Effect of chitosan on thermal stability of horseradish peroxidase

Stefán Bragi Gunnarsson

Faculty of Physical Sciences
University of Iceland
2011

Effect of chitosan on thermal stability of horseradish peroxidase

Stefán Bragi Gunnarsson

90 ECTS thesis submitted in partial fulfillment of a
Magister Scientiarum degree in biochemistry

Advisor
Hörður Filippusson

Faculty Representative
Baldur Símonarson

Faculty of Physical Sciences
School of Engineering and Natural Sciences
University of Iceland
Reykjavik, October 2011

Effect of chitosan on thermal stability of horseradish peroxidase
Effect of chitosan on thermal stability of HRP
90 ECTS thesis submitted in partial fulfillment of a *Magister Scientiarum* degree in biochemistry

Faculty of Physical Sciences
School of Engineering and Natural Sciences
University of Iceland
Hjarðarhagi 2-6
107, Reykjavik
Iceland

Telephone: 525 4700

Bibliographic information:
Stefán Bragi Gunnarsson, 2011, *Effect of chitosan on thermal stability of horseradish peroxidase*, Master's thesis, Faculty of Physical Sciences, University of Iceland, pp. 81.

Printing: Háskólaprent
Reykjavik, Iceland, October 2011

Abstract

Chitin (β-1,4-linked N-acetyl-D-glucosamine) is, along with cellulose, nature's most abundant organic polymer. Chitin is found in the cell walls of fungi and the shells of arthropods such as shrimp, crabs, krill, lobsters and insects. Chitosan (β-1,4-linked D-glucosamine) is the partially deacetylated derivative of chitin. Chitosan contains a free amine group that gives it different properties from chitin and cellulose. Two important properties of chitosan are degree of polymerisation (DP) and degree of deacetylation (DDA) which determine its behaviour in aqueous solutions. Carbodiimides mediate the formation of linkages between amines and carboxylates and can be used to form chitosan-protein conjugates. Chitosan affects thermal stability of proteins in aqueous solutions either as a co-solute or connected to the protein with a covalent bond.

Horseradish peroxidase (HRP) is an oxidoreductase whose main application is in biosensors and in waste water treatment. HRP can form a conjugate with chitosan by using carbodiimide and sulpho-N-hydroxysuccinimide (NHSS). Three different chitosan samples, two oligomers and one polymer were used to form conjugates with HRP and the thermal stability of the conjugates was studied. The effect of two different chitosan samples as co-solutes with the enzyme was also studied. Chitosan with degree of polymerisation of 4-8 proved to increase HRP's thermal stability most. HRP's half life at 65°C doubled after conjugation and the energy of deactivation of the enzyme was increased by 72%. As a cosolute the oligomer increased HRP's half life at 65°C 3.6 fold at a concentration of 5% (w/v).

Útdráttur

Kítín (β-1,4-tengd N-asetýl-D-glúkósamín) er, ásamt sellulósa, algengasta lífræna fjölliðan sem finnst í náttúrunni. Kítin finnst í frumuveggjum sveppa og skeljum liðdýra eins og rækju, krabba, átu, humars og skordýra. Kítósan (β-1,4-tengd D-glúkósamín) er afasetýleruð afleiða kítíns. Kítósan inniheldur frjálsan amínhóp sem veitir kítósani einstaka eiginleika sem eru frábrugðnir eiginleikum kítíns og sellulósa. Tveir mikilvægustu þættir er snúa að eiginleikum kítósans eru stig fjölliðunar, þ.e. hve margar kítósaneiningar hver fjöl- eða fásykra inniheldur, og stig afasetýleringar sem segir til um hve marga frjálsa amínhópa sykran hefur. Þessir eiginleikar ráða hegðun kítósans í vatnslausn. Karbódíimíð geta myndað samgild tengi á milli amína og karboxýlata sem hægt er að nýta við tengingu kítósans við prótein. Kítósan hefur áhrif á stöðugleika próteina gagnvart hita bæði þegar samgild tengi eru á milli kítósans og próteins og þegar kítósan er í lausn með próteini.

Peroxíðasi úr piparrót er oxídóredúktasi sem helst er hagnýttur í lífefnanemum og í hreinsun fráveituvatns. Peroxíðasinn hvatar oxun fenóls og annarra arómatískra efna sem óæskileg eru í fráveituvatni ýmiskonar iðnaðar. Fyrir tilstuðlan karbódíimíðs og N-hydroxýsuccinimíðs eru mynduð samgild tengi á milli peroxíðasa og kítósans. Áhrif tveggja kítósanfáliðaog einnar fjölliðu á hitastöðugleika peroxíðasa voru skoðuð í þessu verkefni. Kítósanfáliða úr 4-8 sykrueiningum reyndist auka stöðugleikann mest. Helmingunartími virkni ensímsins tvöfaldaðist við 65°C þegar kítósan var tengt ensíminu auk þess sem afvirkjunarorka próteinsins jókst um 72%. Helmingunartími ensímsins jókst 3.6 falt við 65°C í 5% kítósanlausn.

Table of Contents

List of Figures

List of Tables

Abbreviations

4-AAP	4-aminoantipyrine
Abs.	Absorbance
BAPNA	Nα-benzoyl-DL-arginine p-nitroanilide
CD	Circular dichroism
Chos	Chitosan
CLEA	Crosslinked enzyme aggregates
CLEC	Crosslinked enzyme crystals
DDA (chitosan)	Degree of deacetylation
DP (chitosan)	Degree of polymerisation
DP (DSC)	Differential power
DSC	Differential scanning calorimetry
EC (enzyme)	Enzyme Commission number
EDAC/EDC	N-(3-Dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IR spectroscopy	Infrared spectroscopy
L-DOPA	L-3,4-dihydroxyphenylalanine
LD_{50}	Median lethal dose
MES	2-[N-morpholino]ethanesulphonic acid
MOPS	3-[N-morpholino]propanesulphonic acid
MW	Molecular weight
NHSS	N-hydroxysulphosuccinimide
NMR	Nuclear magnetic resonance
Tchos	Therapeutic chitosan
TEA	Triethanolamine
TLCK	Nα-tosyl-L-lysine chloromethyl ketone hydrochloride
T_m	Melting point
UV spectroscopy	Ultraviolet-visible spectroscopy

Acknowledgements

First of all I would like to thank Professor Hörður Filippusson for his guidance, patience and support during my time at the Science Institute, especially during the less productive times of my studies. I would also like to thank him, along with Róbert Anton Hafþórsson, for the friendly and positive atmosphere in our lab. It has been a pleasure working at the Science Institute and I would like to thank all my co-workers and the friends I have made there over the last years.

I would like to thank Baldur Símonarson for his very helpful comments and suggestions, both theoretical and grammatical.

I am also very grateful to my family and friends for the support they have shown me.

Finally I would like to thank the Science Institute for having me and its staff for creating a helpful and enjoyable working environment.

1 Introduction

Over the last years and decades, proteins and enzymes have become an essential part of many industrial processes as well as being applied both in cosmetics and in therapeutics. With advances in genetic engineering and in biotechnology the mass production of proteins and enzymes has become more feasible. Enzymes can catalyse reactions under mild conditions that would otherwise depend on polluting, inorganic catalysts and extremes in, for example, temperature, acidity or alkalinity. The use of enzymes is therefore environmentally friendly but the mild conditions, under which they catalyse their reactions, can also be a disadvantage. Enzymes can lose their structure and catalytic activity at temperatures that might be necessary for other components of industrial processes or at physiological conditions when used as drugs. Increase in stability is therefore of great importance and can be accomplished by various methods.

Forming a covalent bond between a protein and a stabilising agent, such as a carbohydrate, has proved to increase thermal stability of proteins. When choosing a stabilising agent it is important to do so with regard to its potential use. Chitosan is both biocompatible and biodegradable which makes it an interesting stabiliser, especially for stabilising protein drugs as it does not cause allergic reactions.

Fannar Jónsson has already used chitosan to improve the stability of trypsin and in this study the effect of chitosan on thermal stability of horseradish peroxidase will be investigated [1].

1.1 Chitin and chitosan

The biopolymer chitin is an amino polysaccharide comparable in structure and availability to the cellulose of plants. Chitin and cellulose are the most abundant organic materials on earth and 10^{11} tons are estimated to be produced annually [2]. Originally the focus of the scientific community was mainly on cellulose but in recent years and decades chitin, and its N-deacetylated derivative chitosan, have received more attention. Chitin contains an acetamido group in the C-2 position instead of a hydroxyl group in cellulose, whereas in chitosan the acetyl group has been removed from chitin leaving behind an amine group (Figure 1.1). The amine group, with pK_a of about 6.3-6.5, is protonated at pH below neutral which gives chitosan very interesting properties, for example mucoadhesiveness, antimicrobial activity and great possibilities in bioconjugation [3, 4].

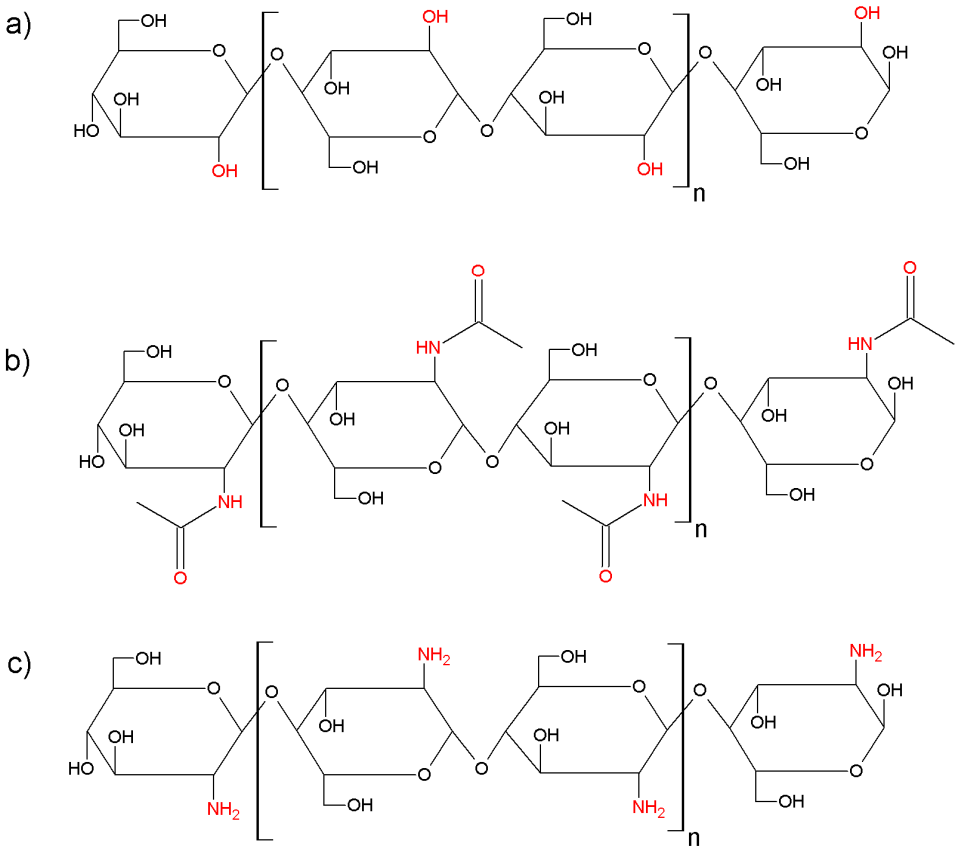


Figure 1.1. *Comparison of the structures of a) cellulose, b) chitin and c) chitosan.*

1.1.1 Sources of chitin

Chitin is found in the cell walls of fungi and the shells of arthropods such as shrimp, crabs, krill, lobsters and insects. The commercial production of chitin is mostly based on waste from the seafood industry. A byproduct of many commercially processed crustaceans is the chitosan-rich shell. The shell's dry weight commonly contains 20-50% chitin and thus can be a resource instead of waste [2]. The hard chitin in the shells of arthropods is called α-chitin while a softer type of chitin, called β-chitin, can be processed from squid.

The shells of shrimp contain another desirable product, the carotenoid astaxanthin. It gives the shells their red colour and when it has been isolated it can be used as an animal feed, giving farm raised salmon a more natural colour [5]. The production of chitin and astaxanthin from waste is therefore both economically and environmentally feasible.

The production of 1 kg of 70% deacetylated chitosan from shrimp requires 6.3 kg of hydrochloric acid, 1.8 kg of sodium hydroxide as well as 1.4 tonnes of water, both for the process itself and for cooling [6].

1.1.2 Properties of chitin

Chitin is a polymer of β-1,4-linked N-acetyl-D-glucosamine units. Chitin functions as a structural factor in arthropods so it is no surprise that it is not soluble in water and is, in

fact, insoluble in most organic solvents. Three forms of chitin polymers exist, named α, β and γ (Figure 1.2). The difference between the forms lies in the packing and polarities of the chains.

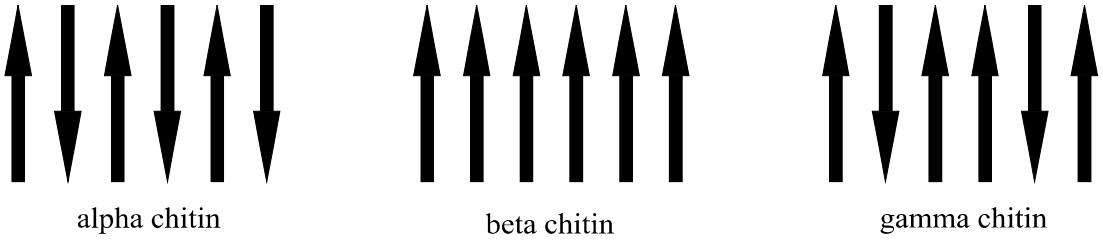


Figure 1.2. *Comparison of the tree types of chitin.*

Alpha chitin's antiparallel packing makes it considerably harder and less soluble than beta chitin which has a parallel packing. This is better illustrated by showing the hydrogen bond network of the two types of chitin in Figure 1.3 and Figure 1.4. Gamma chitin is a mixture of the former two types and is sometimes considered a part of the alpha family.

As can be seen when Figure 1.3 and Figure 1.4 are compared, alpha chitin has both intra- and inter-molecular networks of hydrogen bonds that account for its insolubility and hardness. The network of hydrogen bonds keeps adjacent chains of alpha chitin at a distance of about 0.47 nm [7].

