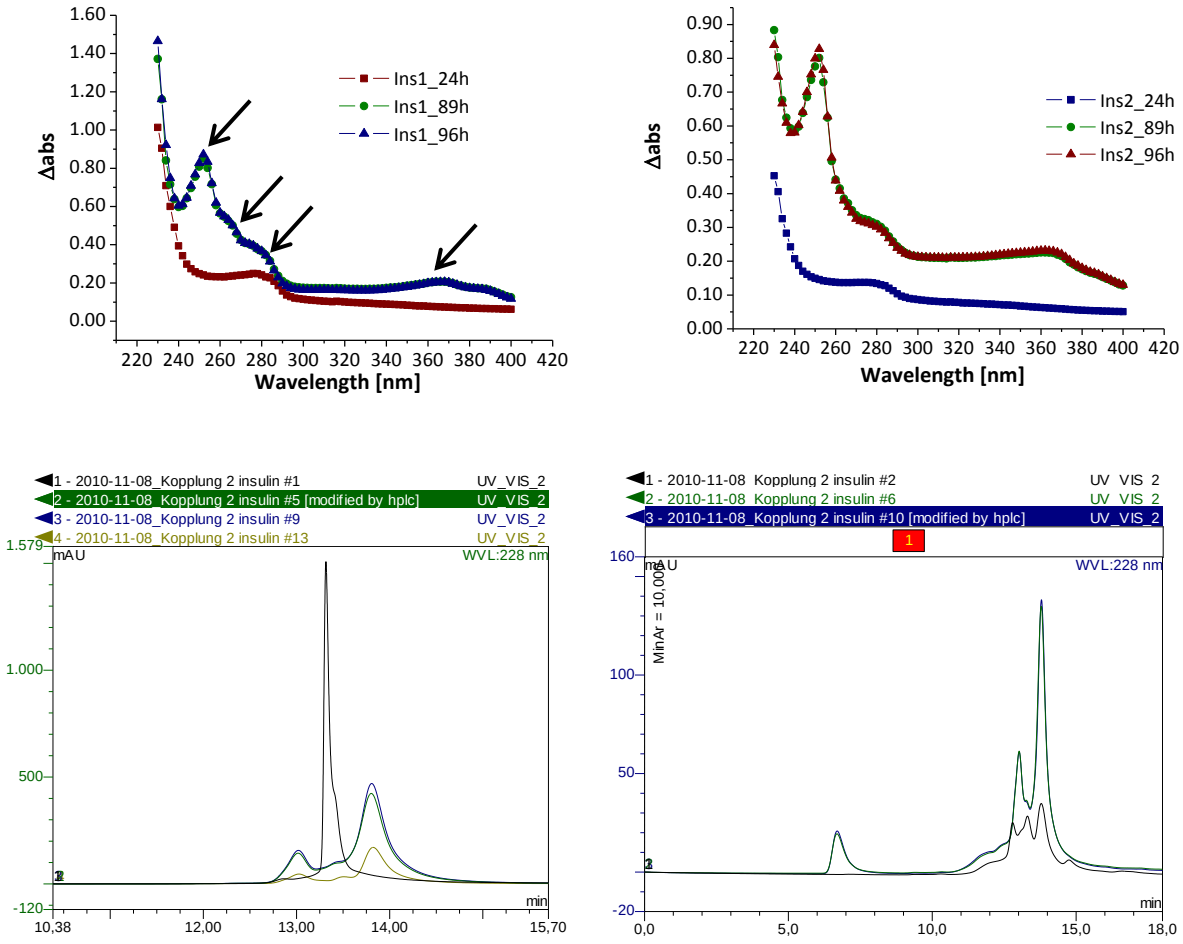


Figure 32: Tyrosinase cross-linking of Insulin Chain B: Wavelength Scans and SEC-HPLC chromatograms (column Zorbax GF 250) of samples Ins 1 (left column) and Ins 2 (right column)

- **Coupling 2: Using Insulin Chain B oxidized from bovine pancreas as model substrate (3)**

Higher enzyme concentrations (6 fold coupling 1) resulted in coupling products after already 48 hours as can be observed in new peaks appearing in Figure 33. The samples Ins 3 and Ins 4 contained the same amount of enzyme that was used as in coupling 1, which didn´t result in coupling products during the reaction time.

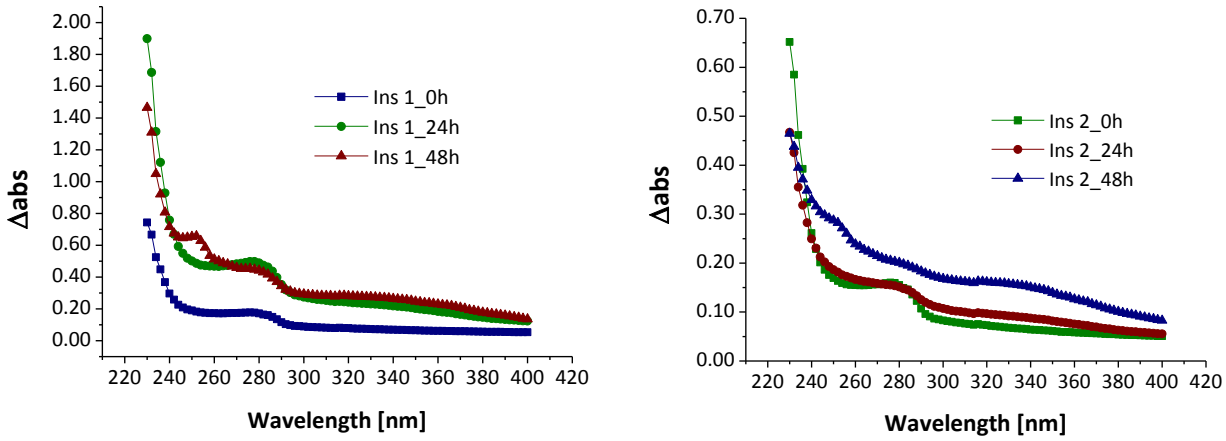


Figure 33: Tyrosinase cross-linking of Insulin Chain B with higher enzyme concentrations: Wavelength Scans of samples Ins 1 (left column) and Ins 2 (right column)

The HPLC chromatograms confirm the results of the platereader with new peaks appearing and peaks shifting to higher molecular weight areas (marked with the black arrow) displayed in Figure 34.

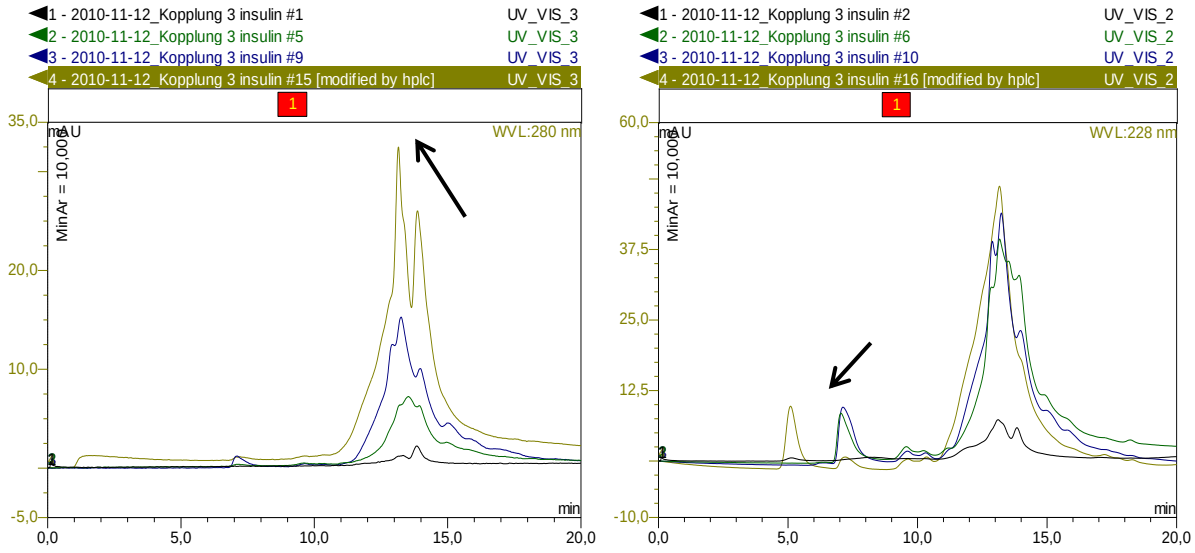


Figure 34: Tyrosine cross-linking of Insulin Chain B with higher enzyme concentrations: SEC-HPLC chromatograms (column Zorbax GF 250) samples Ins1 (left) and Ins 2 (right)

3.3.1.1 Discussion

The successful crosslinking of whey and wheat protein, containing several tyrosine residues, with mushroom tyrosinase has already been reported by Thalmann et al. [20]. Using Insulin Chain B isolated from bovine pancreas as a model substrate confirms the results in the literature with being successfully cross-linked by mushroom tyrosinase. The long reaction time necessary to obtain cross-linking products is probably due to the size of the substrate but can probably be reduced by using higher enzyme concentration. Coupling 1 and 2 have shown that measuring the absorption is a good method to monitor the reaction because the results are very clear. On the other hand it is not possible to say which peak is caused by which compound.

Size exclusion chromatography is very sensitive but the resolution needs to be improved. With this analysis method it is also very difficult to tell how many Insulin Chain B molecules are actually cross-linked.

3.3.2 Using substrates in native form

After successfully crosslinking the model substrate the next step is to test if mushroom tyrosinase can also cope with HSA and mouse IgG1 monoclonal antibody as substrate.

- **Coupling 3: Using HSA as the only substrate (4)**

In coupling 3 no cross-linking products could be obtained under the reaction conditions used. Figure 35 shows the wavelength scans of the sample HSA 1 collected over 72, where no changes can be observed. Figure 36, showing the HPLC chromatogram, confirms the results of the platereader because the retention times for the HSA dimer (9,085 min) and the monomer (9,986 min) don´t shift to higher molecular weight areas over time.

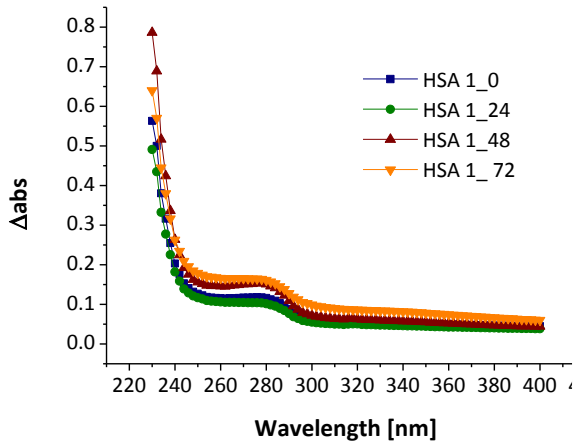


Figure 35: Tyrosinase cross-linking of HSA: Wavelength Scan of sample HSA 1

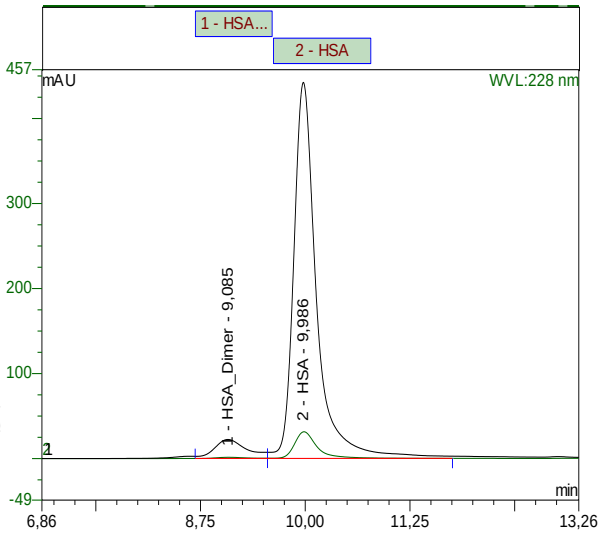


Figure 36: Tyrosinase cross-linking of HSA: SEC-HPLC chromatogram (column Zorbax GF 250) of sample HSA 1

- **Coupling 4: Using Mouse IgG1 monoclonal antibody as the only substrate (8)**

Coupling 4, with mouse IgG1 monoclonal antibody being the only substrate, didn´t result in cross-linking products. Even though Figure 37 shows a slight decrease of % oxygen in solution, in Figure 36 no shifts of the antibody peaks to higher molecular weight areas can be observed. The decrease of oxygen can be explained by the Clark electrode using a certain amount of O_2 for the measurement which especially shows when measuring over a time period of 24 hours.

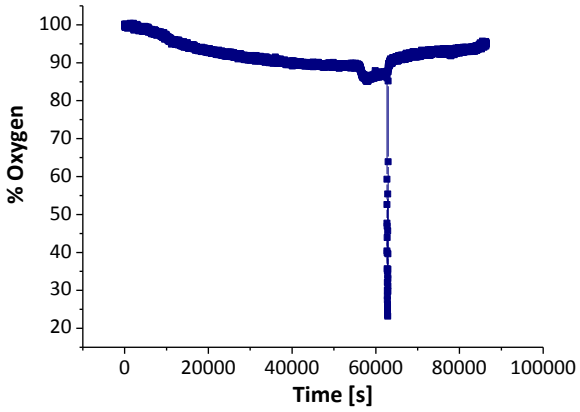


Figure 37: Tyrosinase cross-linking of mouse IgG1 monoclonal antibody: Oxygen decrease of sample AB

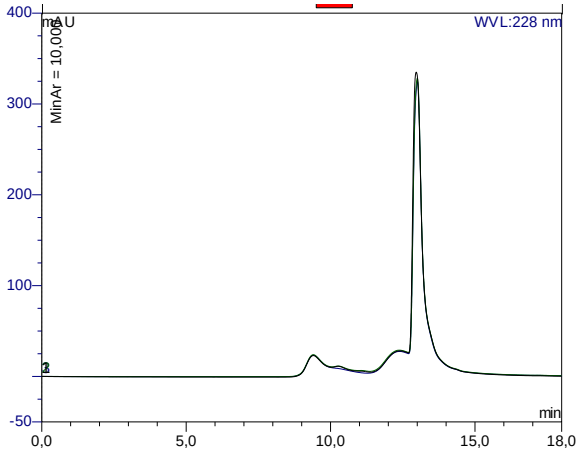


Figure 38: Tyrosinase cross-linking of mouse IgG1 monoclonal antibody: SEC-HPLC chromatogram (column Zorbax GF 250) of sample AB

3.3.2.1 Discussion

Even though coupling 3 and 4 didn´t result in cross-linking products the information obtained is still very valuable. When cross-linking HSA and the mouse AB three types of cross-linking products are possible: HSA-AB, HSA-HSA and AB-AB. After having obtained the above results it can be excluded for following coupling reactions that new peaks or bands appearing are HSA-HSA or AB-AB crosslinks which makes the interpretation a lot easier

3.3.3 Using denatured/modified substrates and linker molecules

Because the use of HSA and AB in their native form didn´t result in cross-linking products new strategies were applied. HSA was denatured and linked with FITC and linker molecules such as Insulin Chain B and phenolic compounds were added to the reaction mixture to facilitate the cross-linking.

- **Coupling 5: Using chemically denatured HSA (10b)**

In Figure 39 and Figure 40 can be observed that using chemically denatured HSA (denaturation time: 24 h) as a substrate resulted in HPLC chromatograms that did not show the characteristic HSA monomer and dimer peaks. In lower molecular weight areas crosslinking seemed to take place but apparently between HSA fragments which occurred throughout the denaturation procedure. In higher molecular weight areas, where cross-linking products would appear, no changes could be observed.

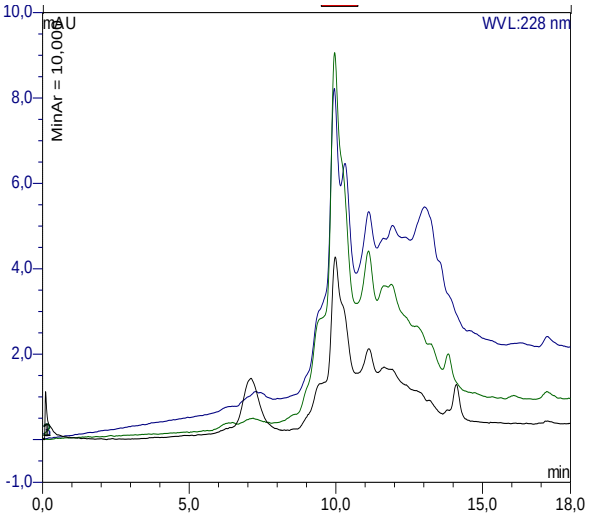


Figure 39: Tyrosinase cross-linking of HSA denatured with GnHCl: SEC-HPLC chromatogram (column Zorbax GF 250)

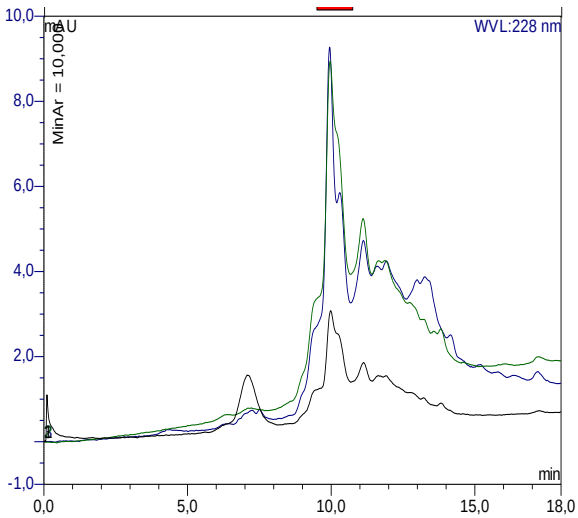


Figure 40: Tyrosinase cross-linking of HSA denatured with Urea: SEC-HPLC chromatogram (column Zorbax GF 250)

- **Coupling 6: Using chemically denatured HSA (11)**

In contrast to coupling 5, in coupling 6 HSA was only incubated with the denaturing agents for 12 hours prior to the actual coupling reaction to avoid the formation of HSA fragments. The decrease of incubation time led to HPLC chromatograms that showed the typical peaks for the HSA monomer and dimer. But again crosslinking of protein fragments occurred and changes in the chromatograms only appeared in smaller molecular weight ranges (retention times 11-15 minutes). In Figure 41 and Figure 42 the HPLC chromatograms of the time points 0, 15 and 18 hours are displayed including Blanks, which are samples that contain chemically denatured HSA after the purification with AEKTA purifier without enzymes.

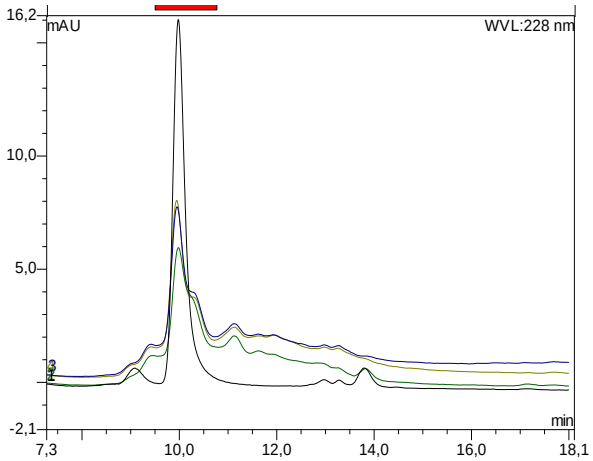


Figure 41: Tyrosinase cross-linking of HSA denatured with GnHCl: SEC-HPLC chromatogram (column Zorbax GF 250) including a blank

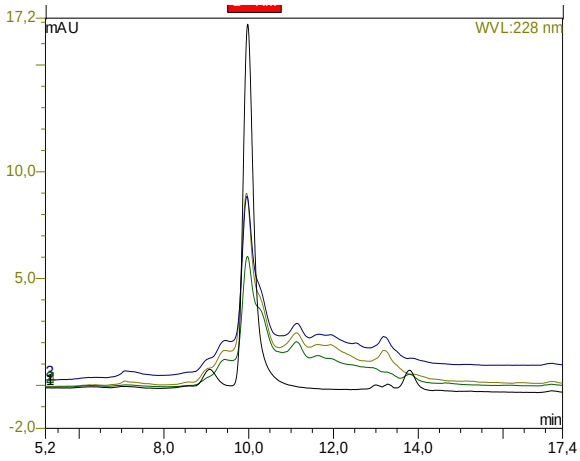


Figure 42: Tyrosinase cross-linking of HSA denatured with urea: SEC-HPLC chromatogram (column Zorbax GF 250) including a blank

The 10 % SDS PAGE shown in Figure 43 confirms the results of the HPLC analysis. The HSA monomer band (orange arrow) has a molecular weight of approximately 55 kDa and the dimer band (red arrow) of approximately 100 kDa. Only below the monomer band changes can be observed, which indicates that protein fragment crosslinking occurred Table 26 contains the samples applied onto the gel.

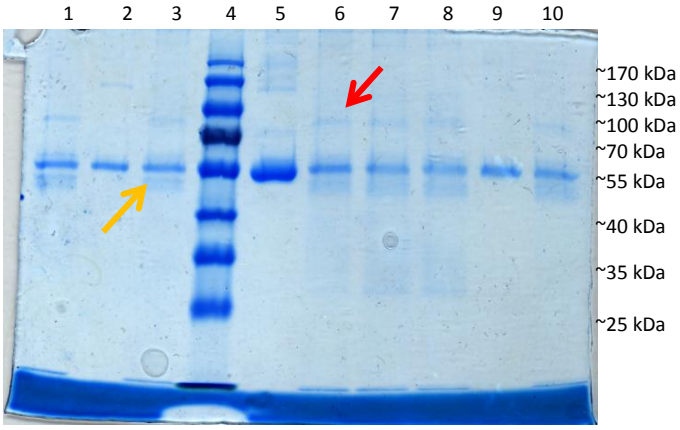


Figure 43: Tyrosinase cross-linking of chemically denatured HSA: 10% SDS PAGE (stained with Coomassie)

Table 26: Samples applied on lanes of 10% SDS gel of coupling 6

Lane	Sample SDS PAGE	Chemically denatured by	Incubation time
1	HSA denatured by urea 0,3 mg/ml		
2	HSA 0h	Urea for 12h	0h
3	HSA 15h	Urea for 12h	15h
4	Fermentas PageRuler Prestained Protein Ladder		
5	HSA 0,5 mg/ml		
6	HSA 18h	Urea for 12h	18h
7	HSA 18h	GnHCl for 12h	18h
8	HSA 15h	GnHCl for 12h	15h
9	HSA 0h	GnHCl for 12 h	18h
10	HSA denatured by GnHCl 0,3 mg/ml		

- **Coupling 7: Using Insulin Chain B as a linker molecule (6)**

Using Insulin Chain B as a linker molecule to crosslink HSA resulted in coupling between Insulin molecules, which is shown in Figure 44 and Figure 45. The HSA monomer and dimer peak stay the same but in the lower molecular weight areas (retention times 11,25-14,66 minutes) new peaks occur.