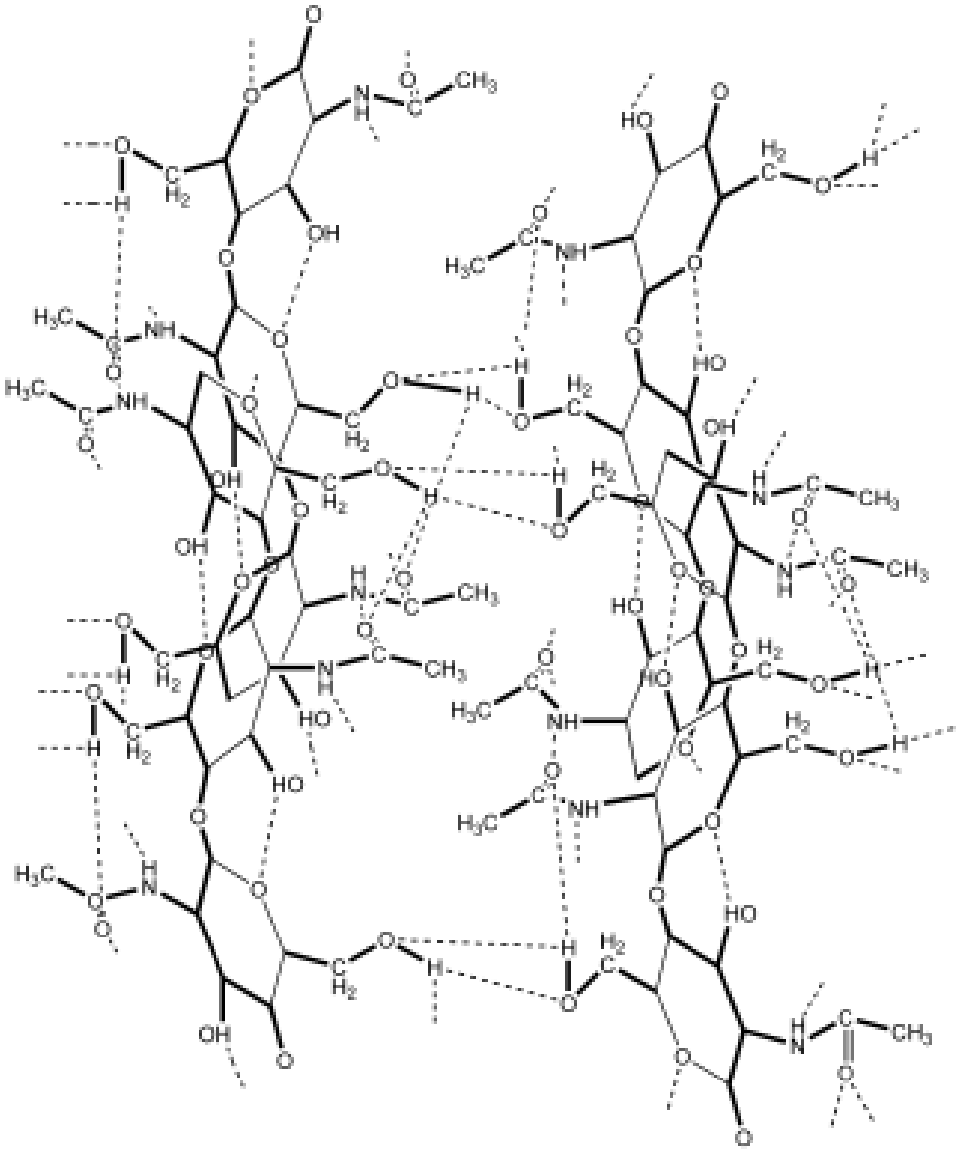


Figure 1.3. *Alpha chitin. Two different chains of chitin packed in antiparallel formation. Dotted lines represent hydrogen bonds [7].*

Alpha chitin is more common than beta chitin in nature, but beta chitin is readily available as a waste from squid processing [8]. Squid's pens contain beta chitin so the raw material is cheap and conversion of beta chitin to alpha chitin is a simple process. Squid pens contain less carotenoids than the exoskeletons of crustaceans so it can be a good raw material if production of carotenoids is not desirable. Beta chitin also contains less minerals, such as calcium carbonate, so less extraction of undesirable materials is necessary [8]. Beta chitin is also reported to be more reactive than alpha chitin which makes it preferable for both enzymatic and chemical modifications of chitin [7].

Figure 1.4 shows that beta chitin has no inter-molecular hydrogen bonds which makes it tough rather than hard. The lack of inter-molecular hydrogen bonds also makes adjacent

chitin chains less compact which makes beta chitin more reactive, since the polymer chains are more accessible.

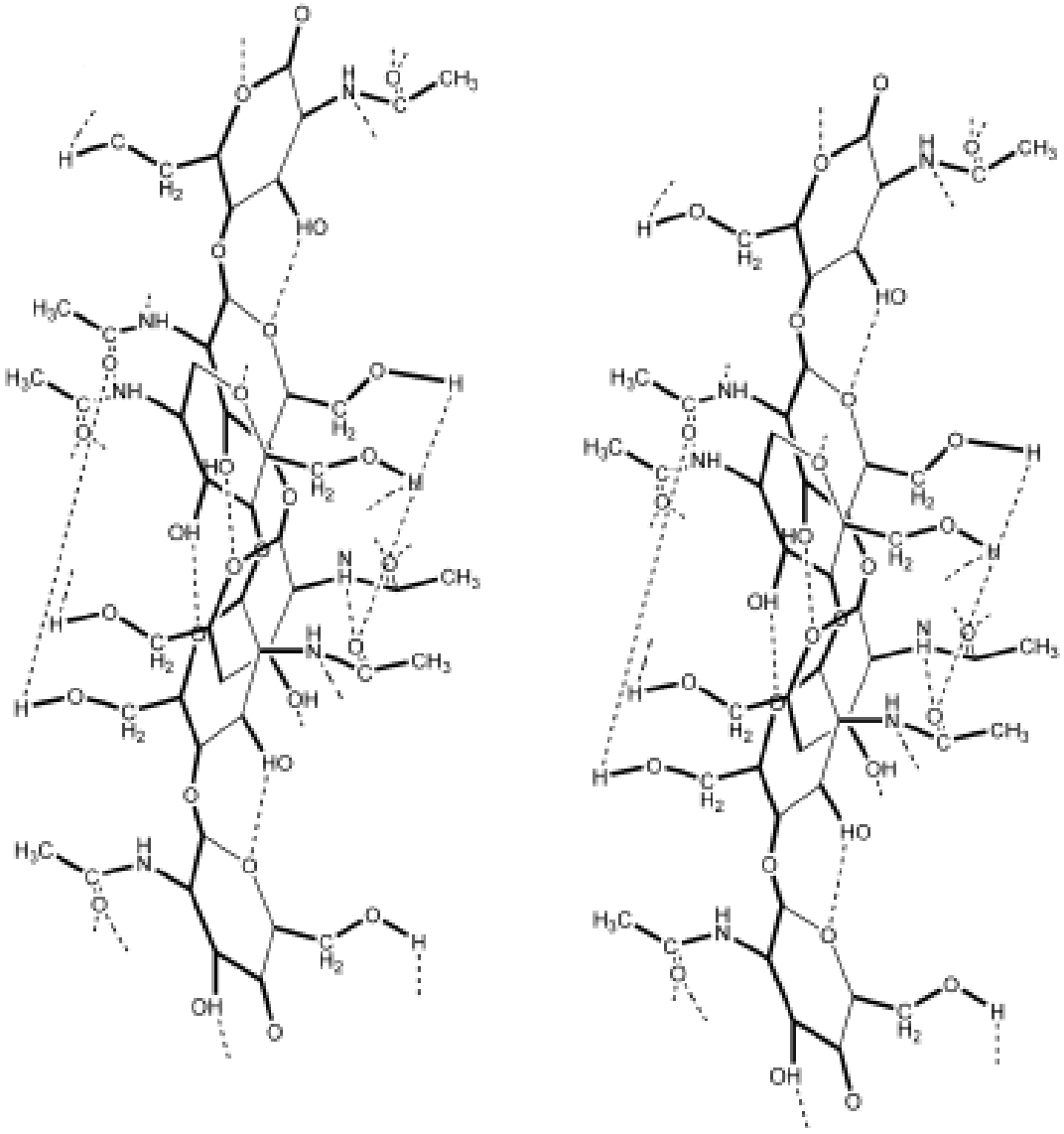


Figure 1.4. *Beta chitin. Two different chains of chitin packed in parallel formation. Dotted lines represent hydrogen bonds [7].*

1.1.3 Production of chitin

When chitin is processed from shells of crabs, shrimp and lobsters it is first treated with dilute hydrochloric acid to remove calcium carbonate. When calcium carbonate has been removed the shells are either ground or cut into small pieces and then boiled in 1 or 2 M sodium hydroxide solution for the decomposition of proteins and pigments. This treatment gives colourless or white chitin with about 10% deacetylation [2].

1.1.4 Chitosan

As stated earlier, chitosan is the deacetylated form of chitin. Due to chitin's insolubility its utilisation is difficult while the relatively soluble chitosan has qualities that can be utilised in many fields, from agriculture to nanotechnology. Utilisation of chitosan in different areas is dependent on its chemical properties. Since the word chitosan is a group noun and covers oligomers and polymers of D-glucosamine and N-acetyl-D-glucosamine in different ratios, its properties must be well defined so the most suitable form is chosen in each case.

1.1.5 Production of chitosan

Chitin is practically insoluble in water because of its acetyl groups, deacetylation of chitin is therefore an important process that produces its derivative, chitosan. After deacetylation an amine group is exposed which gives chitosan much greater solubility than chitin. Chitosan is usually produced by heating alpha chitin up to about 100-160°C for a few hours in 40-50% sodium hydroxide. This process gives chitosan with degree of deacetylation of about 95%. The distinction between chitin and chitosan is somewhat unclear, i.e. at no exact percentage of deacetylation does the polymer stop being chitin and becomes chitosan. Kumar claims that the typical degree of deacetylation of chitosan is above 65% [6].

In organisms such as fungi, chitosan is found in the cell walls. Chitin is made in these organisms by enzymes called chitin synthases and the chitin is then deacetylated by other enzymes, called chitin deacetylases [9]. These enzymes are difficult to produce in industrial scale quantities but their usage would be of great benefit for the environment due to milder conditions during the production.

1.1.6 Properties of chitosan

After the deacetylation of chitin a chitosan polymer is produced. The chitosan polymer usually has a very high molecular weight, normally referred to as the degree of polymerisation (DP), i.e. how many sugar units make up the chitosan chain. Different degrees of polymerisation are needed for different tasks so it is very important to be able to control and analyse the molecular weight. Higher degree of polymerisation leads to less solubility of the chitosan sample and increased viscosity of its solution [10]. Another factor affecting the solubility and general properties of chitosan is the degree of deacetylation (DDA). The degree of deacetylation indicates how many free amine groups each chitosan molecule contains, usually indicated with either a percentage (0-100%) or a fraction (0-1).

Chitosan with degree of deacetylation of about 45-55% is necessary for good solubility. Determination of the degree of deacetylation is a very important part of chitosan chemistry and can be assayed by many different ways. Kurita mentions elemental analysis, hydrolysis of acetamide groups, titration of free amine groups, dye absorption, spectroscopies (IR spectroscopy, UV spectroscopy, CD and NMR), enzymatic degradation and pyrolysis [2].

In addition to the degree of polymerisation and the degree of deacetylation, other factors also control the solubility of chitosan. A suitable solvent must be chosen since chitosan usually does not dissolve in water. The solvent can be hydrochloric acid or a dilute organic acid. Chitosan samples can be very different so suitable solvent conditions must be chosen

for each case. In this study the chitosan oligomers dissolved quickly in weakly acidic phosphate and MES solutions while the polymer required a lower pH (around pH 3) to dissolve, but after dissolution the pH could be brought up to weakly acidic (pH 6.0-6.5) without the polymer precipitating.

Chitosan is only soluble when its amine groups are protonated. These amine groups have pK_a value of 6.3 so they lose their proton at pH between 6.0 and 6.5 which explains their solubility in weakly acidic solutions.

The degree of polymerisation can be analysed by a few different methods. HPLC has been used for this type of analysis, as well as light scattering and viscometry. Viscometry is a simple way to determine the molecular weight of chitosan. Jia and Shen used previously determined Mark Houwink equation (Equation 1.1) parameters.

Equation 1.1

$$[\eta] = KM^a$$

Where η is the intrinsic viscosity, K and a are constants that depend on the solvent and the polymer and M is the molecular weight. They found that when treated with 85% phosphoric acid for up to 35 days, the degree of polymerisation, and subsequently the viscosity, decreased significantly [10].

In this study two oligomers and one polymer were used. The polymer at a concentration of 5 mg/mL in a solution was not easily transferable with a micropipette while solutions of the oligomers at concentrations ten times higher were very easy to transfer.

1.1.7 Applications of chitosan

As was said earlier chitosan is a natural polymer with unique features that stem from its free amine groups. Chitosan has many possibilities both in industrial processes as well as being used in cosmetics, agriculture and pharmaceutical drugs. Chitosan is both biocompatible and biodegradable. It has great mucoadhesive properties since its cationic amine groups adhere to the negatively charged mucus [11]. Chitosan is neither irritant nor does it cause allergy, it can be consumed in big doses and in mice the LD_{50} dose was found to be more than 16 g/kg/day [12].

Since chitosan is broken down by lysozyme, its biodegradability can be used to release drugs that are trapped inside the chitosan polymer. Biodegradation of chitosan occurs either chemically or enzymatically. Acid catalysed degradation is the primary chemical degradation process while enzymes able to hydrolyse glucosamine-glucosamine, glucosamine-N-acetyl-glucosamine and N-acetyl-glucosamine-N-acetyl-glucosamine linkages catalyse the enzymatic degradation [13]. Chitosan has been injected into rat muscle and complete degradation was not achieved in six months. This can be used for localised drug treatment and for drugs that the body excretes rapidly. Chitosan can also be used for localised function of drugs in the gastrointestinal tract since chitosan was found not to be broken down in the stomach or small intestine of rats, but by microorganisms in

the colon the chitosan is effectively degraded. This could allow for a localised drug delivery to the colon [12, 14, 15]. Pan *et al.* formed chitosan nanoparticles that contained insulin and were effective at keeping blood glucose levels normal in diabetic mice [16].

Freier *et al.* reported on lysozyme degradation of chitosan *in vitro* under physiological conditions with regard to pH and enzyme concentration. They found that the degree of deacetylation controlled the rate of degradation where DDA of 40-60% gave complete degradation after 28 days while both non-acetylated and completely acetylated chitosan and chitin showed very little degradation. Low degree of acetylation also gave the best cell adhesion, because of the high percentage of free, protonated amine groups, and it was concluded that chitosan with 20% deacetylation gave fast degradation and good cell adhesiveness [17].

Chitosan is also being investigated for possible use in DNA delivery due to the great possibilities gene therapy could offer. The positively charged chitosan binds to negatively charged DNA and the biocompatibility of chitosan gives DNA a carrier that protects it from degradation during its delivery while minimising inflammatory response and with appropriate nitrogen to phosphate ratio the chitosan-DNA complex will be feasible for cellular uptake [18]. Trapani *et al.* formed chitosan nanoparticles with dopamine that could cross the blood-brain barrier of rats. Chitosan can therefore be used as a carrier for drugs used to treat central nervous system diseases [19].

The mucoadhesiveness of chitosan has been utilised to prolong the activity of topically applied ocular drugs. It has also been used to make more effective and controlled nasally delivered drugs [12]. Onishi *et al.* found that a 50% randomly deacetylated chitosan with molecular weight of about 1.1×10^5 was readily excreted from the body after it had been conjugated with fluorescein isothiocyanate and injected intraperitoneally. Urine and tissue analysis were conducted and showed that most of the conjugate was found in the kidneys as soon as one hour after administration [20]. Maezaki *et al.* reported that when three grams of chitosan per day were added to the diets of eight healthy males their blood cholesterol levels dropped significantly. The positively charged chitosan can bind to negatively charged bile acids and is then subsequently excreted which decreases the cholesterol pool in the body and subsequently lowers cholesterol [21].

Immobilisation of enzymes is a very important aspect of utilisation of enzymes both in biotechnology as well as in industries. To be able to keep the enzymes immobilised on a solid surface is profitable both economically and with regard to enzyme stability. Immobilisation of enzymes mimics their natural environment where enzymes are usually attached to cellular membranes. Immobilisation can also eliminate the need for purification procedures when the reaction product must be free of enzymes and other contaminants.

When support material for immobilisation is chosen it is important that it is non-toxic, cheap, has high affinity to proteins, is hydrophilic, stable and has reactive groups that can be conjugated to proteins. Chitosan meets all these criteria and has therefore been used for many different purposes, ranging from support material for biosensors to incorporation of protein drugs for localised administration.

Luo *et al.* reported a glucose biosensor based on chitosan-glucose oxidase-gold nanoparticles where hydrogel of chitosan with incorporated gold nanoparticles and glucose

oxidase was formed around gold disk electrodes. This produced a glucose biosensor with good sensitivity and stability [22].

In a review article Krajewska collected well over one hundred different applied immobilisation examples for chitosan. These examples are divided into nine different groups depending on the field of their application. The first one is the food industry where chitosan based support material is used for production of both maltose and glucose syrup from starch, removal of hydrogen peroxide from food, for the hydrolysis of starch in ethanol production, the debittering of citrus juice and many more. The second group contains industrial applications other than the food industry, including the production of laundry detergents and enzymatic hydrolysis of collagen and keratin in cosmetics. Into the third group go chitosan-immobilised enzymes in medicine where, for example, tyrosinase is used to produce L-DOPA. Biosensors make up the fourth group where enzymes are used to detect many different biochemicals in solution. The other groups are enzyme reactors for biosensing, separation, purification and recovery of enzymes, environmental, chemical synthesis and immobilisation studies. Enzymatic removal of toxic waste is extremely important and chitosan has been used as support material for the detection and removal of phenols and for treatment of fertilizer effluents [23].

As well as being a support material for enzymes in waste treatment chitosan itself has properties that can be utilised for environmentally friendly processes. The amine and hydroxyl groups of chitosan can form a chelate with metal cations such as copper, mercury, cadmium, iron, nickel, zinc, lead and silver [2] and thus effectively clean waste water of polluting metals [24].

Chitosan has well known antimicrobial activity against fungi and both gram positive and gram negative bacteria [25, 26]. Chitosan suppresses sporulation and spore germination in fungi and its positive charge attracts it to the negatively charged bacterial surface which inhibits growth. Antimicrobial effectiveness of chitosan is dependent on many factors. The positive charge density of chitosan helps attract it to the cell surface so the general rule is that chitosan with higher degree of deacetylation has higher antimicrobial efficiency. The size of the chitosan chain is also of importance but varies between different bacteria. It has been suggested that smaller chitosan molecules can penetrate the cell walls of bacteria and interfere with the synthesis of mRNA while larger molecules of chitosan coat the surface of the bacteria and alter the cell’s permeability. This excellent feature has made chitosan an important food preservative and a wound dressing material [4].

1.2 Horseradish peroxidase

Horseradish peroxidase (HRP; EC 1.11.1.7) is a haem-containing oxidoreductase produced from the roots of horseradish, *Armoracia rusticana*, a perennial plant that belongs to the Brassicaceae family. Along with other members of the Brassicaceae family, like mustard and wasabi, the horseradish root is very popular in cuisine. It also contains a substantial amount of horseradish peroxidase which is commercially produced on a relatively large scale. The first recorded observation of horseradish peroxidase catalysed reactions is in a note written by Louis Antoine Planche in 1810. When a piece of fresh horseradish root was placed in a tincture of guaiac resin (an anti-tubercular preparation [27]) the solution turned blue. This analysis was used to make sure that jalap resin, a laxative, was not contaminated with guaiac resin [28].

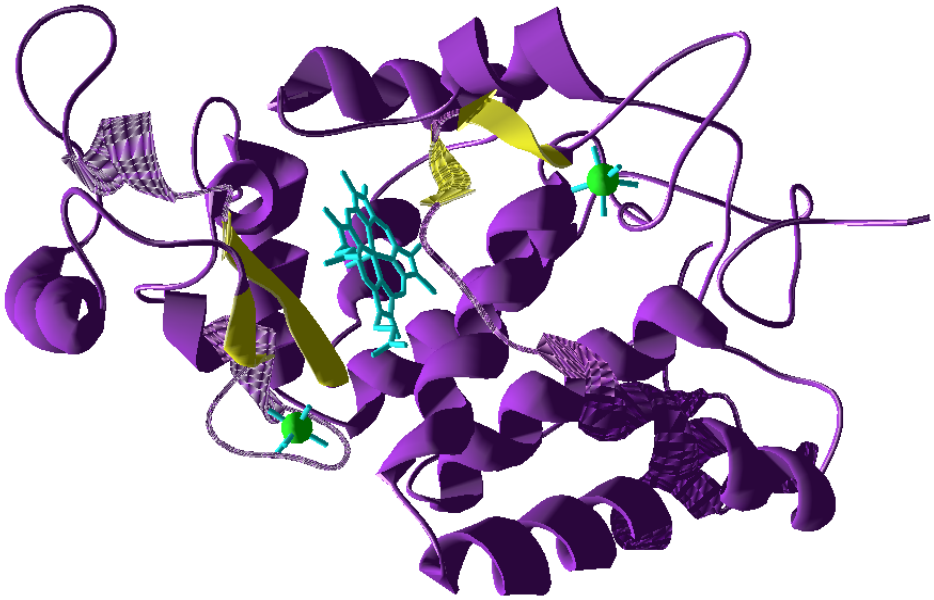


Figure 1.5. *Horseradish peroxidase; haem is light blue, calcium is green (Protein Data Bank ID 1H5A).*

1.2.1 General features

The most abundant of the seven isoenzymes normally referred to as horseradish peroxidase is the C isoenzyme (HRP-C). It consists of 308 amino acid residues [28-30] and its sequence was determined by Welinder in 1976 [30, 31]. HRP-C has molecular mass of 44,100 kDa. In the centre of the enzyme is a haem-group that is critical to its catalytic activity. There are four disulphide bridges in its structure, between cysteine residues 11 and 91, 44 and 49, 97 and 301, and 177 and 209. Two calcium ions are incorporated into the enzyme's structure, one proximal and one distal to the catalytic centre, and both play a part in the enzyme's stability as well as its catalytic activity. The haem group is attached to the

protein chain at His170 with a coordinate bond between a nitrogen atom of the histidine and the iron of haem (Figure 1.6).

Horseradish peroxidase is a glycoprotein with nine possible *N*-glycosylation sites and eight of those are occupied. Depending on the source of the enzyme its total carbohydrate content is between 18-22% [28].

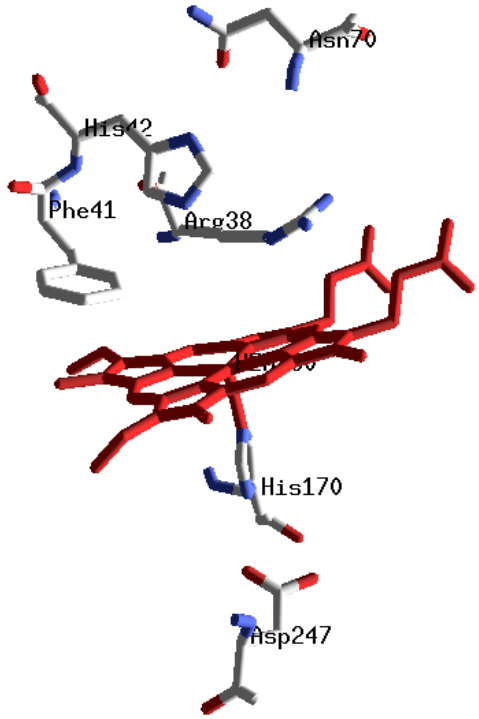


Figure 1.6. *The catalytic centre of HRP. Haem (red) and amino acids that take direct part in the catalytic activity.*

1.2.2 Biological roles of peroxidases

The functions of peroxidases in plants include regulation of hydrogen peroxide concentration and, due to their usual catalytic activity, they probably take part in cross-linking reactions where radicals are produced from reducing substrates which makes substrate polymerisation likely. The formation of lignin, a necessary component of plants' cell walls, from its phenolic monomer starting material utilises a peroxidase. The wounding of plant tissue increases expression of peroxidases to encourage cross-linking to repair damage [28].

1.2.3 Haem

Haem consists of an iron atom centred in a protoporphyrin IX ring. The protoporphyrin IX ring is a porphyrin derivative consisting of four pyrrole rings linked by methane bridges (Figure 1.7). Haem is responsible for the oxygen binding ability of haemoglobin and

myoglobin. Oxygen binds to the iron atom of haem and is transferred to the tissues where it is either used directly for respiration or bound to myoglobin in the muscles. Haem gives blood its red colour. In haemoglobin and myoglobin the iron atom is normally in the Fe(II) oxidation state both when it is bonded to- and free of oxygen [32].

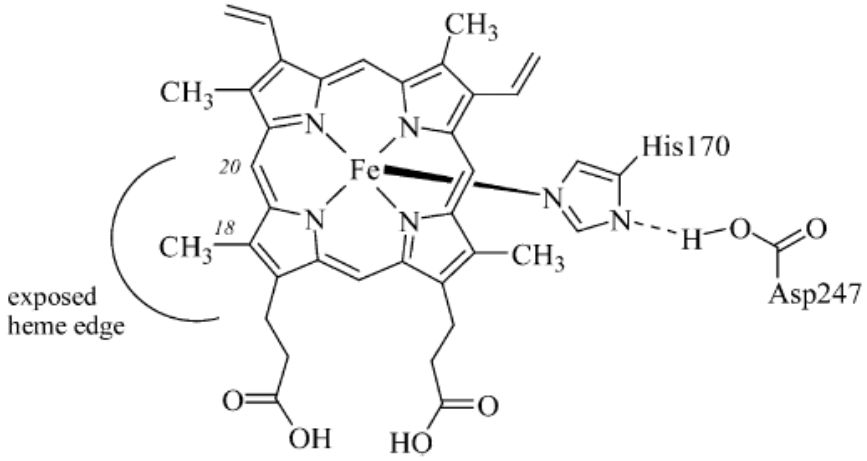


Figure 1.7. *The haem group, amino acids His170 and Asp247 and the exposed haem edge where reducing substrates can access the catalytic centre [28].*

1.2.4 Catalytic activity

Horseradish peroxidase catalyses the oxidation of aqueous aromatic compounds (for example phenol) by hydrogen peroxide. The general sequence of reactions for horseradish peroxidase is as follows:

$$E + H_2O_2 \rightarrow E_1 + H_2O \quad (1)$$
$$E_1 + PhOH \rightarrow E_2 + PhO\cdot \quad (2)$$
$$E_2 + PhOH \rightarrow E + PhO\cdot + H_2O \quad (3)$$

E is the enzyme in its resting state, E_1 (compound I) is the enzyme after oxidation by hydrogen peroxide. Compound I then binds to phenol (PhOH) at the exposed haem edge (Figure 1.7) and subsequently oxidises it and releases the phenol radical (PhOH·) into the solution and is then in the E_2 (compound II) state. Compound II then oxidises another phenol molecule and by doing so returns to its resting state (E) [33]. When the enzyme has formed a bond with hydrogen peroxide but not broken the O-O bond the hydrogen peroxide-enzyme complex is called compound 0. Too high concentration of hydrogen peroxide leads to formation of a reversibly inhibited enzyme, called compound III. When the concentration of hydrogen peroxide decreases the enzyme slowly returns to its resting state.

During the catalytic cycle of HRP its haem-iron takes two different oxidation states while the enzyme itself goes through five different oxidation states [34]. In its resting state the enzyme contains Fe(III) and when the enzyme's catalytic cycle begins the iron forms a bond with one of the two oxygen atoms of hydrogen peroxide. When the bond is formed the iron becomes Fe(IV), water is released as a product of the reaction and compound I is

formed. For the second step of the reaction a reducing substrate (such as phenol) is needed. Compound I is reduced and a radical is formed (PhO· for example) producing compound II. Compound II then undergoes a similar reaction, producing another radical and returning the enzyme to its resting state and the iron back to Fe(III). Both compound I and compound II are very powerful oxidants with redox potentials of about 1 V [28, 34].

The roles of individual amino acid residues have been determined, usually by site directed mutagenesis. Histidine 170 is connected to the iron of haem via the Nε2 atom of the imidazole ring. Aspartic acid 247 keeps the imidazolate character of His170 by accepting protons from the Nδ of the imidazole ring. In haemoglobin and myoglobin histidine is close to the haem iron and at neutral pH it has a neutral charge and is available as a proton acceptor. Thereby it acts as a proton trap, preventing protons from accessing the iron [32]. In a His170Ala mutant no formation of compound I and II is observed since alanine's side chain, a methyl group, cannot form a bond with the iron [28].