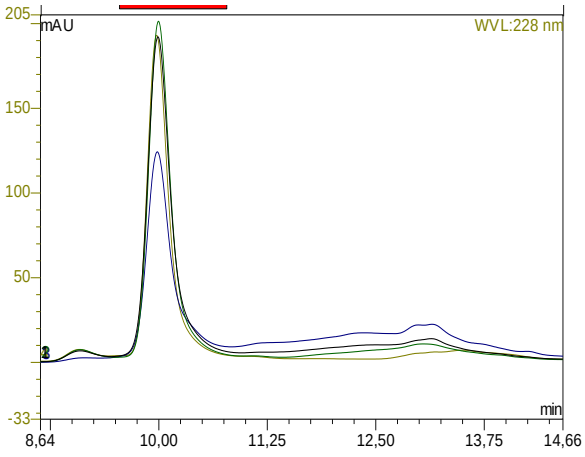


Figure 44: Tyrosinase cross-linking of HSA using Insulin Chain B as a linker molecule: SEC-HPLC chromatogram (column Zorbax GF 250) of Ins 1

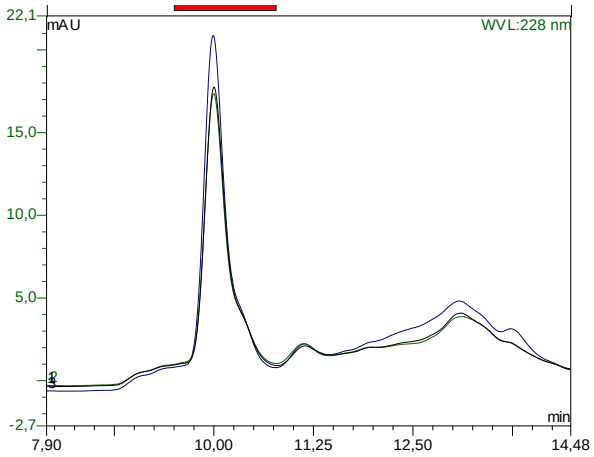


Figure 45: Tyrosinase cross-linking of HSA using Insulin Chain B as a linker molecule: SEC-HPLC chromatogram (column Zorbax GF 250) of Ins 2

The wavelength scans displayed in Figure 46 and Figure 47 show a peak at 280 nm which is the absorption peak of both HSA and Insulin Chain B. The scans were collected over 72 hours but no new peaks could be detected. Even though Insulin Chain B cross-linking occurred, the typical pattern as displayed in Figure 32 cannot be observed due to the low concentrations of Insulin Chain B in the solution.

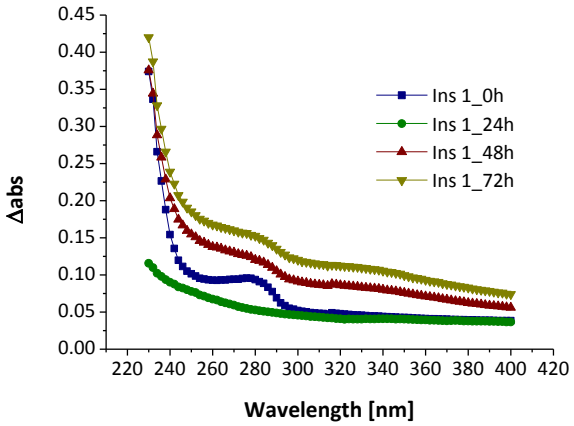


Figure 46: Tyrosinase cross-linking of HSA using Insulin Chain B as a linker molecule: Wavelength Scan of sample Ins 1

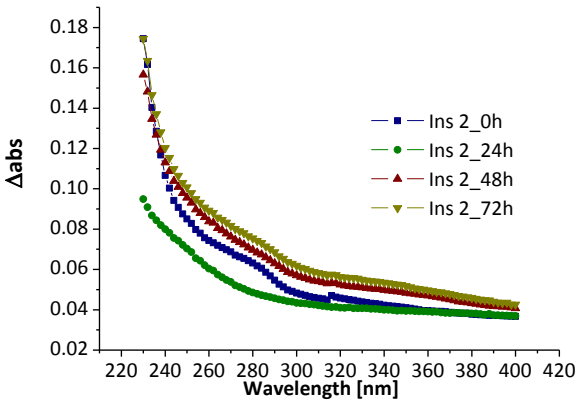


Figure 47: Tyrosinase cross-linking of HSA using Insulin Chain B as a linker molecule: Wavelength Scan of sample Ins 2

- **Coupling 8: Using Insulin Chain B as a linker molecule with activated HSA/AB (9b)**

HSA and AB were incubated with the enzyme before adding Insulin Chain B, to activate the substrates and create higher reactivity. This procedure didn´t lead to the desired HSA-HSA and AB-AB cross-linking products. The HPLC chromatogram of HSA, displayed in Figure 48 and of the AB, displayed in Figure 49, stay identical over time.

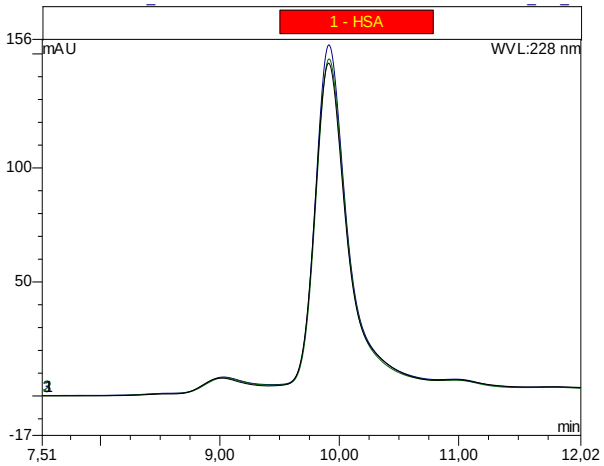


Figure 48: Tyrosinase cross-linking of activated HSA using Insulin Chain B as a linker molecule: SEC-HPLC chromatogram (column Zorbax GF 250)

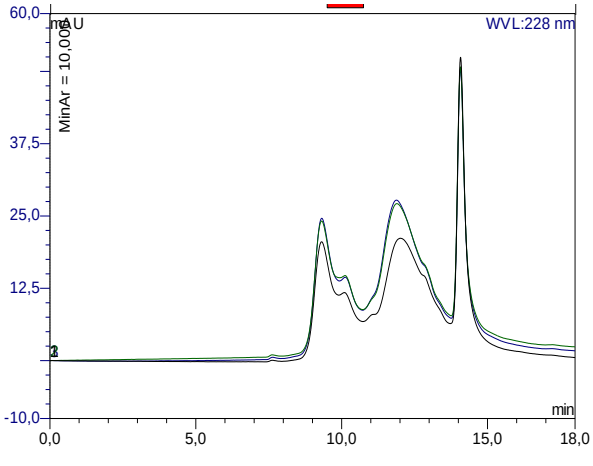


Figure 49: Tyrosinase cross-linking of activated AB using Insulin Chain B as a linker molecule: SEC-HPLC chromatogram (column Zorbax GF 250)

The oxygen content in solution, as shown in Figure 50, is decreasing over time, again due to the oxygen consumption of the Clark electrode but also due to the internal crosslinking of the linker molecule Insulin Chain B. The orange arrow marks the time point when Insulin Chain B was started to be added.

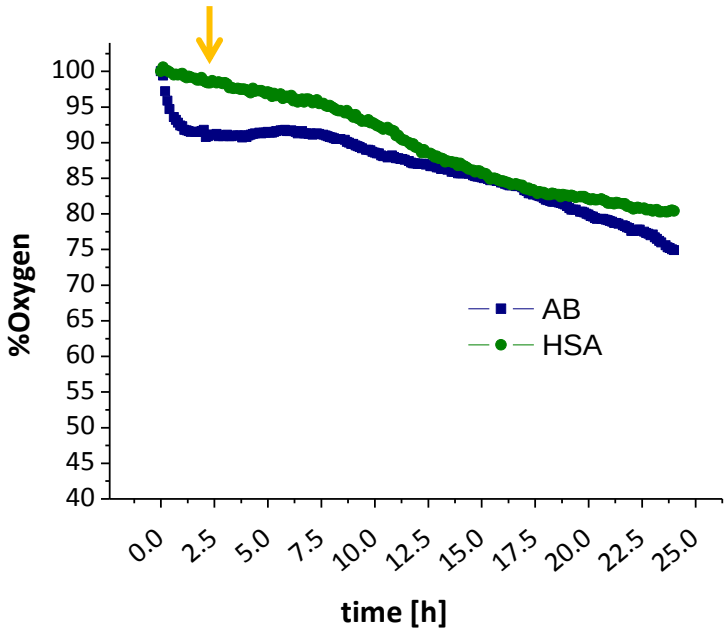


Figure 50: Tyrosinase cross-linking of HSA/AB using Insulin Chain B as a linker molecule: Monitored oxygen consumption over 25 hours

This time also an SDS PAGE analysis was performed. The result, which confirms the outcome of the previously mentioned analysis methods, is displayed in Figure 51. The orange arrow marks the band of the mouse IgG1 monoclonal antibody and the green arrow the band of the human serum albumin monomer. The molecular weights calculated from this gel differ from the ones that can be found in literature, which are 150 kDa for the antibody and 67 kDa for human serum albumin. Insulin chain B cannot be seen on the gel because its molecular weight (3, 4 kDa) is too low. In Table 27 more information is given about which samples were applied onto which lane.

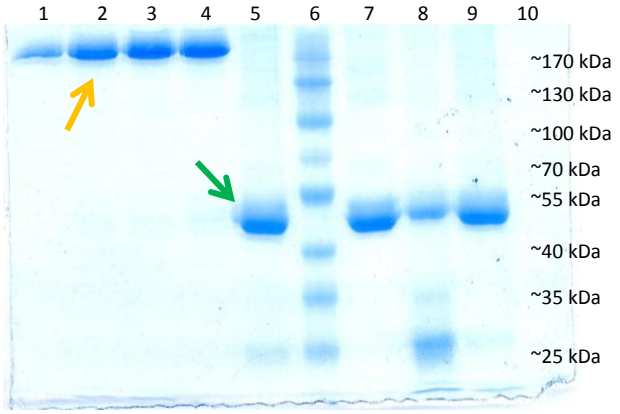


Figure 51: Tyrosinase cross-linking of HSA/AB using Insulin Chain B as a linker molecule: 8% SDS PAGE (stained with KANG)

Table 27: Samples applied on lanes of 10% SDS gel of coupling 8

Lane	Sample SDS PAGE	Activation time	Incubation time
1	Mouse lgG1 0,036 mg/ml		
2	AB 1_24	2h	24h
3	AB 2_24	6h	24h
4	AB 3_24	24h	24h
5	HSA 0,5mg/ml		
6	Fermentas PageRuler Prestained Protein Ladder		
7	HSA 1_24	2h	24h
8	HSA 2_24	6h	24h
9	HSA 3_24	24h	24h
10	Insulin Chain B 0,5 mg/ml		

- **Coupling 9: Using low molecular weight phenolic compounds as linker molecules (15)**

The use of low molecular weight phenolic compounds as linker molecules to obtain HSA-AB cross-linking products brought diverse results. The HPLC chromatograms of the experiment where caffeic acid and p-coumaric acid were used as linker molecules (Figure 52 and Figure 53) show differences between samples taken at 0h (black lines), 18,5 h (blue lines) and 24 h (green lines). The HPLC chromatograms of the experiments where catechol, ferulic acid and guaiacol were used as linker molecules are not shown because no changes could be observed.