Arginine 38 plays a role in formation of compound I by promoting the cleavage of hydrogen peroxide's O-O bond and then donating a proton to the β-oxygen (the oxygen atom not bound to the iron) of the hydrogen peroxide. It subsequently takes part in a hydrogen bond with the ferryl oxygen (the oxygen atom of hydrogen peroxide bonded to the iron of the haem group). It also has functions in stabilisation and binding of ligands to the active site. When Arg38 was replaced with leucine, a nonpolar amino acid, no cleavage of the O-O bond was observed and the formation of compound 0 was not detected [35].

Histidine 42 accepts a proton from hydrogen peroxide during the formation of compound I and takes part in the formation of important stabilising hydrogen bonds near the active site. It is also important in the binding and stabilisation of ligands. Asparagine 70 takes part in the catalytic activity of the enzyme by maintaining the basicity of His42 through a hydrogen bond. When a His42Ala mutant was produced the rate of compound I formation decreased by a factor of 10^6 [36]. The formation of compound I for an Asn70Val mutant was 30-fold slower than for the wild type enzyme [28].

Phenylalanine 41 plays its part by preventing access of reducing substrates to the ferryl oxygen of compound I at the exposed haem edge (Figure 1.7). When mutants with alanine, leucine or threonine instead of phenylalanine were made the transfer of oxygen increased since phenylalanine shields the ferryl oxygen from the substrate more efficiently than the other amino acids and therefore encourages oxidation instead of oxygenation[28, 37].

1.2.5 The two calcium ions

Figure 1.8 shows two calcium ions in the structure of HRP. The calcium ions play roles both in structural stability and in the enzyme's activity. One calcium ion is distal to the active site of the enzyme while the other is proximal to the active site (Figure 1.8). Both calcium ions are seven-coordinate with oxygen-donor ligands of amino acid residues in the ions' surroundings. The distal calcium ion is in direct interaction with His42 and the proximal calcium ion is in direct interaction with His170, both important in the catalytic activity of the enzyme [28].

Calcium depletion of HRP results in 40% decrease in the enzyme's activity [28] and when one mol of calcium is added to one mol of calcium-free HRP the activity increases to about 80% of the wild type activity. This indicates that the two calcium ions are not equally important and that calcium depletion with EDTA does not remove both calcium ions. Further addition of calcium to the calcium-free HRP does not increase the activity [38]. Howes *et al.* suggested that calcium depletion of HRP only leads to removal of one calcium ion, the proximal one (located near - and interacting with - His170), and that has great effect on the whole structure and activity of the haem pocket (the hole in the middle of the protein where the haem group is) which leads to the instability and decreased activity [39].

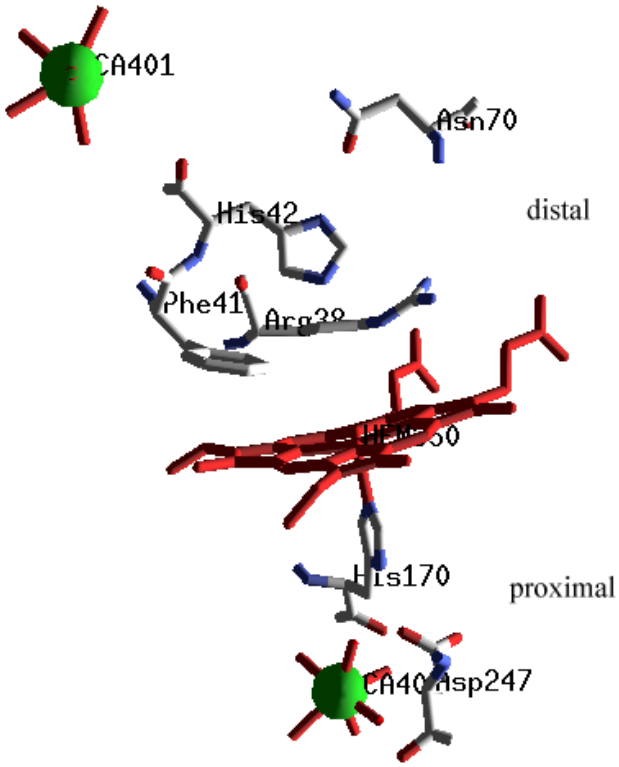


Figure 1.8. *Positions of the calcium ions (proximal and distal) and the catalytically important amino acids.*

1.2.6 Aspartic and glutamic acid residues

Carbodiimides facilitate the conjugation of carboxyl groups found on the surface of proteins and amine groups on chitosan. Aspartic acid and glutamic acid side chains contain carboxyl groups that make the residues hydrophilic and increases the possibility of them being on the hydrophilic surface of the protein rather than in the hydrophobic protein core. Horseradish peroxidase contains twenty one aspartic acid residues and seven glutamic acid residues. The positions of these residues are shown in Figure 1.9.

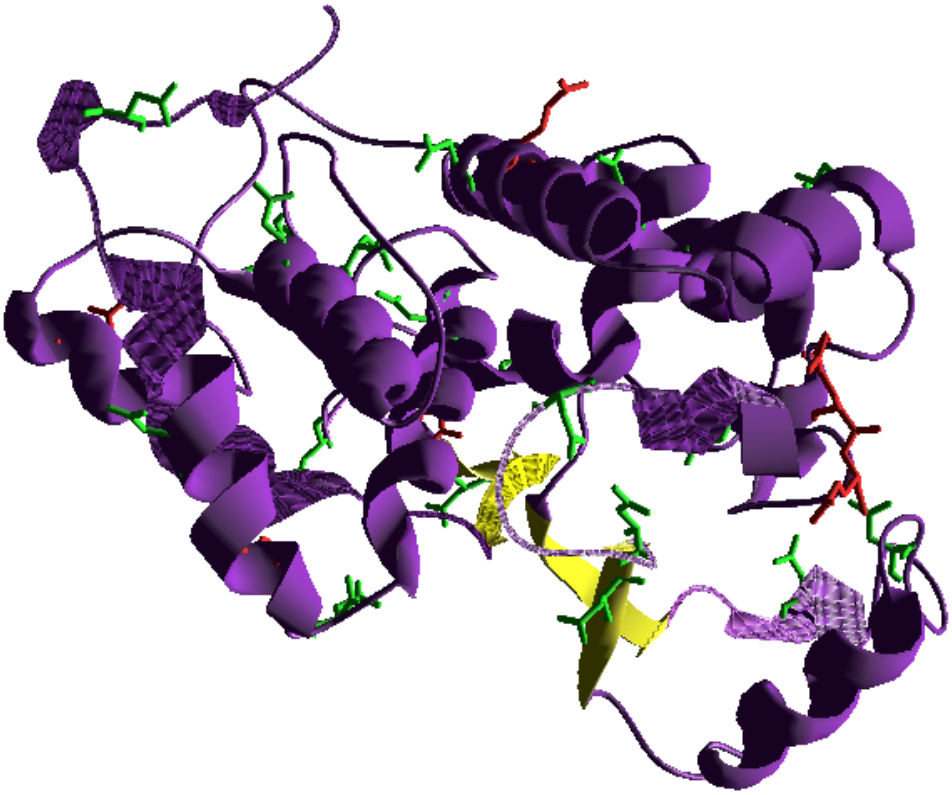


Figure 1.9. *Aspartic acid residues (green) and glutamic acid residues (red) on the surface of HRP.*

1.2.7 Stability of horseradish peroxidase

Many articles on the stability of horseradish peroxidase can be found in the literature. Thermally induced inactivation is usually observed at 60°C and above. Miland *et al.* reported that incubation of HRP at 65°C for 10 minutes in 10 mM phosphate buffer, pH 7.0, reduced the catalytic activity significantly and its relative activity (v/v_0) was about 35%. After 30 minutes the relative activity was around 10% while a modified acetylated HRP retained about 50% of its initial activity [40].

Haifeng *et al.* studied the effect of the concentration of sodium phosphate incubation buffer on HRP. It was reported that thermal stability decreases as the concentration of the incubation buffer increases. Horseradish peroxidase incubated in 2.5 mM sodium phosphate buffer at 70°C for 30 minutes retained 80% of its initial activity while HRP incubated at the same temperature for the same period of time in 30 mM sodium phosphate buffer only retained about 60% of its initial activity. They also reported that HRP did not show signs of aggregation during its denaturation, proved by differential scanning calorimetry data [41].

Chang *et al.* found that after 30 minutes of incubation at 70°C in 50 mM phosphate buffer at pH 7.0 HRP's relative activity was 20-30%. Addition of 10% sucrose, lactose, glucose and fructose decreased thermal stability at 90°C significantly, compared to native HRP [42].

Hassani *et al.* measured loss of activity at 70°C in 20 mM phosphate buffer at pH 7.0. After 30 minutes of incubation about 10% of the initial activity remained while HRP with citraconic anhydride modified lysine residues in 20% w/v sorbitol retained about 80% of its initial activity [43].

Generally the thermal inactivation of HRP was observed in the temperature-range mentioned in the examples above. This was not always the case. Tams and Welinder reported on the difference in thermal stability of HRP and deglycosylated HRP. Both types of HRP lost all activity during 15 minutes of incubation at 70°C in 50 mM sodium citrate buffer at pH 6.0. It was also shown that thermal stability of HRP and deglycosylated HRP was identical while denaturation by guanidinium chloride was much faster for the deglycosylated HRP [44]. Liu *et al.* found that 30 minutes of incubation at 50°C in 0.1 M phosphate buffer at pH 7.0 gave relative activity of about 70% [45]. Mogharrab *et al.* reported that incubation in 0.2 M phosphate buffer at 70°C for 10 minutes resulted in complete inactivation of the enzyme [46].

Asad *et al.* studied the effect of additives on thermal stability of HRP and recombinant HRP (HRP that is not glycosylated). They found that in 10 mM phosphate buffer at pH 7.0 the half life of HRP at 80°C is 35 minutes. When the thermal stability was measured in 10 mM phosphate buffer, pH 7.0, with the addition of 60% trehalose the half life at 80°C increased to about three hours. Recombinant HRP was considerably less stable against thermal inactivation compared to wild type HRP. The recombinant HRP showed the same behaviour towards concentration of incubation buffer and its half life was 40 minutes at 50°C in 200 mM buffer and 87 minutes in 10 mM buffer. The recombinant enzyme showed significantly greater tolerance towards high concentration of hydrogen peroxide in 200 mM buffer than in 10 mM buffer. It is reported that this is because of the scavenging function of phosphate buffer that protects the unglycosylated enzyme. No difference in hydrogen peroxide tolerance was shown for the native enzyme at these two different concentrations of incubation buffer [47].

Mahmoudi *et al.* found that the addition of histidine and tyrosine to HRP reaction solutions with high concentrations of hydrogen peroxide partly prevented the enzyme from forming its inhibited compound III. This level of hydrogen peroxide concentration does not occur under physiological conditions but in biotechnological and clinical applications the increased peroxide tolerance can be of great benefit [48].

1.2.8 Applications of horseradish peroxidase

One of the most important applications of HRP is its use in biosensors. A biosensor combines a biological molecule, for example an enzyme, and a transducer that produces an electronic signal to indicate the level of activity that the biological molecule is undergoing. The biological molecule can be HRP and the signal can be the presence of glucose, ethanol or hydrogen peroxide [29]. Lomillo *et al.* made a glucose biosensor by creating a system of glucose oxidase and horseradish peroxidase with ferrocyanide as mediator. When the biosensor comes into contact with glucose the glucose is oxidised by glucose oxidase, leaving the enzyme reduced. Glucose oxidase is then oxidised by O_2 which is subsequently reduced to hydrogen peroxide. The hydrogen peroxide oxidises the reduced form of HRP producing oxidised HRP. The oxidised HRP then finally oxidises the ferrocyanide mediator and the signal is measured [49]. This can also be done by spectroscopic methods, for example by using the same setup as Lomillo *et al.* but inserting a chromogenic substrate for the enzymatic reaction instead of a mediator that transduces the electrical signal [50]. Horseradish peroxidase is also very common in enzyme-linked immunosorbent assay (ELISA) as mentioned in part 1.4.4. Many types of peroxidases have been used for removal of phenol and other aromatic compounds from waste water. When peroxidases are in solution with hydrogen peroxide and phenol a free radical phenol is created, forming a solid phenol polymer that can then easily be removed from the waste water [51, 52]. The production of synthetic dyes, for example in colour photography and textile dyeing, results in considerable amount of waste that contains complex aromatic compounds [53].

1.3 Bioconjugation

Biological molecules have very specific, yet diverse, properties. While one protein can have an extremely good affinity towards a certain target molecule, the protein cannot be localised. Another protein that catalyses a fluorescent reaction or a reaction forming coloured products is easily localised but has no affinity towards the target molecule. With the combined qualities of these two proteins the target molecule can easily be found. This is where bioconjugation comes into play. By connecting two different biological molecules together the new molecule ideally possesses a combination of the unique qualities of both its subunits. Since proteins are often unstable in solutions due to concentrations of other solutes, temperature, acidity or alkalinity and other environmental factors it can be of benefit to make bioconjugates with some support material, for example sugars or even the protein itself [54].

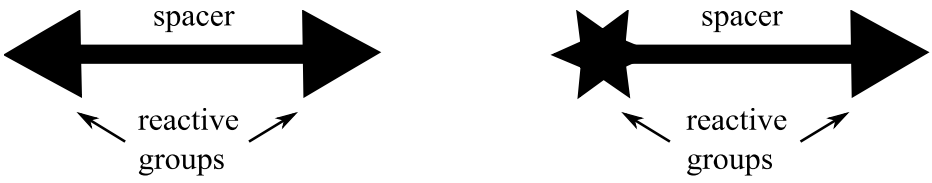
When bioconjugation of proteins is designed, many factors need to be considered. The most important one is to maximise the retention of the protein's activity. Preferably the conjugate has all the activity of the native protein and the added functionality of the molecule conjugated to it. To avoid losing activity the site of conjugation must not be near the active site of the protein, i.e. the catalytic centre of enzymes, the antigen binding sites of antibodies and so on. The active sites of proteins can be protected by adding a reversible inhibitor to the reaction mixture if no reactive agents are available that avoid conjugation near the active site.

Another factor is the type of crosslinking reagent, i.e. the reagent responsible for joining the two biological molecules together. The crosslinkers are usually made up of two reactive groups connected by a spacer. These two reactive groups can either be identical, called homobifunctional crosslinkers, or different, called heterobifunctional crosslinkers. A common problem when homobifunctional crosslinkers are used is the production of unwanted conjugates consisting of two or more molecules of the same protein. To minimise the effect of this problem the conjugation is often carried out in a single step where all the reactants are present in the reaction solution for the whole duration of the reaction and the concentration of the less important conjugation reactant is kept relatively higher than the more important one.

When the single step procedure is not feasible, a two-step procedure is possible by activating one reactant first with the crosslinker before adding the second reactant. Two-step procedures are difficult due to hydrolysis of the activated intermediate. Polymerisation of the first reactant can reduce the efficiency of the conjugation, compared to a single-step procedure.

To limit the polymerisation a heterobifunctional crosslinker can sometimes be used. Ideally the heterobifunctional crosslinker has one reactive group that reacts with a specific group on the first reactant and the other reactive group of the crosslinker is unaffected. Then this activated intermediate can be purified and then the second reactant is added, making a conjugate without the undesired polymerisation. To make this possible the second reactant needs to have a very specific active group that is not found on the first reactant.

An important property of bifunctional crosslinkers is the length of the spacer (Figure 1.10) and what the crosslinker contributes to the conjugate. When making a conjugate to produce an immune response the presence of a linker between the conjugated molecules can interfere with the response since some of the antibodies can have specificity for the crosslinking agent. This problem is eliminated when zero-length crosslinkers are used. Zero-length crosslinkers contribute nothing to the bond itself, they only mediate the formation of the covalent bond.



homobifunctional crosslinker heterobifunctional crosslinker

Figure 1.10. *Homobifunctional crosslinker with identical reactive groups and a heterobifunctional crosslinker with different reactive groups. Reactive groups are separated by a spacer.*

A third possibility exists which is a trifunctional crosslinker. As the name indicates trifunctional crosslinkers contain three different functional groups, each capable of connecting to a different molecule.

1.4 Examples of bioconjugation

The unique and highly selective catalytic properties of enzymes under mild reaction conditions are very favourable for many production processes. Since enzymes work under such mild conditions they can be susceptible to denaturation with increased temperature, extreme acidity or alkalinity or an increase in concentration of another solute. It is therefore very important to find ways to increase stability while maintaining enzyme activity.

1.4.1 Glycosylation

When membrane-associated proteins and proteins destined for secretion have been synthesised in the ribosomes of eukaryotes, oligosaccharides are usually attached to them in a process called glycosylation. The role of oligosaccharides can be to help the protein improve its physicochemical characteristics such as solubility, thermal stability and prevent it from proteolytic enzymes. The oligosaccharides can also have a role in binding to receptors [27]. Because glycosylation plays a role in protein stability it is logical to see if further glycosylation of proteins increases their stability.

Jafari-Aghdam *et al.* found that deglycosylated glucoamylase had worse thermal stability; aggregated more easily because of less hydrophilicity (the saccharides are hydrophilic) but the deglycosylated form was more resistant to alkalinity than the native form. The deglycosylated form was also more sensitive to proteolysis because the oligosaccharides

shield the protein chain from the proteolytic enzyme. Furthermore the deglycosylated protein is more flexible, giving the proteolytic enzyme better access to the site of proteolysis [55].

Pham *et al.* attached glucose to trypsin and chymotrypsin and increased thermal stability significantly (T_m was increased by about 5°C; CD measurements) while also making trypsin more stable towards autolysis [56]. It is therefore clear that glycosylation can increase the stability of proteins against many different threats.

1.4.2 Immobilisation of enzymes

Enzymes can be immobilised on supports or conjugated to other molecules in solution. Immobilisation makes separation of enzymes from their reaction products easy and allows for reuse of enzymes that are often expensive. Porous support materials can be used for enzyme immobilisation and will ideally have a large internal surface with good surface density of reactive groups. The reactive groups of the support material should also be suitable for reactions with common reactive groups on the surface of proteins and be able to form stable covalent bonds with the protein. When the support material has high density of reactive groups a multipoint immobilisation can occur. Multipoint immobilisation can make the protein structure more rigid and therefore more stable towards denaturation.

Figure 1.11. *Immobilised enzymes with a single linker (left) and multipoint immobilised enzyme (right).*

When enzymes are immobilised on a porous support material some factors that reduce activity can be eliminated. When the enzymes are conjugated to the support material it prevents them from aggregating when they start to lose their structure while also preventing proteolysis caused by possible proteases in the solution, since they will also be immobilised [57].

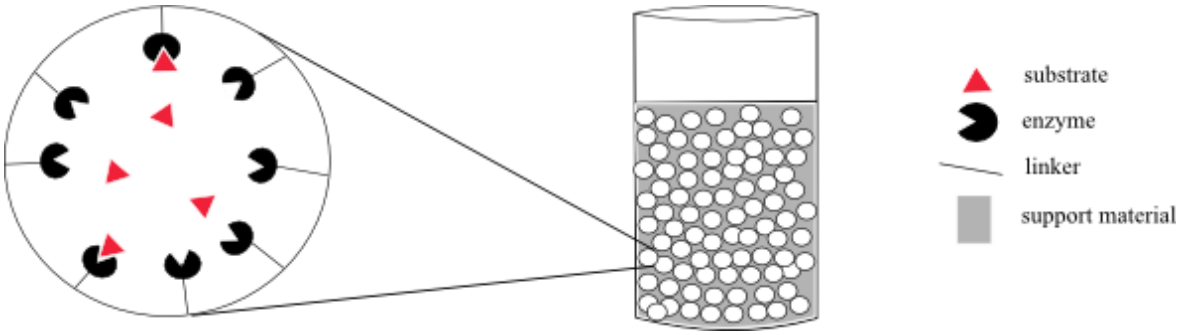


Figure 1.12. *Enzymes immobilised on a porous support material.*

To maintain as much activity as possible when conjugating an enzyme to a solid support it can be beneficial to include a reversible inhibitor for the enzyme. The inhibitor binds to the active site of the enzyme and prevents active groups near the active site from participating in the immobilisation bond formation.

Tardioli *et al.* reported that aminated glucoamylase showed significant increase in thermostability when immobilised on glyoxyl-agarose support. Amination of aspartic and glutamic acid residues on the enzyme surface leads to a significant increase in thermostability compared to regular glucoamylase immobilised on the same type of support material. This increase is attributed to improved covalent immobilisation that makes the enzyme more rigid [58].

1.4.3 CLECs and CLEAs

Crosslinked enzyme crystals (CLECs) are one form of immobilised enzymes. In this case the enzyme acts as both the catalyst and the support material. Inter-molecular crosslinks between enzyme molecules are made with bifunctional crosslinkers and this, as before, leads to a more rigid structure and increased stability [54]. This method requires pure, crystallised enzyme samples to form the crosslinked enzyme. Another method, described by R.A. Sheldon, precipitates the enzyme from aqueous buffer and the enzyme aggregates are then crosslinked, forming CLEAs or crosslinked enzyme aggregates [59, 60].

Crosslinked enzyme crystals and aggregates can show reduced activity when their crosslinked units are too large, decreasing the access of substrate to the enzyme's active sites. When CLEAs are formed with low concentration of the target enzyme in the buffer solution or when the enzyme is vulnerable to the high concentration of crosslinking agent addition of other proteins, such as bovine serum albumin, to the solution can increase the activity of the product [61].

1.4.4 ELISA

Enzymes are widely used as detection components in assay systems because of their unique ability to catalyse reactions with easily detectable characteristics. This makes assays such as the well known enzyme-linked immunosorbent assay (ELISA) possible. Enzymes that can catalyse reactions resulting in the formation of a coloured product are conjugated to an antibody. The antibody-enzyme conjugate is then introduced to a surface where a mixture of molecules has been immobilised, perhaps the antigen, and then the surface is washed. If the antigen is present, immobilised on the surface, the antibody part of the antibody-enzyme conjugate will bind to the antigen and when the substrate of the enzyme is added, a colour signal can be used to verify the presence of the target molecule.

1.5 Carbodiimides

Carbodiimides are zero-length crosslinkers that mediate the formation of amide linkages between carboxylates and amines or phosphoramidate linkages between phosphates and amines. They are probably the most used zero-length crosslinkers since they can form conjugates between two protein molecules, a peptide and a protein or any other biomolecules containing these active groups. Carbodiimides are therefore ideal for conjugating the amine group of chitosan to the carboxyl group of proteins.

Carbodiimides consist of a central carbon atom that is made electron deficient by adjacent nitrogen atoms (Figure 1.13). Properties of the R and R´ groups dictate the solubility of carbodiimides. Water soluble carbodiimides are used for most biochemical conjugations while water insoluble carbodiimides are used for peptide synthesis and other conjugations of water insoluble molecules [62].

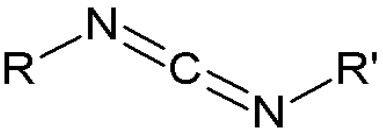


Figure 1.13. *The general structure of carbodiimides.*

One of the nitrogen atoms adjacent to the central carbon atom can accept a proton from a carboxylic acid, leaving the carboxylate negatively charged. The carboxylate then attacks the central carbon atom of the carbodiimide forming an unstable O-acylisourea. This O-acylisourea can then either be hydrolysed or form an amide bond with a nucleophilic amine. The formation of an amide bond can be made more efficient with the addition of the water-soluble sulpho-N-hydroxysuccinimide (NHSS). NHSS reacts with the O-acylisourea to form a NHSS-ester intermediate that is more soluble and more stable than the o-

acylisourea. NHSS is a good leaving group when an nucleophilic amine attacks the carbonyl group of the ester, forming an amide bond [62].

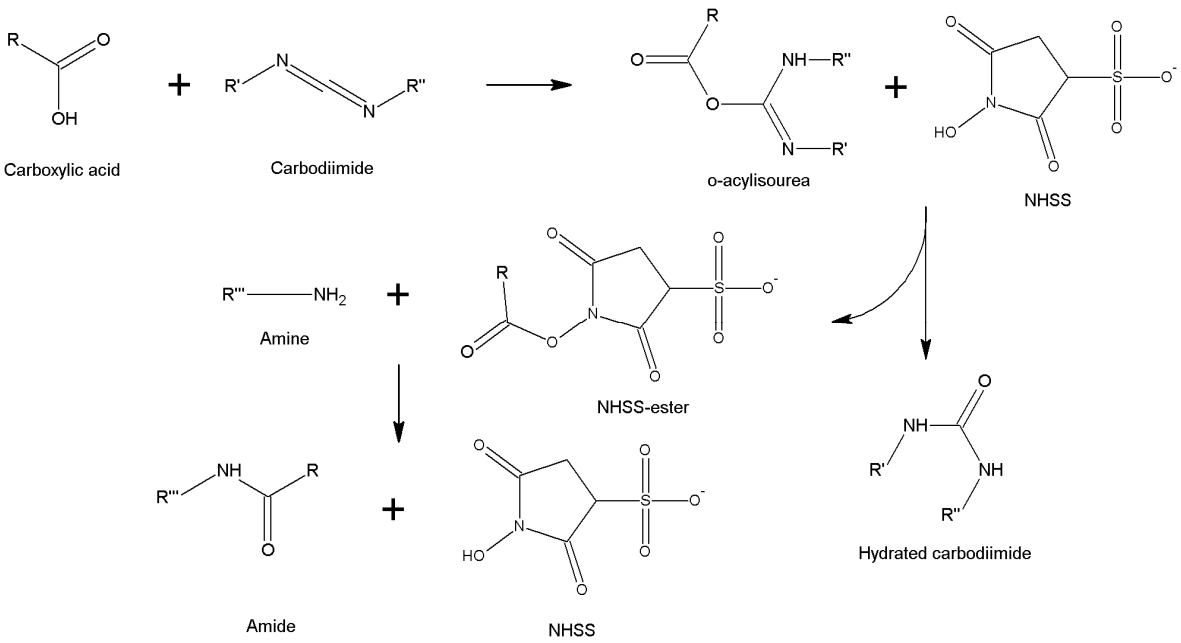


Figure 1.14. *Conjugation of a carboxylic acid to an amine, forming an amide, with the assistance of a carbodiimide and NHSS.*

Figure 1.14 shows how the O-acylisourea intermediate is formed with the activation of a carboxylate group with a carbodiimide. Then the O-acylisourea intermediate is reacted with NHSS forming an NHSS-ester intermediate that is more efficient in reacting with the amine. This conjugation method is suitable for conjugation of the amine groups of chitosan with the carboxylates of proteins (aspartic acid and glutamic acid).

1.5.1 EDAC

EDAC, also called, EDC or N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride is the most popular water-soluble carbodiimide for conjugating biological carboxylates and amines. It has a molecular weight of 191.71 g/mol and is therefore easily removed from protein conjugate reactions with dialysis or gel filtration. The efficiency of EDAC is based on reaction pH with carboxylate activation most successful at pH 3.5-4.5 but amide bond formation optimum at pH 4-6. EDAC is hydrolysed at acidic pH so compromises must be made when deciding the reaction conditions, and pH in the range of 4.7-6.0 is usually chosen.

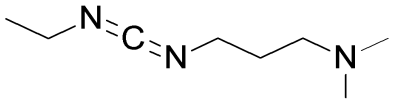


Figure 1.15. *Structure of EDAC.*

1.6 NHSS

NHSS or sulpho-N-hydroxysuccinimide is a water soluble crosslinker that is often used along with carbodiimide for the conjugation of carboxylates and amines. When the carbodiimide has activated the carboxylate to make an O-acylisourea the formation of amide bond is often unsuccessful because of hydrolysis of the ester bond. NHSS can react with the O-acylisourea and make a more stable NHSS ester. NHSS is also a good leaving group when a nucleophilic amine attacks the carbonyl of the ester bond.

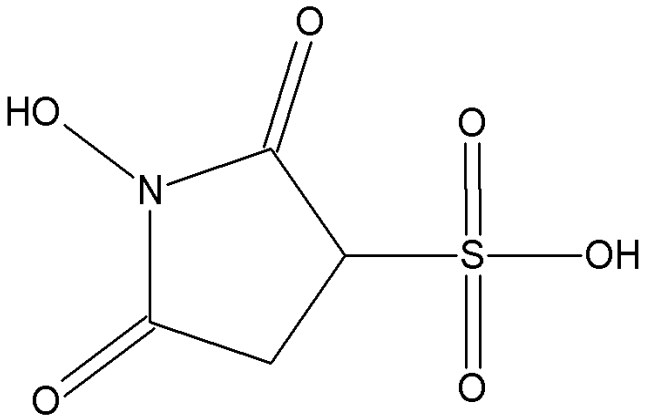


Figure 1.16. *Structure of NHSS.*

NHSS contains an SO_3^- group (Figure 1.16) that makes it water-soluble compared to N-hydroxysuccinimide that is water-insoluble and is often used in peptide synthesis. NHSS has a molecular weight of 115.09 g/mol and is therefore, just like EDAC, easily removed from reaction solutions by dialysis or gel filtration.