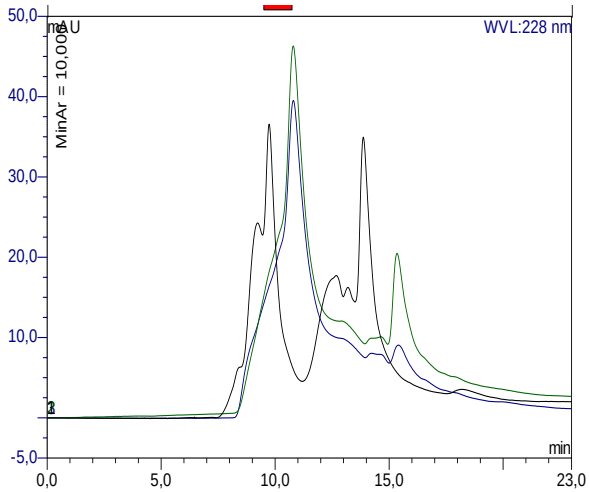


Figure 52: Tyrosinase coupling of HSA and AB using low molecular phenolics as linker molecules: SEC-HPLC chromatogram (column Zorbax GF 250) of caffeic acid-sample

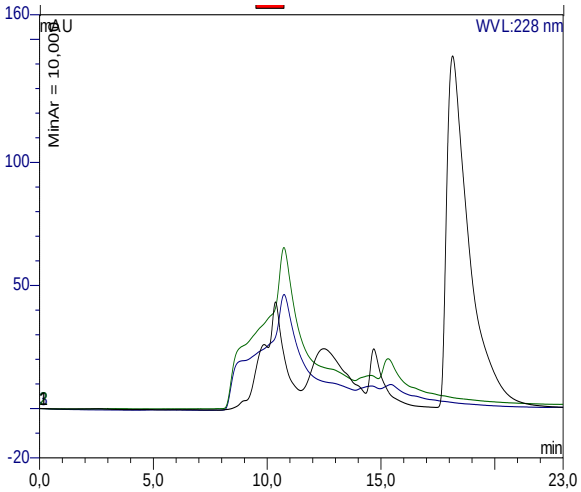


Figure 53: Tyrosinase coupling of HSA and AB using low molecular phenolics as linker molecules: SEC-HPLC chromatogram (column Zorbax GF 250) of p-coumaric acid- sample

SDS PAGE analysis displays the results more clearly. The bands in lanes 2 a, b and 9, marked with the green arrow, show differences to the samples taken at time point 0h shown in lanes 1 and 8. The HSA band at ~ 55 kDa starts to smear and above the original AB band at ~170 kDa new bands appear which proposes that cross-linking products were produced throughout the reaction. Table 28 contains the samples applied onto the gel. The descriptions printed in Italic are lanes that have no relevance for the described coupling reaction.

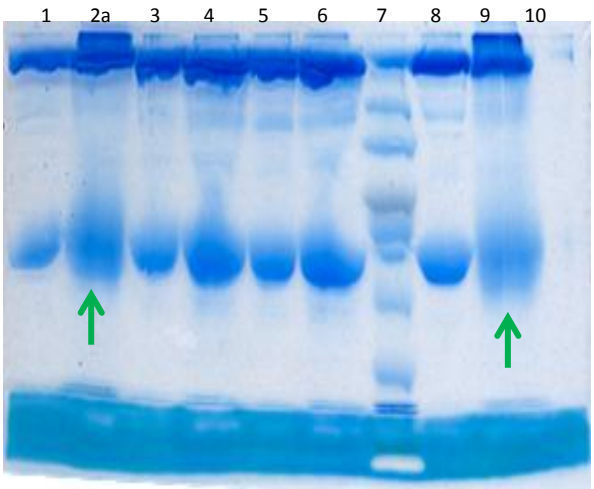


Figure 54 Tyrosinase coupling of HSA and AB using low molecular phenolics as linker molecules: 8% SDS PAGE 1 (stained with KANG)

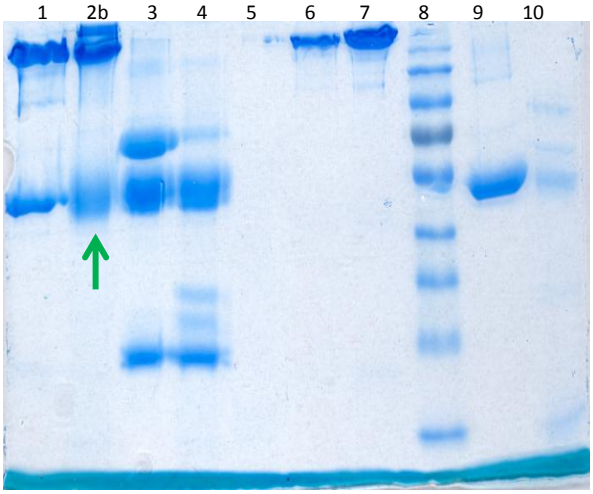


Figure 55: Tyrosinase coupling of HSA and AB using low molecular phenolics as linker molecules: 8% SDS PAGE 2 (stained with KANG)

Table 28: Samples applied on lanes of 8% SDS gels of coupling 9

Lane	Sample SDS PAGE 1	Linker molecule	Sample SDS PAGE 2	Linker molecule	Incubation time
1	AB + HSA 0h	Caffeic acid 2mM	AB + HSA 0h	p-coumaric acid 2 mM	0h
2 a/b	AB+ HSA 24h	Caffeic acid 2mM	AB + HSA 24h	p-coumaric acid 2 mM	24h
3	AB + HSA 0h	Ferulic acid 2 mM	*DTT 0h*		0h
4	AB + HSA 24h	Ferulic acid 2 mM	*DTT 24h*		24h
5	AB+ HSA 0h	Guaiacol 2 mM	FAF 99 2 mg/ml		0h
6	AB+ HSA 24h	Guaiacol 2 mM	AB 0,36 mg/ml		24h
7	Fermentas PageRuler Prestained Protein Ladder		AB 0,9 mg/ml		
8	AB+ HSA 0h	catechol 2 mM	Fermentas PageRuler Prestained Protein Ladder		0h
9	AB+ HSA 24h	catechol 2 mM	HSA 0,5 mg/ml		24h
10	HSA 0,5 mg/ml		Mushroom tyrosinase 1 mg/ml		

- **Coupling 10: Changing concentration of low molecular weight phenolic compounds (16)**

To check whether the linker molecule concentration is the limiting component in the reaction, the concentration was doubled. This change did slightly increase the product formation as can be observed in lanes 2 a/b and 4 a/b (marked with the green arrow) in Figure 56 and Figure 57. HSA essentially fatty acid free was used for the first time because the more purified protein could enhance the coupling product formation. The orange arrows show the SDS PAGE profiles of the protein variants. Table 29 contains the sample information.

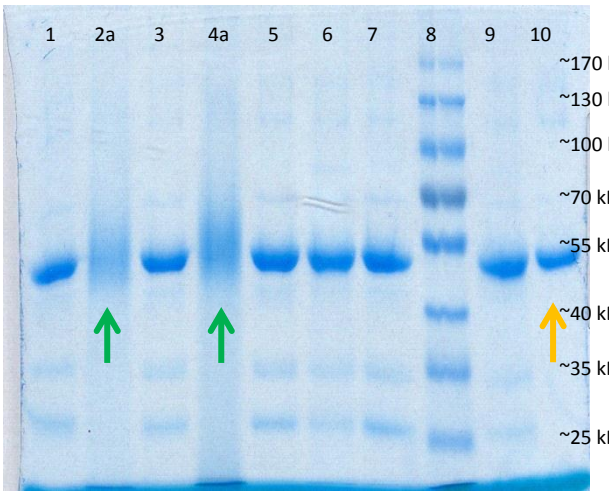


Figure 56: Tyrosinase coupling of HSA and AB using 4 mM of low molecular phenolics as linker molecules:8% SDS PAGE 1 (stained with KANG)

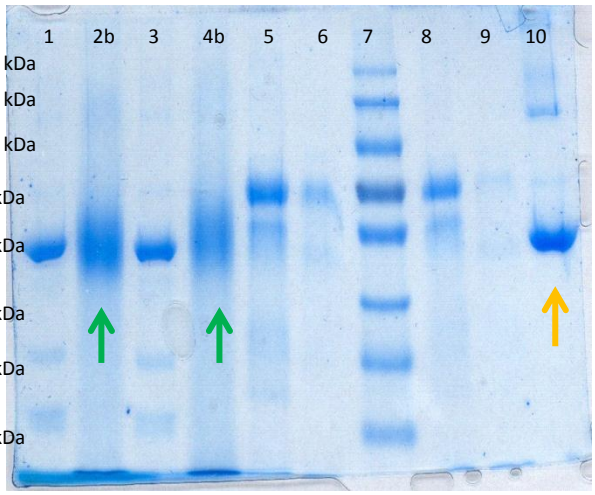


Figure 57: Tyrosinase coupling of HSA and AB using 4 mM of low molecular phenolics as linker molecules: 8% SDS PAGE 2 (stained with KANG)

Table 29: Samples applied on lanes of 8% SDS gels of coupling 10

Lane	Sample SDS PAGE	Linker molecule	Sample SDS PAGE 2	Linker molecule	Incubation time
1	HSA FAF 0h	caffeic acid 2 mM	HSA FAF p-coumaric acid 2 mM 0h	p-coumaric acid 2 mM	0h
2a/b	HSA FAF 24h	caffeic acid 2 mM	HSA FAF p-coumaric acid 2 mM 24h	p-coumaric acid 2 mM	24h
3	HSA FAF 0h	caffeic acid 4 mM	HSA FAF p-coumaric acid 4 mM 0h	p-coumaric acid 4 mM	0h
4a/b	HSA FAF 24h	caffeic acid 4 mM	HSA FAF p-coumaric acid 4 mM 24h	p-coumaric acid 4 mM	24h
5	HSA FAF 0h	guaiacol 2 mM	*HSA FAF DTT 20 mM 0h*		0h
6	HSA FAF 24h	guaiacol 2 mM	*HSA FAF DTT 20 mM 24h*		24h
7	HSA FAF 0h	guaiacol 4 mM	Fermentas PageRuler Prestained Protein Ladder		0h
8	Fermentas PageRuler Prestained Protein Ladder		*HSA FAF DTT 10 mM 0h*		
9	HSA FAF 24h	guaiacol 4 mM	*HSA FAF DTT 10 mM 24h*		24h
10	HSA 0,5 mg/ml		HSA FAF 1 mg/ml		

- **Coupling 11: Elucidation of compositions of new bands on gel (18)**

Coupling 11 was performed to find out more about the new bands appearing on the SDS gel. It did result in more details when coupling products are formed but didn´t reveal if it was a homo- (AB-AB) or heteromolecular (HSA-AB) coupling. To explain it in more detail the example of p-coumaric acid shown in Figure 59 is used. The homomolecular coupling of HSA leads to a smear in the same molecular weight range (red arrow). All the other substrate combinations (green arrows) give the same pattern, which is various bands above around 170 kDa, but it is still not possible to tell if HSA and AB are being cross-linked or if it is just a homomolecular coupling of the AB. The results of the coupling with caffeic acid which don´t differ from p-coumaric acid are shown in Figure 58. Table 30 explains which samples were applied onto which gel.