1.7 Differential scanning calorimetry

Differential scanning calorimetry is a technique that measures the difference in internal energy of two samples that the same amount of heat is applied to. The samples are in two very well insulated cells that contain exactly the same volume of solution. The two samples are identical in every way except for the component that is to be measured. One sample is therefore called the reference sample and the other is the unknown sample. The temperatures of these two samples are measured and the temperature difference is equal to the energy that the unknown component absorbs or emits. The difference can be due to various chemical changes such as crystallisation, oxidation, freezing, melting or denaturation of proteins.

In 1962 Emmett S. Watson and Michael J. O'Neill applied for a patent for differential microcalorimeter. The design of the instrument was based on an already recognised technique in thermal analysis but their instrument required smaller samples and gave more accurate results. In 1997 MicroCal introduced a new microcalorimeter that could measure samples of just 0.5 mL and with sensitivity of about 50 µg of protein. This instrument keeps the two samples at the same temperature and measures how much energy is needed to compensate for the energy absorbed or emitted by the unknown component. This

difference is called DP (differential power) and is measured in mCal/min. The data acquired in these measurements can give the heat capacity of the sample at each temperature and when the function of the excess heat capacity is integrated it equals the enthalpy of the protein‘s denaturation (Equation 1.2).

Equation 1.2

$$\Delta H(cal) - \int_{T(N)}^{T(D)} Cp \cdot \delta T$$

To be able to determine the enthalpy accurately it is very important that the sample solutions are homogenous in every way except for the component to be measured. First the reference sample is put in both cells to acquire a baseline for the instrument. Next the unknown sample is measured against the reference sample (Figure 1.17).

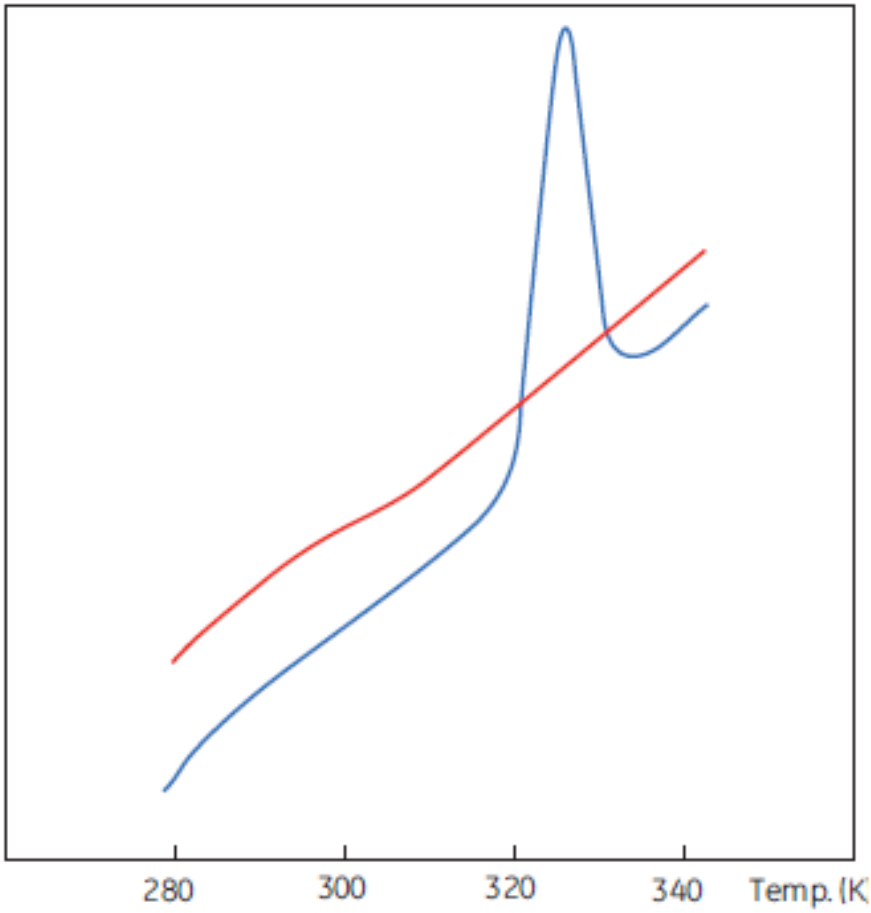


Figure 1.17. *Baseline (red) and a sample with barnase (blue). Figure from DSC: Theory and practice [63] edited to show temperatures. The y-axis shows heat capacity.*

When the data for the unknown sample has been acquired it is necessary to subtract the baseline from the data to see what effect the unknown component has on the heat capacity of the solution.

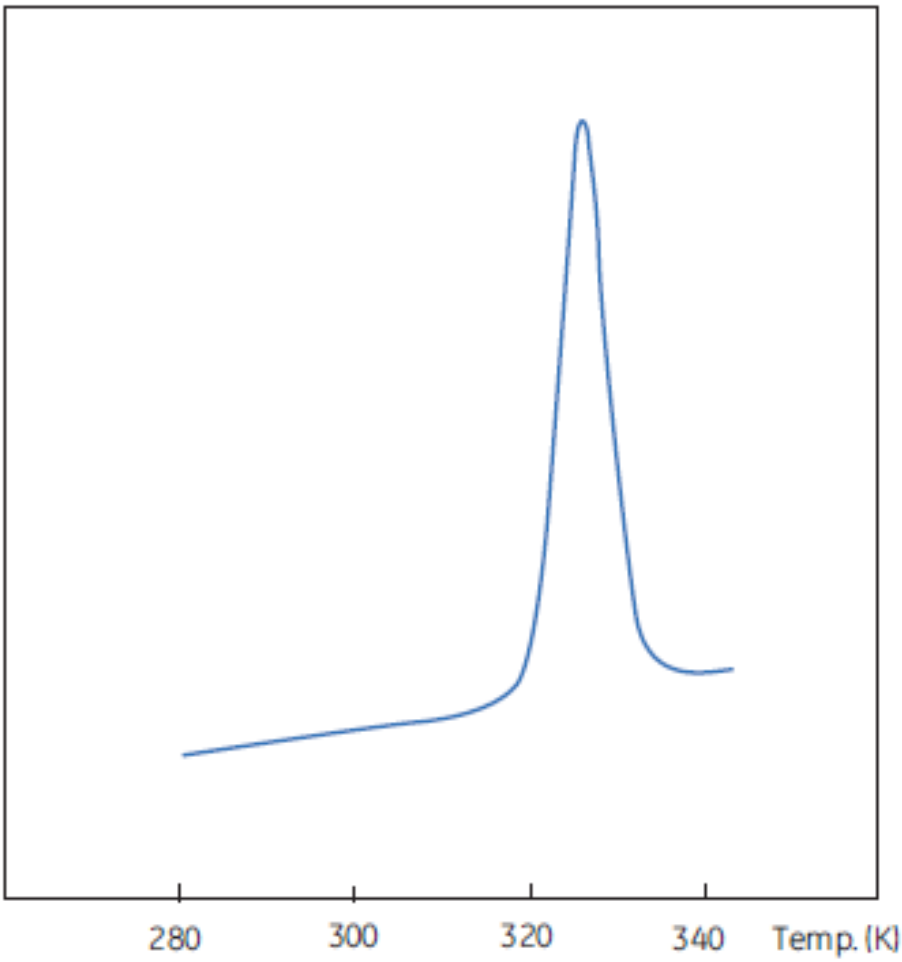


Figure 1.18. *Data for barnase after subtraction of baseline. Figure from DSC: Theory and practice [63]. The y-axis shows heat capacity.*

When the area under the curve is integrated it gives the enthalpy for the reaction given that the concentration of the sample is known. This method can therefore give information about the stability of a molecule such as a protein. When a protein undergoes denaturation many weak non-covalent bonds are broken, such as intramolecular hydrogen bonds. Differential scanning calorimetry is the best way to measure the strength of the total of these bonds. When the temperature increases, the hydrogen bonds are broken and the energy needed to break these bonds is directly related to the difference in heat necessary to raise the temperature of the protein sample compared to the reference sample. To make sure the solutions in the sample cell and the reference cell are identical except for the protein in the sample cell dialysis is sometimes necessary.

Denaturation of proteins is a complicated reaction on molecular level. Hydrophobic amino acids on the inside of proteins in aqueous solutions become exposed when the protein loses its structure and this can lead to exothermic aggregation of denatured proteins. The calorimeter can of course not distinguish between the endothermic breakages of

intramolecular bonds so the data can be very chaotic. Aggregation can sometimes be prevented by altering the pH or the ionic strength of the solution, slower or quicker scan rate or by decreasing the concentration of the protein [64, 65].

1.8 Gel filtration

Small molecules can be separated from larger molecules using gel filtration. Columns can be packed with porous material such as Sephadex G-25 which consists of cross-linked dextran. This solid column matrix along with an appropriate solution can be used for the separation of small and large molecules since larger molecules have access to smaller volume of the column solution. This causes the larger molecules to travel through the column quicker than the small molecules. This can be used for buffer exchange or removal of small molecules such as salts from protein solutions. When using reactive agents such as carbodiimides that can cause crosslinking of proteins it is important to be able to stop the reaction when appropriate. By using a desalting column the horseradish peroxidase (MW 44.000 g/mol) travels through the column quicker than the EDAC (MW 192 g/mol) and can therefore be removed with a simple and rapid process. PD-10 desalting columns from GE Healthcare are pre-packed and ready to use. They contain Sephadex G-25 medium and Figure 1.19 shows their desalting capacity using bovine serum albumin (MW 67,000 g/mol) and sodium chloride (MW 23.0 g/mol and 35.5 g/mol respectively).

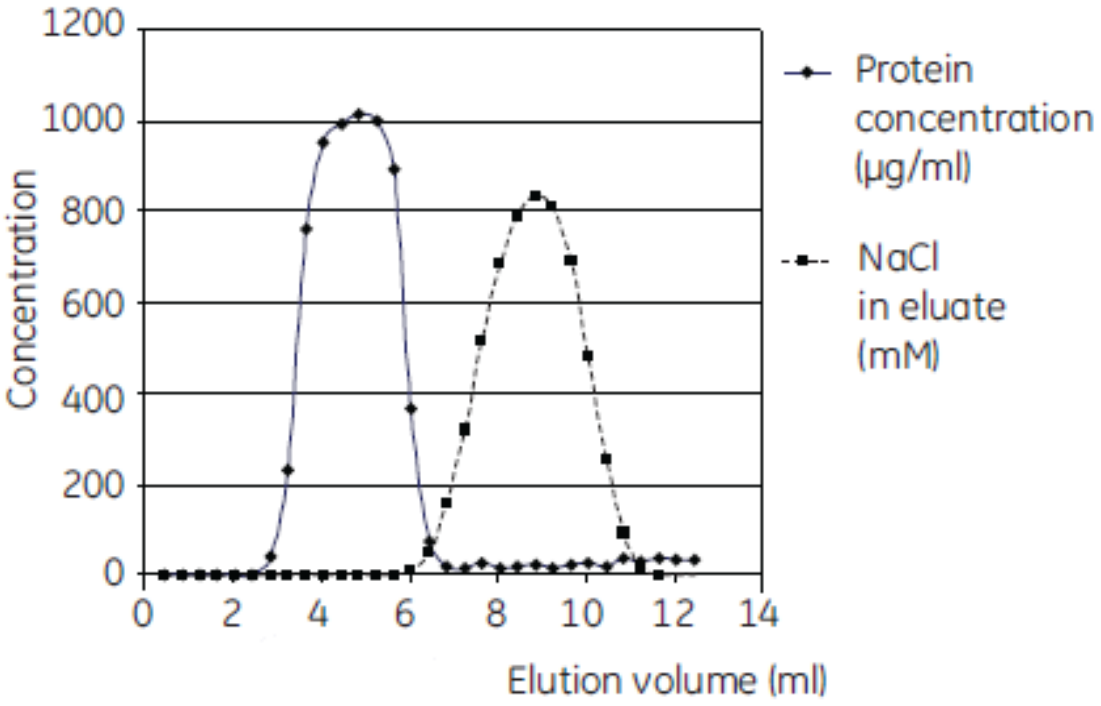


Figure 1.19. *Desalting capacity of PD-10 desalting column with bovine serum albumin and sodium chloride [66].*

1.9 Dialysis

Another method to separate large and small molecules is dialysis. Semipermeable membranes are used to make a tube containing a solution of large and small molecules. The membrane of the tube has pores of a predefined size that allows molecules of a certain maximum size to diffuse through the membrane. The tube is then placed in a large volume of appropriate buffer solution. Molecules larger than the pores are trapped inside the tube and given enough time diffusion of small molecules equilibrates their concentration inside and outside of the tube (Figure 1.20). If a tube containing 2.5 mL of sample is placed in 1000 mL solution the concentration of molecules small enough to escape the tube can be decreased 400-fold. This can be repeated if more purity is necessary.