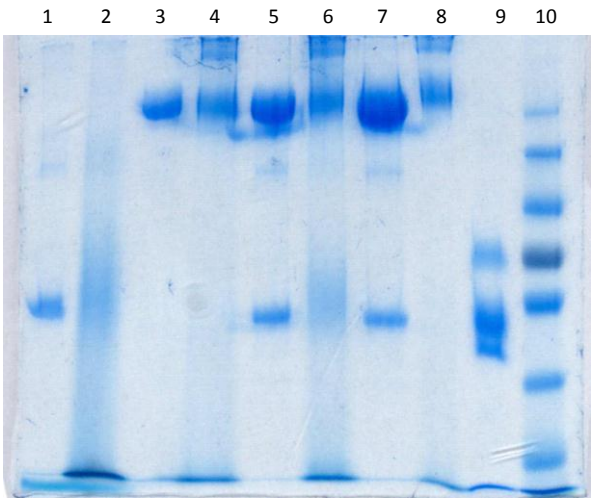


Figure 58: Tyrosinase coupling of HSA and AB using low molecular phenolics as linker molecules: 7,5% SDS PAGE 1 (stained with KANG)

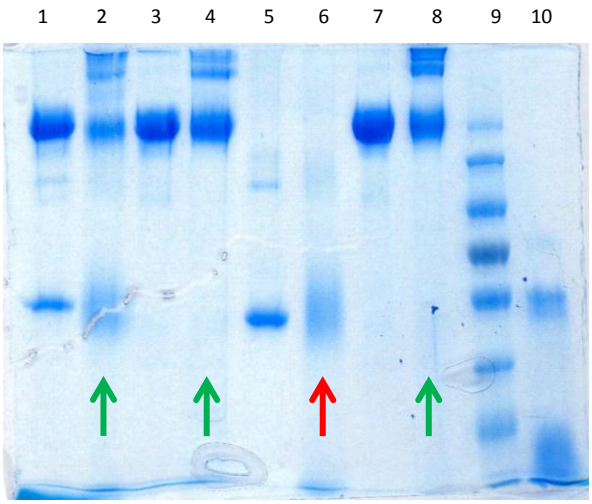


Figure 59: Tyrosinase coupling of HSA and AB using low molecular phenolics as linker molecules: 7,5% SDS PAGE 2 (stained with KANG)

Table 30: Samples applied on lanes of 7,5% SDS gels of coupling 11

Lane	Sample SDS PAGE 1	State	Sample SDS PAGE 2	State	Incubation time
1	HSA FAF CA 0	Native	HSA FAF + AB PCA 0	native	0h
2	HSA FAF CA 24	native	HSA FAF + AB PCA 24	native	24h
3	AB CA 0	native	HSA FAF den + AB PCA 0	denatured by temperature	0h
4	AB CA 24	native	HSA FAF den + AB PCA 24	denatured by temperature	24h
5	HSA FAF + AB CA 0	native	HSA FAF PCA 0	native	0h
6	HSA FAF + AB CA 24	native	HSA FAF PCA 24	native	24h
7	HSA FAF den + AB CA 0	denatured by temperature	AB PCA 0	native	0h
8	HSA FAF den+ AB CA 24	denatured by temperature	AB PCA 24	native	24h
9	*DTT 24*		Fermentas PageRuler Prestained Protein Ladder		
10	Fermentas PageRuler Prestained Protein Ladder		*DTT 24*		

Because the 7,5 % SDS gel still didn´t bring the desired resolution, selected samples were applied onto a 6% SDS gel displayed in Figure 60 with the samples being listed in Table 31. Also HSA denatured by temperature was used as a substrate for coupling 11, but the results are the same as for the non-denatured substrates. The only difference is that precipitates form throughout the reaction, which indicates that bigger compounds are formed which are not soluble anymore.

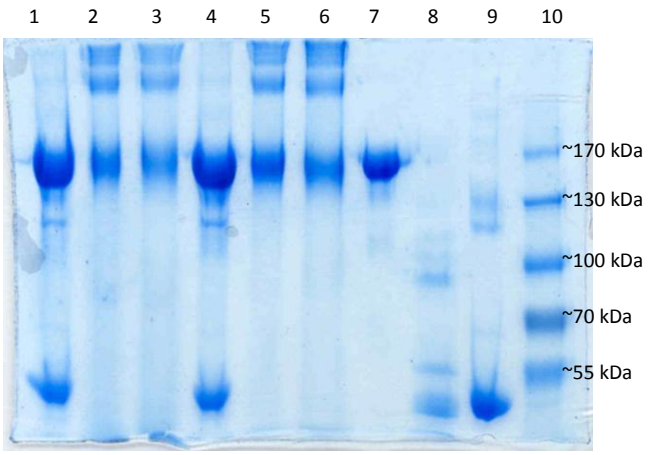


Figure 60: Tyrosinase coupling of HSA and AB using low molecular phenolics as linker molecules: 6% SDS PAGE 3 (stained with KANG)

Table 31: Samples applied on lanes of 6% SDS gels of coupling 11

Lane	Sample SDS PAGE 3	State	Incubation time
1	HSA FAF den +AB CA 0	denatured by temperature	0h
2	HSA FAF den +AB CA 48	denatured by temperature	48h
3	AB CA 48	native	48h
4	HSA FAF +AB PCA 0	native	0h
5	HSA FAF den +AB PCA 48	denatured by temperature	48h
6	AB PCA 0		
7	AB 0,54 mg/ml		
8	tyrosinase 1 mg/ml		
9	HSA 1 mg/ml		
10	Fermentas PageRuler Prestained Protein Ladder (SMO 0671)		

- **Coupling 12: Using FITC labelled HSA for better analysis (20)**

The first coupling where HSA labelled with FITC was used to obtain more information about how the substrates are cross-linked brought useful results. The samples were applied onto a 6% SDS gel displayed in Figure 61 with Table 32 giving more information about the samples. This time a new molecular weight marker was used that covers a range from 300-40 kDa.

On this high resolving gel differences can be observed between the sample where HSA was used in the native form and the one where HSA was denatured with temperature prior to the enzymatic treatment. Lane 2, marked with the green arrow, shows three defined bands above the AB band whereas lane 5, marked with the red arrow, shows faded bands between 180 and 300 kDa, but the thickest band right at the start of the gel. This indicates that when using denatured HSA as a substrate big cross-linking products (>>300 kDa) or even coagulates are formed, which is undesired, because the perfect coupling product would be a single HSA molecule linked with a single AB molecule.

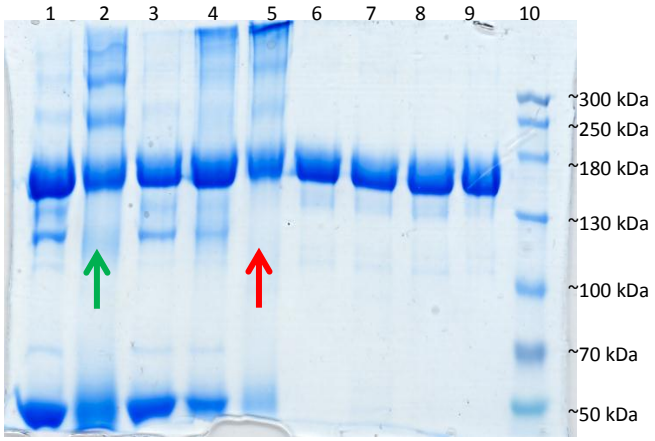


Figure 61: : Tyrosinase coupling of HSA and AB using FITC labeled HSA: 6% SDS PAGE 1 (stained with KANG)

Table 32: Samples applied on lanes of 6% SDS gel of coupling 12

Lane	Sample SDS gel	State	HSA/AB ratio	Incubation time
1	HSA FAF + AB_0h	native	1:3	0h
2	HSA FAF + AB_22h	native	1:3	22h
3	HSA FAF + AB_22h	native	1:4	22h
4	HSA FAF den + AB_0	denatured by temperature	1:4	0h
5	HSA FAF den + AB_22h	denatured by temperature	1:4	22h
6	*9_0*			
7	*9_22h*			
8	*10_22h*			
9	*11_22h*			
10	Spectra Multicolor High Range Protein Ladder (SM 1851)			

To achieve even better resolution a 4%/6% gradient SDS gel was casted. Figure 63 displays the gel after it was stained with KANG staining. Prior to staining it was scanned with a fluorescence scanner where the image displayed in Figure 62 was obtained. Table 33 contains information about the samples applied onto the gel. The gel didn´t run ideally but the bands with a high molecular weight were still well resolved and the fluorescence scan showed that FITC didn´t lose its fluorescent activity after the prior treatment. New insights on how the gel should be treated before fluorescence were gained which are that the dye front needs run off completely because it interferes with the imaging process and that acidic fixation of the gel decreases the fluorescence intensity of FITC to a minimum due to its pH sensitivity.

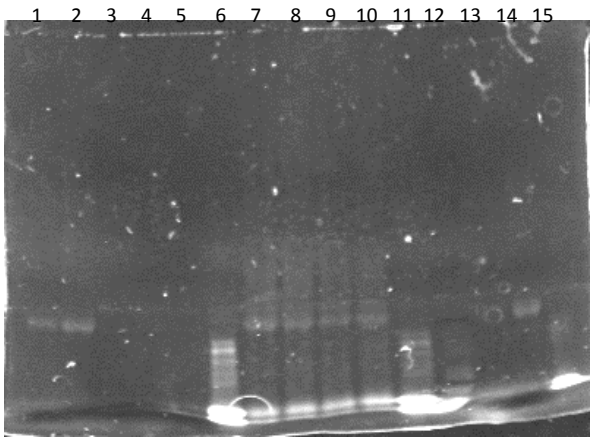


Figure 62: : Tyrosinase coupling of HSA and AB using FITC labeled HSA: Fluorescence Scan of 4%/6% SDS PAGE 2

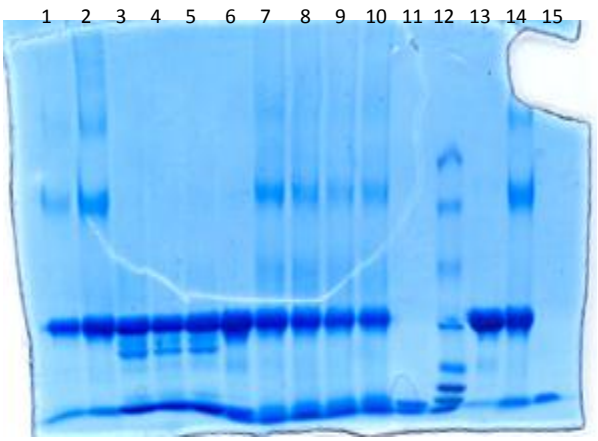


Figure 63: : Tyrosinase coupling of HSA and AB using FITC labeled HSA: 4%/6% Gradient SDS PAGE 2 (stained with KANG)

Table 33: Samples applied on lanes of gradient SDS gel of coupling 12

Lane	Sample SDS gel	HSA/AB ratio	Incubation time
1	*11_24*		
2	AB_22h		22h
3	*12_0*		
4	*12_24*		
5	*13_24*		
6	HSA FITC + AB_0h	1:2	0h
7	HSA FITC + AB_22h	1:2	22h
8	HSA FITC + AB_22h	1:3	22h
9	HSA FITC + AB_22h	1:4	22h
10	HSA FITC + AB_22h	1:4	22h
11	FITC FAF 1mg/ml		
12	Spectra Multicolor High Range Protein Ladder (SM 1851)		
13	AB_0h		0h
14	AB_22h		22h
15	FITC FAF 1mg/ml		

- **Coupling 13: Changing the molar ration of HSA/AB (21)**

Coupling 13 had the goal of obtaining a SDS gel and a fluorescent scan of higher quality to gain more information about the new bands as well as the investigation of how the change of the HSA: AB molar ratio and the drop wise addition of linker would affect the coupling product formation.

Due to the high sensitivity of fluorescence, the linkage of HSA to FITC prior to the coupling reaction resulted in important information. First of all a band (red arrow) can be seen at approximately 220 kDa on the fluorescence scan, that is only slightly visible with the KANG staining, but is very likely to be the desired coupling product. Additionally coupling 13 revealed that the HSA/AB ratio 2:1 (green arrows) results in the highest concentration of coupling product formed and that extending the reaction time to 24 hours doesn´t increase product formation. The drop wise addition of caffeic acid didn´t change the amount of product formed (orange arrow). In Figure 64 and Figure 65 the SDS PAGE and the fluorescence scan containing all these results are displayed in Table 34 giving additional information on which samples were applied.

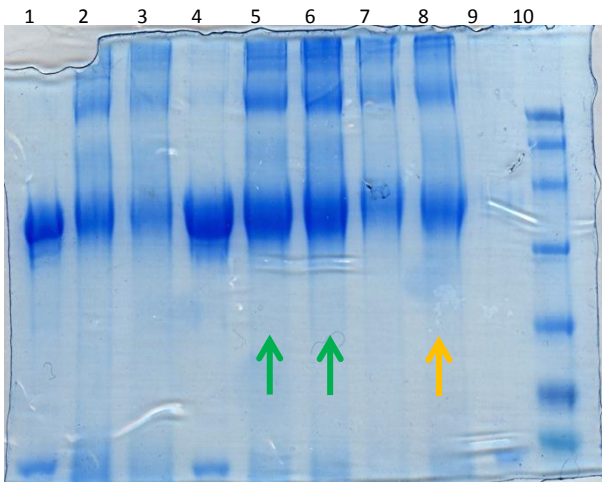


Figure 64: Tyrosinase coupling of HSA and AB using different HSA/AB ratios: 6% SDS PAGE 1 (stained with KANG)

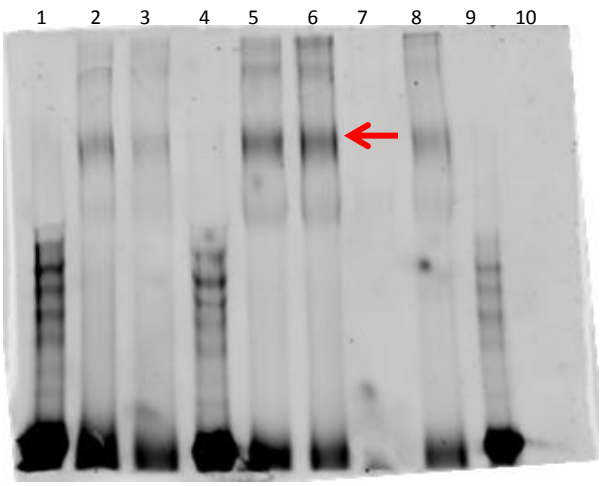


Figure 65: Tyrosinase coupling of HSA and AB using different HSA/AB ratios: Fluorescence scan of 6% SDS PAGE 1

Table 34: Samples applied on lanes of 6% SDS gel of coupling 13

Lane	Sample SDS gel	HSA/AB ratio	Incubation time
1	FAF FITC + AB 0h	5:1	0h
2	FAF FITC + AB 24h	5:1	24h
3	FAF FITC + AB 48h	5:1	48h
4	FAF-FITC + AB 0h	2:1	0h
5	FAF FITC + AB 24h	2:1	24h
6	FAF FITC + AB 48h	2:1	48h
7	AB 48h		
8	FAF FITC+ AB 48h	5:1	48h
9	HSA FITC 0,5mg/ml		
10	Spectra Multicolor High Range Protein Ladder		

Figure 66 shows a 5, 5/6 % gradient gel with defined bands and a good resolution. The HSA band is still visible on the gel but at the same time the product bands can be seen individually. Figure 67 shows a comparison of both the SDS gel and the fluorescence scan. The green frame marks the band that is only visible with fluorescence.

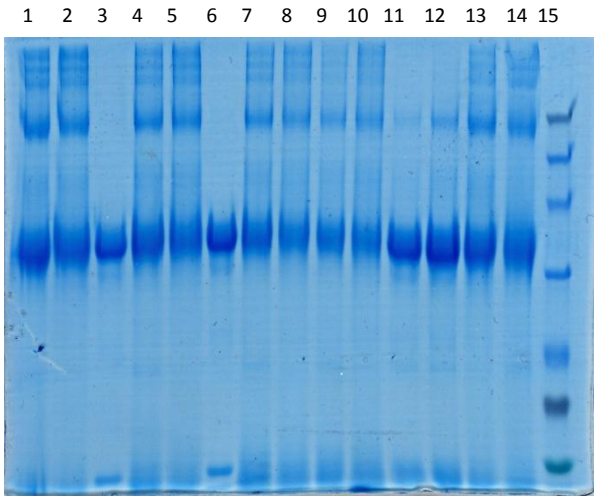


Figure 66: Tyrosinase coupling of HSA and AB using different HSA/AB ratios: 5,5/6% gradient SDS PAGE 1 (stained with KANG)

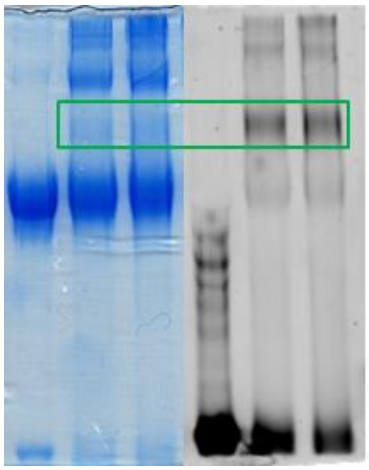


Figure 67: Tyrosinase coupling of HSA and AB using different HSA/AB ratios: Comparison of the bands visible on the stained gel and on the fluorescence scan

Table 35: Samples applied on lanes of gradient SDS gel of coupling 13

Lane	Sample SDS gel	HSA/ AB ratio	Remarks/incubation time
1	FAF FITC + AB 24	2:1	24h
2	FAF FITC + AB 48	2:1	48h
3	FAF FITC + AB 0	3:1	0h
4	FAF FITC + AB 24	3:1	24h
5	FAF FITC + AB 48	3:1	48h
6	FAF FITC + AB 0	4:1	0h
7	FAF FITC + AB 24	4:1	24h
8	FAF FITC + AB 48	4:1	48h
9	FAF FITC + AB 24	5:1	24h
10	FAF FITC + AB 48	5:1	48h
11	FAF FITC + AB CA drops 17	5:1	10x addition of 2µl of caffeic acid/ 17h
12	FAF FITC + AB CA drops 48	5:1	10x addition of 2µl of caffeic acid/ 48h
13	AB 48		48h
14	AB 48		48h
15	Spectra Multicolor High Range Protein Ladder (SM 1851)		

- **Coupling 14: Display all possibilities on one gel (22)**

Coupling 14 gave the final proof that the desired product was only formed when both HSA and AB were present. All possible substrate combinations were applied onto one SDS PAGE displayed in Figure 68 with the corresponding fluorescence scan shown in Figure 69. Table 36 contains the samples applied onto the gel.

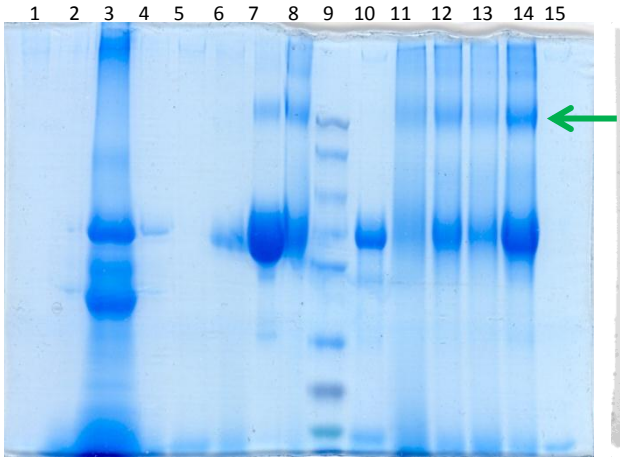


Figure 68: Tyrosinase coupling of HSA and AB: 5,5/6% gradient SDS PAGE 1 (stained with KANG)

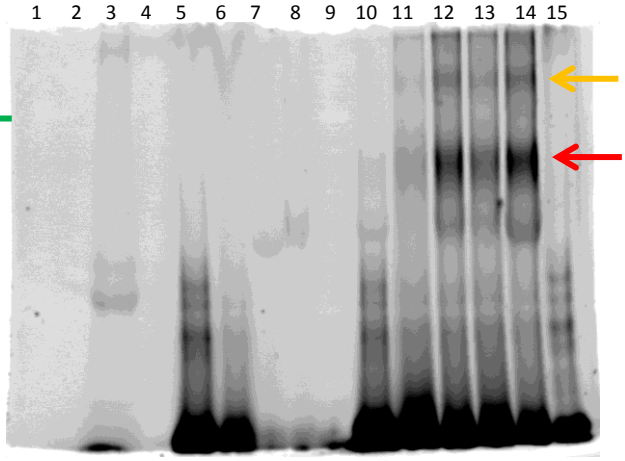


Figure 69: Tyrosinase coupling of HSA and AB: Fluorescence Scan of gradient SDS PAGE 1

Figure 70 displays an overlay of the fluorescence scan and the SDS PAGE stained with KANG. It shows once again that HSA-AB coupling band (red arrow) can only be seen on the fluorescence scan.

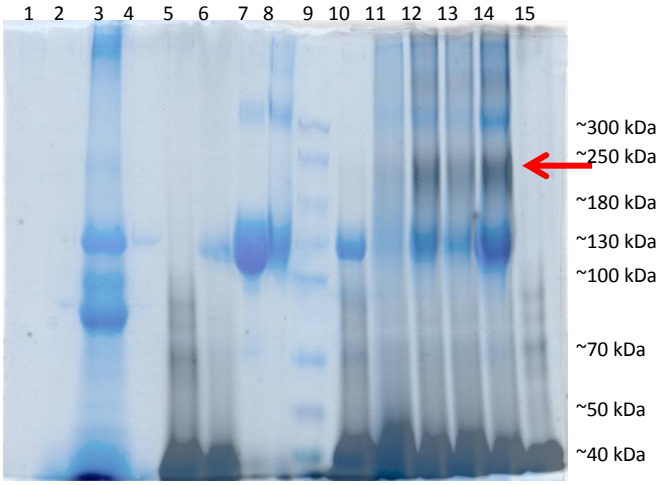


Figure 70: Tyrosinase coupling of HSA and AB: overlay of fluorescence scan and KANG staining of the gradient SDS gel 1

Table 36: Samples applied on lanes of gradient SDS gel of coupling 14

Lane	Sample SDS PAGE	Incubation time	Remarks
1	*TAM 1*		
2	*TAM 2*		
3	*TAM 3*		
4	*TAM 4*		
5	HSA FITC Blank 48	48h	no enzyme added
6	HSA FITC 48	48h	
7	AB Blank 48	48h	no enzyme added
8	AB 48	48h	
9	Spectra Multicolor High Range Protein Ladder		
10	HSA FITC + AB Blank 48	48h	no enzyme added
11	HSA FITC + AB 8:1 48	48h	HSA/AB ratio 8:1
12	HSA FITC + AB 4:1 48	48h	HSA/AB ratio 4:1
13	HSA FITC + AB 6:1 48	48h	HSA/AB ratio 6:1
14	HSA FITC preincubated 48	48h	HSA FITC was preincubated with CA and AbT for 3h
15	HSA FITC 0,8 mg/ml		

3.3.4 Discussion

Measuring the change of the intrinsic fluorescence has proven that some sort of unfolding is taking place when incubating HSA with GnHCl and urea. On the contrary coupling 5 and 6 showed that HSA is either refolding during the purification process or that the structure of HSA is still not accessible enough for mushroom tyrosinase. The 24 hour incubation period apparently destroys the characteristic structure of human serum albumin and 12 hours don´t have enough effect. Ahmad et al. [34] report that GnHCl induced unfolding is reversible. As the purification and concentration steps nearly take 2 hours, there is enough time for HSA to regain its original folding state. It is not an option for the chaotropic agent to remain in solution because they also attack the structure of mushroom tyrosinase. This leads to the conclusion that the chemical denaturation is not suitable for this kind of application. The only possible way would be to irreversibly unfold human serum albumin with very high concentrations of denaturing agents, which is not desired because the protein should more or less keep its original folding characteristic and only open up its structure a little bit.