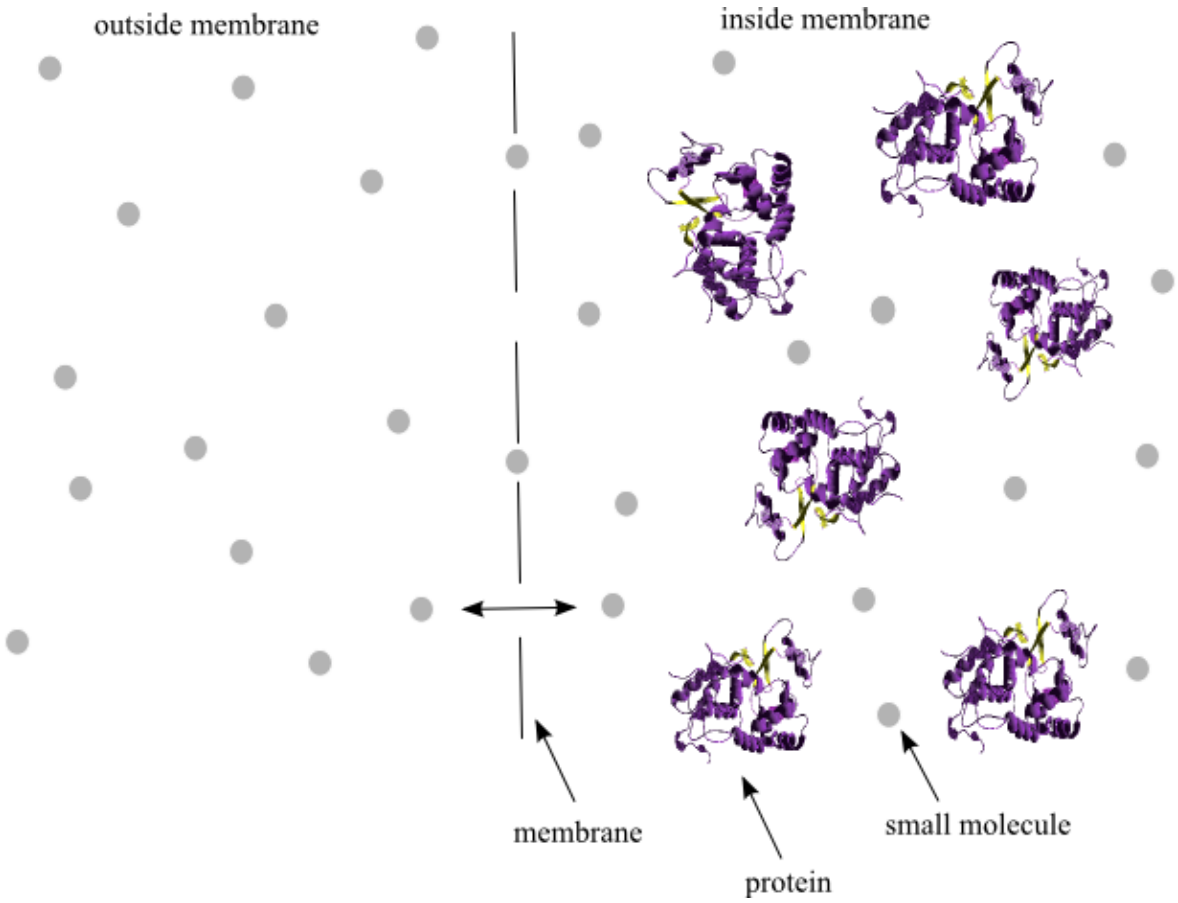


Figure 1.20. *Dialysis can be used to separate small and large molecules, for example in desalting and buffer exchange.*

1.10 Quantification of carbohydrates

Reducing carbohydrates can be quantified by phenol-sulphuric acid reaction reported by Dubois *et al.* in 1956. The method uses concentrated sulphuric acid to break up poly- and oligosaccharides to monosaccharides that are dehydrated and can then react with phenol forming an orange product that can be quantified by measuring the absorbance of the solution (Figure 1.21) [67].

The method described by Dubois *et al.* to determine the amount of carbohydrates using phenol and sulphuric acid was carried out as follows: A sample containing carbohydrate, either a standard or an unknown sample, with volume of 2.0 mL was added to a test tube. Then about 100 mg of phenol dissolved in water were added and finally 5 mL of concentrated sulphuric acid were added rapidly to make sure the solution was sufficiently mixed. The solution becomes very hot and is allowed to stand for 10 minutes at room temperature to cool down. Then it is placed in a water bath for 10 to 20 minutes and then the absorbance is determined at 490 nm in the microplate reader, with 300 µL in each well.

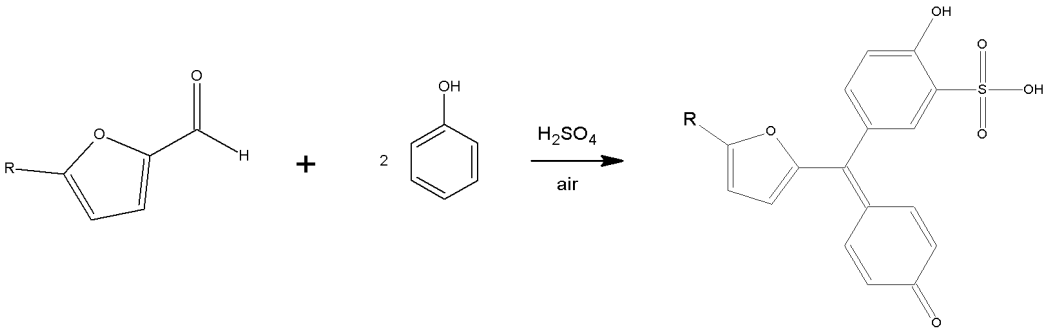


Figure 1.21. *The reaction of a dehydrated monosaccharide with phenol and sulphuric acid for colorimetric quantification of reducing sugars.*

Masuko *et al.* optimised the method and found that the best results were obtained by adding the sulphuric acid to the carbohydrate sample, then immediately the phenol followed by incubation at 90°C for five minutes before cooling the sample to room temperature in a water bath and finally measuring the absorbance at 490 nm [68].

1.11 Quantification of proteins

Coomassie Brilliant Blue G-250 (Figure 1.22) is a protein dye that is usually used for quantification of proteins. It has a red colour at low pH when all nitrogens are protonated. When it binds to protein it does so in its anionic form and has a blue colour with maximum absorbance at 595 nm [69].

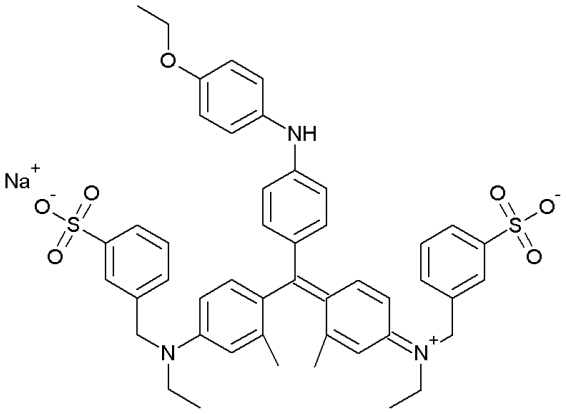


Figure 1.22. *Structure of Coomassie G-250.*

To quantify proteins in a solution it is necessary to obtain a standard curve that unknown samples can be compared with. If the protein solution to be quantified is homogenous, i.e. no other proteins are in the solution, most accuracy will be obtained if the protein in its pure form is used to produce the standard curve. If more than one type of protein is in the solution other methods are necessary. Usually a solution of bovine serum albumin with known concentration is used as standard and an assumption made that the ratio of amine-containing side chains of the unknown protein sample and the bovine serum albumin sample are the same.

Bovine serum albumin has a rather high ratio of amine groups and is therefore not the ideal protein for producing a standard curve, especially if the unknown protein is low in amine groups. This can be corrected by using a mixture of five parts bovine serum albumin and three parts immunoglobulin G.

2 Materials and methods

2.1 Materials

Horseradish peroxidase, N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride (EDAC), sulpho-N-hydroxysuccinimide (NHSS), 2-[N-morpholino]ethanesulphonic acid monohydrate (MES), 3-[N-morpholino]propanesulphonic acid (MOPS), 4-aminoantipyrine (4-AAP), hydrochloric acid, Tween 20, Coomassie Brilliant Blue G, methanol and sodium chloride, trypsin from bovine pancreas, Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), triethanolamine (TEA), calcium chloride and Nα-benzoyl-DL-arginine p-nitroanilide (BAPNA) were purchased from Sigma. Potassium dihydrogen phosphate, sulphuric acid, phenol, hydrogen peroxide, perchloric acid and boric acid were purchased from Merck. Sodium hydroxide was purchased from Acros Organics, PD-10 desalting columns were purchased from GE Healthcare, 96 well microplates were purchased from Thermo Fisher Scientific and potassium acetate was purchased from BDH Chemicals. Porous membrane tubes with molecular weight cut off of 12.-14.000 Da were purchased from Spectrum Medical Industries. Whatman No. 4 filter paper was purchased from Whatman International. The chitosan samples were supplied by Genís ehf. Tchos (therapeutic chitosan) with the batch number G0601217K is enzymatically degraded chitosan currently being tested for therapeutic application (Appendix B. Chitosan certificates).

2.2 Methods

2.2.1 Spectrophotometry and microplate reader

Two spectrophotometers were used in this study. The first is a Heλios α from Thermo Electron Corporation with its program Vision. Heλios α is a spectrophotometer that can measure absorbance at any given wavelength between 190.0 and 1,100.0 nm and can both scan the wavelength spectrum or measure the change in absorbance over time at a fixed wavelength. The Heλios α spectrophotometer was used for single absorbance measurements, for example at 280 nm for rough estimation of protein concentration.

The other spectrophotometer used was a ThermoMax microplate reader from Molecular Devices using the SOFTmax software. Microplate readers can measure up to 96 samples simultaneously in 96-well microplates that hold up to 300 µL of sample solution in each well. By measuring the change in absorbance (A) of a solution over time, the rate of formation of a compound, with defined molar extinction coefficient (ε), concentration (c) and distance the light travels through the solution (b), can be calculated using Beer-Lambert law (Equation 2.1 and Equation 2.2).

Equation 2.1

$$A = \varepsilon \cdot b \cdot c$$

Equation 2.2

$$\frac{\Delta A}{\Delta t} = \varepsilon \cdot b \cdot \frac{\Delta c}{\Delta t}$$

The wells can hold up to 300 µL of solution but the reactions were carried out with total volume of 250 µL making the distance that the light travels through 0.7025 cm [70]. This has to be accounted for in activity calculations. The rate is measured as v_{max} and is equal to the slope of the line formed when change in absorbance is plotted versus time. To calculate the enzyme units (U) per litre reaction solution Equation 2.3 is used.

Equation 2.3

$$\frac{Enzyme\ unit\ [\mu mol/min]}{V\ [L]} = \frac{\frac{\Delta A}{\Delta t\ [min]} \cdot 10^6 \left[\frac{\mu mol}{mol}\right] \cdot V_{total}[\mu L]}{\varepsilon\ [M^{-1}cm^{-1}] \cdot b\ [cm]\ \cdot V_{sample}\ [\mu L]}$$

Equation 2.3 gives enzyme activity as µmol per minute in one litre of reaction solution. To transform this to SI units the enzyme unit must be divided by 60 to get µkatal per second. The molar extinction coefficient for the product of this reaction is 10,730 $M^{-1}cm^{-1}$ [71]. The decrease in activity during heating was then used to estimate the denaturation of the enzyme.

2.2.2 Desalting

Using a desalting column is the easiest way to separate small molecules from larger ones. PD-10 desalting columns from GE Healthcare contain Sephadex G-25 Medium with pores small enough to exclude large molecules, such as proteins, but large enough to let smaller molecules through and therefore making larger volume of the column available for the smaller molecules. This causes the large molecules to travel quickly through the column while the small molecules travel slower. Figure 2.1 shows the desalting capacity of PD-10 column using bovine serum albumin and sodium chloride.

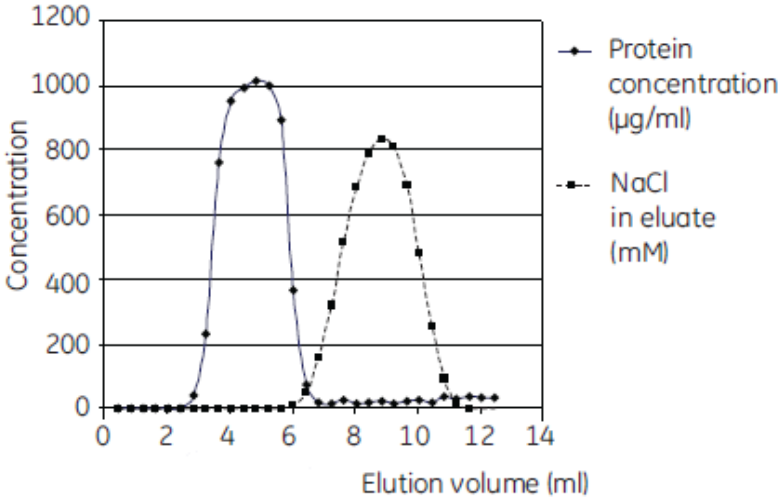


Figure 2.1. *Desalting capacity of PD-10 desalting column [66].*

The desalting column is first washed with about 20 mL of deionised water and then equilibrated with about 20 mL of the protein solution buffer. Then the protein sample with volume up to 2.5 mL is added. Additional buffer is added if the protein sample is less than 2.5 mL and the solution is allowed to enter the column. When the sample has been loaded to the column 2.5 mL of buffer is added and the eluate is collected (elution volume 2.5-5.0 mL in Figure 2.1).

2.2.3 Dialysis

Dialysis is another method to separate small and large molecules. Dialysis depends on diffusion through a semi-permeable membrane of long tubes with pores large enough to allow small molecules to diffuse through the membrane while blocking larger molecules from leaving the tube. The tube, containing a few millilitres of protein solution, is placed in one litre of buffer solution with the properties desired for the protein in the tube. The dialysis is carried out for 24 hours and the small molecules diffuse through the membrane making the concentration of small molecules equal inside and outside the tube. The large volume solution can be renewed if extra purity of the protein solution is required. Protein sample of 2.5 mL dialysed in 1 L of buffer would, theoretically, result in 400-fold decrease in small-molecule-concentration.

When the HRP-chitosan conjugate samples had been desalted on the PD-10 desalting column the 2.5 mL sample was placed in a dialysis tube and placed in 1 L of 10 mM MES buffer, pH 6.5, and the solution was stirred for 24 hours before the sample was removed from the tube and used.

2.2.4 Thermal inactivation of horseradish peroxidase

Samples of HRP were incubated in 3 mL of 10 mM MES buffer, pH 6.5, for up to 60 minutes, depending on temperature and enzyme sample. During the incubation 100 µL samples were removed from the incubation solution with five minutes intervals and cooled on ice until activity measurements were carried out.

2.2.5 Pre-treatment of microplates

The microplates used for the activity measurements were pre-treated for one hour with 0.1% Tween 20 solution in 50 mM phosphate buffer pH 7.4 with 0.9% sodium chloride. This solution makes the microplate wells hydrophilic, preventing the formation of meniscus that can affect the precision of the measurements by giving different wells varying length that the light travels through (b in Equation 2.2).