Due to the chemical denaturation not bringing the desired results the next step was to use linker molecules. The idea behind using Insulin Chain B as a linker molecule was that mushroom tyrosinase would oxidize the small peptide and that the o-quinone would further react with amino and sulfhydryl groups of HSA and AB. To avoid the intermolecular crosslinking of Insulin Chain B, it was added drop wise and in very low concentrations, to keep the probability of two Insulin Chain B molecules meeting and reacting to a minimum.
The results of coupling 7 and 8 show that the affinity of mushroom tyrosinase towards Insulin Chain B is much greater than towards the larger substrates. Even with the preactivation of HSA and AB no HSA-HSA and AB-AB cross-linking products could be obtained. This indicates that both substrates, additionally to being very big, have no side chains sticking out that contain reactive amino acid residues accessible for Insulin Chain B. Another reason for the results could be that Insulin Chain B is too big to act as a linker molecule because it cannot interact that easily with AB or HSA molecules.

With the knowledge that Insulin Chain B is not able to react with either human serum albumin or the mouse IgG1 monoclonal antibody another concept described by Thalmann et al. [20] was used. They report that the use of low molecular weight phenolic compounds enables the enzymatic cross-linking of substrates, which are not accepted by mushroom tyrosinase in their native form. Coupling 9 and 10 confirm those results but only for the linker molecules caffeic acid, p-coumaric acid and catechol. This is interesting because all 5 linker molecules are accepted by mushroom tyrosinase which can be easily seen by the change of colour of the reaction mixture to different red and brown shades around thirty minutes into reaction time.

Interestingly the two phenolic compounds guaiacol and ferulic acid, whose use didn´t result in cross-linking products, are the ones that carry an ether group in ortho position of the hydroxyl group, which could be the reason for the lack of reactivity with amino acid residues of the protein due to steric hindrance. Another explanation could be that the three phenolics, that successfully link the two substrates, oligomerise after being oxidized. This would increase the length of the molecule resulting in a better accessibility.

When human serum albumin is used as the only substrate, it doesn´t result in a defined new band in higher molecular weight areas on the SDS gel but in a smear in the range of approximately 50-65 kDa. This derives from a different number of phenolic compounds linking to the protein creating a narrow size distribution. That proves on the one hand that the oxidized phenolic compounds are actually able to react with larger proteins and on the other hand that no HSA-HSA cross-linking occurs. No difference could be observed when using HSA or HSA essentially fatty acid free, which indicates that the fatty acids bound to the protein surface don´t interfere with the enzymatic reaction.

When AB and HSA are present in solution new bands appear above the original AB band. Those compounds are not very well resolved in a 8 % SDS gel because the grid is too finely meshed. This is the reason why no estimations about molecular weight of newly formed compounds or number of bands can be taken.

To analyse coupling 11 7,5 and 6% SDS gels were used which improved the resolution in high molecular weight areas but only to an extend which didn´t enable the classification of the new bands. The most valuable result of the coupling was that the new band pattern above the original AB band appears when either AB and HSA or only AB are present in the reaction mixture, which left one question unanswered: do the new bands derive from homomolecular coupling of AB or from heteromolecular coupling of AB and HSA.

Coupling 12, 13 and 14 helped to prove that mushroom tyrosinase was able to successfully crosslink human serum albumin and mouse IgG1 monoclonal antibody in the presence of the low molecular weight linker molecule caffeic acid. The final clue was to link the fluorescence marker FITC to HSA which made the low concentrated coupling product of an approximate size of 220 kDa visible on the fluorescent scan. When all possible substrate combinations (HSA only, AB only, HSA+AB), including a blank (HSA+AB without AbT), were displayed on a 5, 5/6 % gradient SDS gel the above mentioned band was only visible on the lane where the reaction mixture of HSA + AB + AbT was applied. The

combination of the high resolution of the gradient gel and the sensitivity of the fluorescence enabled the analysis of this result.

To analyse it in a little more detail Table 37 contains the calculated molecular weights of possible coupling products taking into account that mouse IgG1 monoclonal antibody has a MW of ~150 kDa and human serum albumin of ~66 kDa.

Table 37: Predicted and measured molecular weight of possible substrate

Substrate combination	Predicted molecular weight [kDa]	Measured molecular weight [kDa]
AB-AB	300	300
HSA-HSA	132	-
AB-HSA	216	220
AB-AB-HSA	366	370
AB-HSA-HSA	282	-

Taking Figure 68 and Figure 69 as a reference, the band marked with the red arrow has an estimated molecular weight of 220 kDa, which is perfectly matching the calculated weight of the HSA-AB coupling product. On the KANG stained image the most visible band is marked with the green arrow and lies at the same height as the 300 kDa marker band so it can be categorized as the AB-AB coupling band. To estimate the molecular weight of the band marked with the orange arrow is a little more difficult because it already exceeds the weight range of the marker. But being positioned a little further up the 300 kDa marker band and being visible only at the fluorescence scan it could possibly be an AB-AB-HSA coupling product with approximately 370 kDa.

It is very interesting to observe that when HSA is the only substrate present in the solution along with the linker molecule and AbT, no successful cross-linking of two HSA molecules takes place even though the oxidized linker molecules apparently react with amino acid residues in the protein backbone. Otherwise when HSA shares the solution with the AB plus the already mentioned components it is successfully cross-linked with the AB.

This phenomenon must derive from the AB forming a very reactive component with the linker molecule which can further react with either another AB or another HSA molecule. HSA on the other hands needs to react with the oxidized linker molecule in a way that hinders it from further reacting with other molecules, thus actually linking two proteins together. The mechanism behind the cross-linking using a phenolic linker molecule is, that the e.g. caffeic acid is oxidized by mushroom tyrosinase forming a reactive quinone. This compound can then further react with free amino- and sulfhydryl groups present in lysine and cysteine residues. Depending on where these groups are located in the protein, meaning if it is exposed on the surface or buried in the folded structure, the

phenolic compounds can react accordingly. In the case of human serum albumin it seems that the phenolic compound is small enough to access the mentioned groups in the proteins, but after reacting with it, it is sterically hindered from reacting with another HSA molecule. Therefore the outcome of a cross-linking experiment with HSA, phenolic compound and AbT present in solution leads to different numbers of phenolics linked to the HSA-surface.

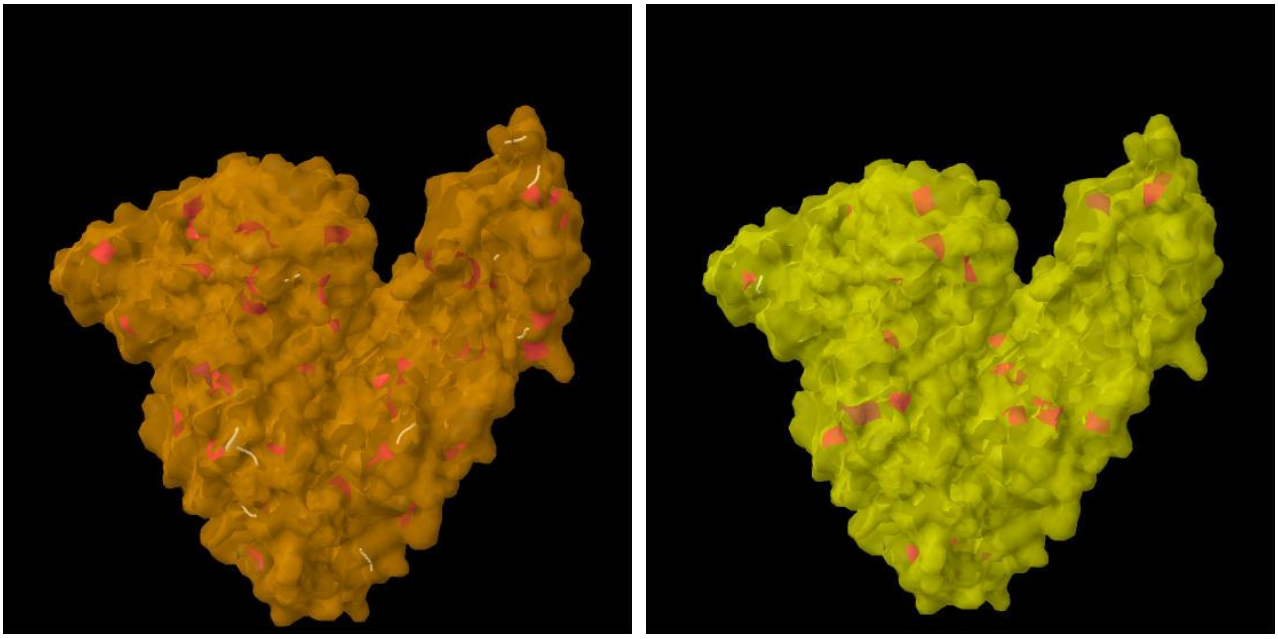


Figure 71: HSA structure with lysine residues in pink (left) and with cysteine residues in pink (right) [46]

More studies on the reaction mechanism of the phenolic compounds with the AB and HSA need to be performed to be able to explain this phenomenon in more detail and with structural proof.

Table 38: Summary of outcomes of performed cross-linking reactions

Substrate 1	Substrate 2	Cross-linker	Cross linking products	Remarks
Insulin Chain B	-	-	yes	
Native HSA	-	-	no	
AB	-	-	no	
Chemically denatured HSA	-	-	no	
Native HSA	-	Insulin Chain B	no	Insulin Chain B cross-linking
Activated HSA	-	Insulin Chain B	no	Insulin Chain B cross-linking
Activated AB	-	Insulin Chain B	no	Insulin Chain B cross-linking
HSA	AB	caffeic acid/p-coumaric acid/ catechol	yes	New bands above 170 kDa, smear in low MW areas
HSA	AB	guaiacol/ferulic acid	no	
HSA	-	caffeic acid/p-coumaric acid	no	Smear in low molecular weight areas
HSA	-	guaiacol	no	
AB	-	caffeic acid/p-coumaric acid	yes	New bands above 170 kDa and precipitation
HSA denatured by temperature	AB	caffeic acid/p-coumaric acid/ catechol	yes	New bands above 300 kDa and precipitation
HSA-FITC	AB	caffeic acid	yes	AB-HSA product band at 220 kDa

Attempts have been made to increase the formation of HSA-AB coupling products by changing the molar ration of HSA/AB, decreasing the AB concentration in solution. In the end the formation of the desired product depends on the probability of a reactive AB molecule, thus linked to a phenolic compound, meeting a HSA molecule in solution and reacting with it rather than coupling with another AB molecule. One way to increase the probability of the two desired molecules reacting is to change the molar ratio to have more HSA molecules present in solution. Interestingly the best HSA/AB ratio was 2:1, which means that not only the amount of HSA molecules present has an influence on product formation. Other strategies like preincubation of the AB with AbT before the drop wise addition of HSA need to be tested to improve the outcome of the enzymatic cross-linking reaction.

3.4 Tryptic digestion followed by MALDI-TOF

With the tryptic digestion followed by MALDI-TOF it was possible to proof on a protein sequence level that HSA and AB are present in the coupling product band cut out of the SDS PAGE for the analysis. As displayed in Table 39 the 3 most abundant peptides originate from human serum albumin and the mouse IgG1 monoclonal antibody.

Table 39: Protein/Peptide summary of Spectrum Mill Search

Group (#)	Spectra (#)	Distinct peptides (#)	Protein MW (Da)	Species	Protein name
1	148	24	69367	Homo Sapiens	Serum albumin preprotein
2	119	10	51973	Mus musculus	Igh protein
3	9	7	533780	Mus musculus	Monoclonal antibody A12-d6.3 heavy chain precursor

3.4.1 Discussion

The outcome of this analysis was the proof on a molecular basis that the 220 kDa band cut out from the SDS gel derived from the coupling of human serum albumin and mouse IgG1 monoclonal antibody. It is a qualitative analysis, which doesn´t contain information on how many HSA molecules coupled with how many AB molecules or which specific amino acid residues were responsible for the reaction. But it can be said that HSA and the AB were present in this specific bands which confirms the estimations made after the fluorescent analysis.

4 Conclusion

The aim of this work was to successfully cross-link human serum albumin with mouse IgG1 monoclonal antibody using mushroom tyrosinase. These conjugates can be used for the production of nanospheres which find a very broad application in targeted drug delivery. The enzymatic method has several advantages when compared to the chemical procedure used at the moment such as the avoidance of toxic compounds.

The characterization experiments revealed that the activity of mushroom tyrosinase is very sensitive to temperature changes but is not affected by varying pH-values. The optimum working conditions for AbT were found to be 25°C and a pH value of 6,8.

The activity is highly dependent on the chosen substrate but in general it can be stated that the diphenolase activity is 8x higher than the monophenolase activity. The specific activity towards L-tyrosine, representing the monophenolase activity, is **2,23 U/mg** and towards L-DOPA, representing the diphenolase activity, **13,38 U/mg.**

The results of the oxygen uptake confirmed the outcome of the Dopachrome and the MBTH assay. When L-tyrosine is present in solution mushroom tyrosinase uses **0,4 ± 0,2 mg O_2/(min*g_{enzyme})** on the other hand, when L-DOPA serves as a substrate AbT consumes **2,0 ± 1,0 mg O_2/(min*g_{enzyme})** so 5x as much.

Insulin Chain B oxidized from bovine pancreas was successfully cross-linked gaining insights on reaction conditions that are suitable for mushroom tyrosinase. Because HSA and AB could not be cross – linked when used in their native states, the plasma protein was partially unfolded using chaotropic agents and high temperature. The denaturation experiments showed that the use of the high temperature method is more suitable for this sort of application. This way human serum albumin is irreversibly denatured and the solution does neither require a purification- nor a concentrating step after the denaturation process.

After the screening of 5 different linker molecules concerning their ability to enable the cross-linking of the two compounds, it was possible to obtain HSA-AB coupling products. Caffeic acid, catechol and p-coumaric acid are the three low molecular weight phenolic compounds identified to successfully link HSA and AB together.

Possible explanations for the differences in reactivity and cross-linking success of the compounds used are sterical hindrance and occurring oligomerisation. Some compounds tend to oligomerise after being oxidized, which enables them to act as linker and as spacer at the same time. Therefore they show a higher accessibility which helps obtaining more cross-linking product.
Furthermore the unreactive compounds contain an ether group in ortho position which could be the reason for sterical hindrance during the reaction with the protein.

Labeling human serum albumin with FITC prior to the enzymatic cross-linking, proved to be the analysis method of choice to detect even very low concentrations of the desired coupling product. Due to the high sensitivity of fluorescence, low concentrated protein bands that cannot be detected with conventional staining methods are still visible on fluorescence scan.

Experiments have shown that HSA as the only substrate cannot be successfully cross-linked using phenolic linker molecules even though the linker molecules are binding to the protein. When adding AB to the reaction mixture the coupling product HSA-AB is obtained. Due to this fact it is very likely that the linker molecules are only able to form a reactive compound when binding to the AB. This compound further reacts with other substrates present in solution forming the desired coupling product HSA-AB as well as AB-AB di- and oligomers.

To increase the yield of the desired coupling product the HSA/AB ratio used in the reaction was changed to augment the probability of an activated AB molecule meeting a HSA molecule and reacting with it. The ratio with the highest product yield was found to be 2:1.

Further investigation has to be performed to increase the yield of the desired coupling product and to avoid the building of the unwanted side-products throughout the reaction. Apart from optimizing the outcome of the cross-linking reaction, studies need to be carried out to further elucidate the mechanism of how HSA and AB are linked together. When the amino acid residues taking part in the reaction are identified it should be possible to state which part of the antibody is linked to the HSA surface. This is especially interesting because the conjugated antibody needs to fulfill its original purpose of recognizing and binding the complementary antigen.

5 Declaration

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Graz, am …… ……..

(Unterschrift)

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

…… ……..

date (signature)

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7 Abbreviations

%	percent
(v/v)	volume per volume
(w/v)	weight per volume
°C	degree Celcius
µm	micrometer
AB	mouse IgG1 monoclonal antibody
AbT	tyrosinase f. *Agaricus bisporus*
APS	ammonium persulfate
C	catechol
c	concentration
CA	caffeic acid
d	pathlength
ddH_2O	double destilled water
dH_2O	destilled water
DLS	dynamic light scattering
e.g.	exempli gratia
EtOH	ethanol
f	dilution factor
FA	ferulic acid
FAF	fatty acid free
FITC	fluoresceinisothiocyanate
g	gram
GA	guaiacol
GnHCl	guanidin hydrochloride
H_2O	water
HCl	hydrochloric acid
HSA	human serum albumin
HSA-FAF	human serum albumin essentially fatty acid free
HSA-FITC	human serum albumin linked to FITC
IgA/D/E/M/G	immunoglobulin A/D/E/G/M
Ins	Insulin Chain B oxidized from bovine pancreas
kDa	kilo Dalton
M	mole per liter
mAbs	monoclonal antibodies
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
MBTH	3-methyl-2-benzothiazoninone hydrazine
mg	milligram
min	minute
ml	milliliter
mM	millimole per liter
MW	molecular weight
NCBI	National Center for Biotechnology Information
nm	nanometer

NPs	nanoparticles
o-	ortho
O_2	oxygen
p-	para
PAGE	polyacrylamide gel electrophoresis
PCA	p-coumaric acid
PDB	protein database
PLA	poly lactic acid
RA	rheumatoid arthritis
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulfate
t	time
TAE	Tris-acetate EDTA
TEMED	tetramethylethylenediamine
U	Unit
U/mL	Units per milliliter
V	Volt
V_{total}	total volume
Δabs/Δt	change of absorbance per minute
ε	molar extinction coefficient
λ	wavelength
µL	microliter
µmol	micromol

8 Equipment

AEKTA purifier	Amersham Pharmacia Biotech, Model
analytic balance	Sartorius 2004 MP
analytic balance	KERN PB
analytic balance	DENVER instrument, S-4002
centrifuge	Eppendorf mini spin (F-45-12-11)
centrifuge	Hettich EB 3S
centrifuge	Heraeus, Biofuge primo
electrophoresis power supply	BIO-RAD, PowerPAC 1000
electrophoresis power supply	BIO-RAD, PowerPac HV
HPLC	Dionex
	Solvent Rack SOR-100
	P680 HPLC Pump
	UltiMate 3000 Autosampler
	Thermostated Column Compartment TCC 100
	UV-VIS Detector: UVD170U
magnetic stirrer	IKA RCT basic
Oximeter	Rank Brothers LTD, Dual digital Model 20
pH-meter	METTLER TOLEDO, Seven Easy
Photometer	HITACHI, Spectrophotometer U2001
pipette	Carl Roth GmbH
pipette	Soccorex, ACURA 825
plate reader	TECAN infinite M200
scanner	HP Scanjet 4890
thermomixer	Eppendorf Thermomixer comfort
vortex	JANKE & KUNKEL, IKA, VF2
Zetasizer	MALVERN, Nano Series

9 Chemicals

2,2',2'',2'''-(Ethane-1,2-diyldinitrilo)tetraacetic acid	Carl Roth GmbH
2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS)	Carl Roth GmbH
3,4 dihydroxyphenylalanine	Sigma-Aldrich
37% formaldehyde	Carl Roth GmbH
3-methyl-2-benzothiazolinone hydrazone (MBTH)	Sigma-Aldrich
40 % Acrylamide-, Bisacrylamide-stock solution 37,5:1	Carl Roth GmbH
Acetic acid	Carl Roth GmbH
Acetone	Carl Roth GmbH
Aluminium sulfate 18 hydrate	Merck
Ammoniumperoxodifsulfat (APS)	Merck
Broad Range SDS PAGE Standard	BIORAD
Bromphenol blue	Merck
Caffeic acid	Sigma Aldrich
Catechol	Merck
Charcoal	Merck
Coomassie Blue G-250	BIORAD
Coomassie Brilliant Blue R-250	BIORAD
Di-sodiumhydrogenphosphate dehydrate	Carl Roth GmbH
Ethanol	Carl Roth GmbH
Ferulic acid	Sigma Aldrich
Fluoresceinisothiocyanate (FITC)	Sigma Aldrich
Glycerol	Carl Roth GmbH
Glycin	Carl Roth GmbH
Guaiacol	Sigma Aldrich
Guanidine hydrochloride	Merck
Human Serum Albumin	Sigma-Aldrich
Human Serum Albumin (essentially fatty acid free)	Sigma-Aldrich
Hydrochloric acid	Carl Roth GmbH
Insulin Chain B	Sigma-Aldrich
L-tyrosine	Fluka
Methanol	Carl Roth GmbH
Mouse monoclonal antibody	EXBIO
N, N´-dimethylformamide (DMF)	Merck
N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED)	Acros Organics

PageRuler Prestained Protein Ladder	Fermentas
P-coumaric acid	Sigma Aldrich
Phosphoric acid	Sigma Aldrich
Polyethylene glycol (PEG)	Sigma Aldrich
Silver nitrate	Ögussa
Sodium carbonate anhydrate	Carl Roth GmbH
Sodium di-hydrogen phosphate 2-hydrate	Carl Roth GmbH
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH
Sodiumthiosulfateanhydrate	Merck
Triton X-100	Carl Roth GmbH
Tyrosinase mushroom	Sigma-Aldrich
urea	Carl Roth GmbH

10 Appendix

Composition of Sodium phosphate buffer and Tris Acetate buffer

0,1 M Sodium phosphate-Buffer (pH 6,8)		m [g] used for 1 L
	49 mM Na_2HPO_4	6,96 g
	51 mM NaH_2PO_4	7,96 g
1 X Tris-Acetate-EDTA (TAE) (pH 8)		
	Tris	4,8 g
	Na-EDTA	0,4 g
	Acetic acid	For pH adjustment

Results of Spectrum Mill Search after tryptic digestion

Run #	Run Name	Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Protein pI	Species	Database Accession #	Protein Name
1	BTH-6	1	148	24	471.23	49	2.30e+007	69367.1	5.92	Homo sapiens	4502027	serum albumin preproprotein
1	BTH-6	2	119	10	184.91	27	4.68e+007	51976.3	8.12	Mus musculus	19353759	Igh protein
1	BTH-6	3	9	7	126.07	23	1.59e+007	53378.0	7.12	Mus musculus	290877931	monoclonal antibody A13-d6.3 heavy chain precursor
1	BTH-6	4	6	4	69.93	7	2.73e+006	77080.4	6.97	Homo sapiens	37747855	Transferrin
1	BTH-6	5	40	3	59.93	20	1.47e+007	25881.5	6.85	Sus scrofa	242253868	trypsinogen
1	BTH-6	6	7	3	56.64	5	5.37e+006	66067.0	8.15	Homo sapiens	11935049	keratin 1
1	BTH-6	7	7	3	53.30	83	8.46e+007	11391.8	6.28	Mus musculus	2222728	variable region of immunoglobulin kappa chain
1	BTH-6	8	3	2	41.06	4	2.77e+006	100344.0	5.10	Pan troglodytes	114667507	Keratin 10
1	BTH-6	9	9	2	38.71	15	3.35e+006	27107.2	5.19	Homo sapiens	119614650	hCG1749481, isoform CRA_l

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☺