2.2.6 Activity assays

The activity of horseradish peroxidase was measured at room temperature, pH 7.0. Phosphate buffer solution was prepared by dissolving 2.72 g KH_2PO_4 in 90 mL deionised water and the pH was adjusted to 7.0 with the addition of 4 M sodium hydroxide and the solution filled up to 100 mL with deionised water. Two millilitres were removed from this solution and replaced with the same volume of 0.3% hydrogen peroxide solution. This solution was made fresh daily. Phenol solution was made by dissolving 3.24 g phenol crystals in 100 mL deionised water making the phenol concentration 0.34 M. A solution of 4-aminoantipyrine was made by dissolving 0.100 g of 4-AAP in 100 mL of deionised water, making a solution with concentration 0.0050 M. One part phenol solution and one part 4-AAP solution were mixed shortly before each activity measurement was carried out.

To microplates, pre-treated with Tween 20 solution, 125 µL of phosphate buffer-hydrogen peroxide solution was added as well as 115 µL of the phenol-4-AAP mixture. To start the reaction 10 µL of enzyme sample was added and the absorbance at 490 nm was measured at time intervals of 10 seconds for five minutes [72]. The concentration of enzyme was adjusted to give v_{max} of about 40 mOD/min. This was achieved by adding 10 µL of 1 mg/mL HRP solution to 3 mL of incubation buffer and then 10 µL of the incubation solution were used for the activity assay (see Table 2.1). The slope of the line, v_{max}, was determined by finding the average of four measurements made simultaneously. Very good consistency was observed in these activity measurements.

Table 2.1. *Composition of the 250 µL reaction solution in each microplate well. Initial concentrations show the concentration of stock solutions and the final concentration shows each component's concentration after mixing in each microplate well.*

Reaction component	**Initial concentration [M]**	**Volume used [µL]**	**Final concentration [M]**
Phosphate	0.20	125*	0.10
H_2O_2	$1.7 \cdot 10^{-3}$	125*	$8.5 \cdot 10^{-4}$
4-AAP	$5.0 \cdot 10^{-3}$	57.5	$1.2 \cdot 10^{-3}$
Phenol	0.34	57.5	$7.8 \cdot 10^{-2}$
HRP	$7.6 \cdot 10^{-8}$	10	$3.0 \cdot 10^{-9}$

* The same solution contained phosphate and hydrogen peroxide.

The reaction is shown in Figure 2.2 [73-75].

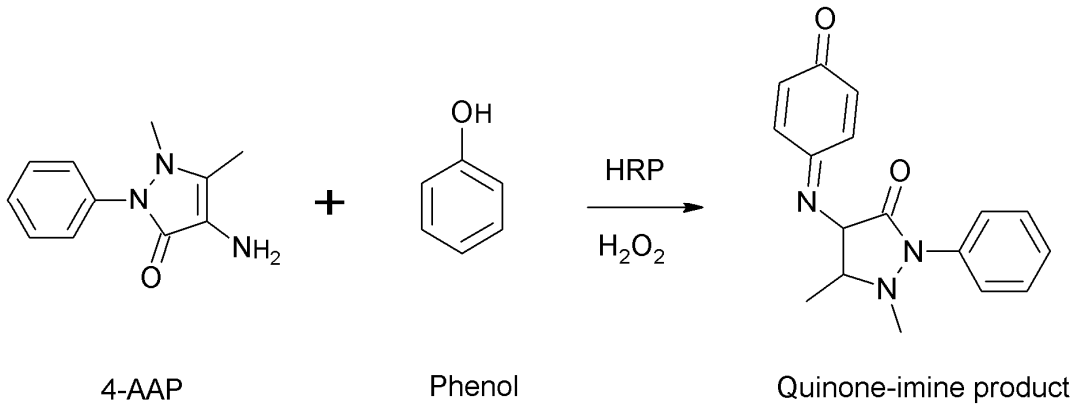


Figure 2.2. *Formation of the quinone-imine dye product formed by the oxidation of 4-AAP and phenol by hydrogen peroxide catalysed by HRP.*

The activity of native HRP and its conjugates was also assayed at different pH values to determine if the conjugation affected the enzyme's performance. This was done over the range of pH 2-12 with one pH unit intervals. A buffer containing 50 mM acetate, 50 mM phosphate and 50 mM borate was made and the pH adjusted to the desired value.

2.2.7 Chitosan-enzyme conjugation

The conjugation of horseradish peroxidase was performed by preparing an 8 mg/mL solution of each chitosan oligosaccharide and 5 mg/mL of the polymer in 10 mM MES buffer, pH 6.5. Then 2 mg HRP were added to 2 mL of the chitosan solution, along with 4 mg of NHSS and 8 mg of EDAC. The solution was shaken gently overnight at room temperature and then desalted on a PD-10 desalting column to remove unreacted EDAC and NHSS along with the unconjugated chitosan. The first 2.5 mL of the desalted solution were collected and dialysed for 24 hours in 10 mM MES buffer pH 6.5 to leave only the conjugated enzyme-chitosan complex in the sample used for measurements of thermal stability, protein concentration, pH activity, carbohydrate content and differential scanning calorimetry.

2.2.8 Quantification of proteins

Protein concentration was determined using Bradford protein assay, modified by Zaman and Verwilghen [69]. Five grams of Coomassie Brilliant Blue G-250 were dissolved in 100 mL methanol and 900 mL of deionised water to make a stock solution. To prepare the dye-solution 200 mL of the stock solution were diluted with 1800 mL of deionised water, 84 mL of perchloric acid and filled up to 2000 mL with deionised water. This solution was left overnight and then filtered with Whatman no. 4 filter paper.

The assay was then carried out by adding 25 µL protein samples to 275 µL dye-solution and the dye and the protein were allowed to bind for 10-15 minutes at room temperature. Then the absorbance was measured at 650 nm using the microplate reader. Standard curve was produced by preparing a 0.1 mg/mL solution of HRP that was then diluted to produce ten different concentrations in the range 0-0.1 mg/mL. Unknown samples were then diluted to be covered by the standard curve.

2.2.9 Quantification of carbohydrates

The method originally described by Dubois *et al.* [67] and optimised by Masuko *et al.* [68] was used. First 1 mL of carbohydrate solution was added to a test tube. Then 2.5 mL of concentrated sulphuric acid was added rapidly before adding 100 µL of 80% (w/w) phenol dissolved in water. This solution was shaken for a few seconds before being incubated in a 90°C water bath for five minutes. The sample was then removed from the incubator and placed in room temperature water bath for five minutes. Then 300 µL of each sample was placed in each well of a microplate and eight different measurements made for each sample to give good accuracy. Standard curves were made for each chitosan sample in the concentration range 0-3.0 mg/mL.

2.2.10 Differential scanning calorimetry

The calorimeter used in this research is a VP-DSC Microcalorimeter from MicroCal and the data is processed with Origin 7, a program made for this type of calorimeters. In the spring of 2008 the University of Iceland Science Institute installed the calorimeter along with an Isothermal Titration Calorimeter (ITC 200). ITC is a very sensitive technique that can measure the thermodynamics of a system when small amounts of two reactants are titrated together. MicroCal was founded in the USA in 1977 and has ever since been a pioneer in the development of microcalorimetry. In 2008 General Electric bought MicroCal and it now functions under GE Healthcare.

The VP-DSC Microcalorimeter consists of two lollipop shaped cells, made from a tantalum alloy, that are surrounded by insulating material and sensors to control and measure extremely small temperature changes. Very narrow access tubes are used to get solutions into the cells using a syringe. The two cells have volumes of about 0.5 mL and when the solutions are in place another syringe that is specially designed to remove excess solution, is used to make sure that the volumes of solutions in the two cells are equal. When filling the cells it is extremely important to make sure that no air bubbles are in the cells. Small air bubbles dramatically change the heat capacity of the solutions and the data shows very large shifts in DP. A large negative value indicates air bubbles in the sample cell while large positive value indicates air bubbles in the reference cell. A special degassing apparatus, called ThermoVac, from MicroCal can be used to degas samples. When sufficiently degassed (for about an hour with stirring) the samples are easy to load and measure.

Scans are designed depending on the nature of the sample to be measured. Operating temperatures between -10 °C and 130 °C can be chosen along with the scan rate (usually about 60-90 K/h), number of scans and if upscans (from low to high temperature) and downscans (from high to low temperature) are necessary. If the denaturation of the protein is irreversible downscans are not required.

Buffer samples were first degassed for over an hour using ThermoVac at 30°C. Then the buffer was loaded to both sample and reference cells of the VP-DSC calorimeter and up-scans performed from 10°C up to 110°C. The scan rate was 90°C per hour and the pressure was around 24 psi. Since the thermal history of the calorimeter is essential for reproducibility of the data the first scan was discarded and scans 2 and 3 were used as the

baseline. If the two scans were consistent the protein was loaded to the sample cell. The protein was dissolved in degassed buffer and then placed in the ThermoVac for a few minutes. If no formation of air bubbles was observed on the walls of the sample tube the protein was loaded when the temperature of the calorimeter was between 25°C and 15°C during its cooling period. The data are then interpreted using Origin.

3 Results

3.1 Thermal stability determination

Thermal stability measurements of the effect chitosan has on horseradish peroxidase are presented in two parts. First the effect of chitosan in solution is presented. The concentration of the chitosan oligosaccharides and the chitosan polymer depend on their solubility and the viscosity of their solutions. In the second part the effect of conjugated chitosan on the stability of HRP is presented. The enzyme was incubated at 60, 65 and 70°C for periods up to one hour. During this period samples were taken, cooled on ice and then the activity was measured.

When the enzyme loses its activity it goes through two steps on its way to the inactive denatured state (D). The native enzyme (N) is first unfolded (U) in a reversible reaction and can then become denatured in an irreversible reaction with rate constant k (Equation 3.1).

Equation 3.1

$$N \leftrightarrow U \rightarrow_k D$$

The relative activity (v/v_0) was then calculated and plotted against time (Equation 3.2) and when appropriate the energy of deactivation (E_{da}) was calculated. The relative activity indicates how far the denaturation reaction has proceeded.

Equation 3.2

$$ln\left(\frac{v}{v_0}\right) = -k \cdot t$$

When the rate of deactivation constants (k) for each temperature have been decided they can be used to find the energy of deactivation by applying the Arrhenius equation (Equation 3.3).

Equation 3.3

$$k = A \cdot e^{\frac{-E_{da}}{R \cdot T}}$$

Equation 3.3 gives Equation 3.4.

Equation 3.4

$$\ln(k) = \ln(A) - \frac{E_{da}}{R} \cdot \frac{1}{T}$$

When ln(k) is plotted against 1/T, temperature measured in Kelvin, the slope of the line is equal to the energy of deactivation divided by the gas constant, R. The energy of deactivation gives a measurement of the energy needed to deactivate the enzyme. By comparing the results for free HRP and HRP in chitosan solution or HRP conjugated to chitosan it is possible to indicate the increase in stability accurately. This method depends on the linearity of the deactivation curves and when the linearity is poor different interpretations of the data are necessary. The rate constant of deactivation can also give the half life ($t_{1/2}$) of the enzyme, according to Equation 3.5.

Equation 3.5

$$t_{1/2} = \frac{\ln(2)}{k}$$

3.2 Problems due to incubation buffer

To measure the thermal stability of horseradish peroxidase the enzyme was originally incubated in 10 mM phosphate buffer, pH 7.0, at different temperatures for up to 90 minutes. Asad et al. reported that thermal stability of HRP depends on the concentration of the incubation buffer [47]. This was consistent with the literature where loss of activity was typically measured around 60-80 °C [44, 76, 77]. At first the results were very inconsistent and significant loss of activity occurred even at temperatures below 46°C. Figure 3.1 shows an example of thermal stability curves for HRP at these conditions.

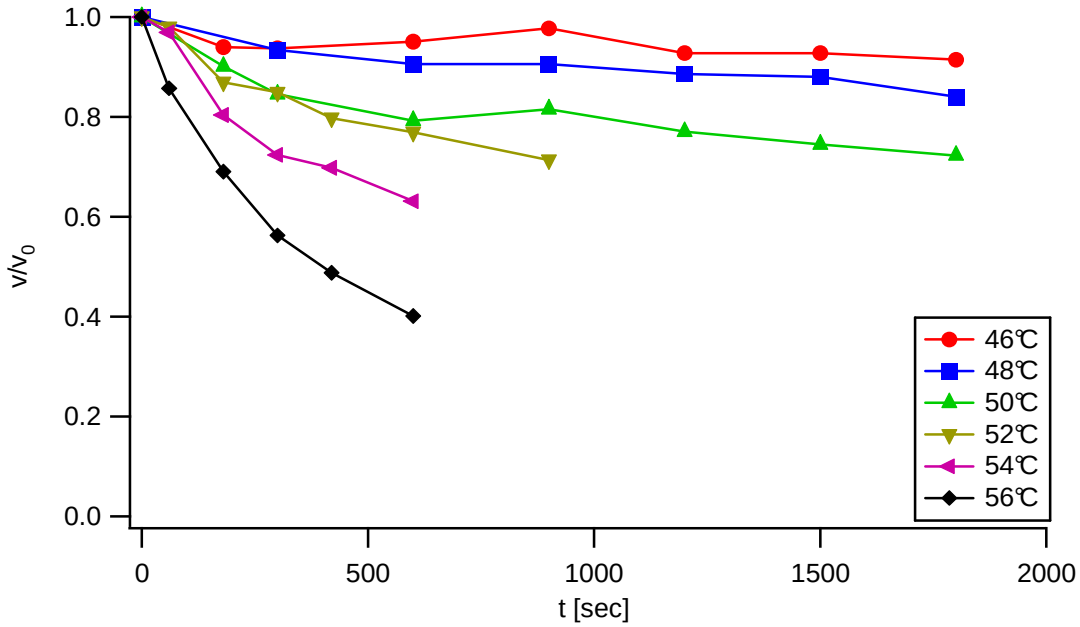


Figure 3.1. *Relative activity of HRP at different incubation temperatures for up to 30 minutes.*

Figure 3.1 shows that after only ten minutes at 56 °C the activity is below 40% of the initial activity. These unexpected results soon pointed to problems with the incubation buffer since the initial activity of HRP was very constant which ruled out the substrate solutions and the enzyme sample. The incubation buffer was prepared each morning and showed a tendency to give more stable results in the afternoon, a trend that was confirmed with 22 hours of constant measurements. When the buffer was kept for a few days it usually gave better thermal stability until a certain maximum was reached, usually after about five days. Degassing did not change this behaviour and the pH remained constant. MES and MOPS buffers were tested and showed similar behaviour. Although MES gave poor thermal stability when freshly made, maximum thermal stability was reached quicker than with phosphate buffer. The maximum thermal stability curves for phosphate and MES were identical. Figure 3.2 and Figure 3.3 show how the two different buffers gave different thermostability curves the days after the buffers were made.

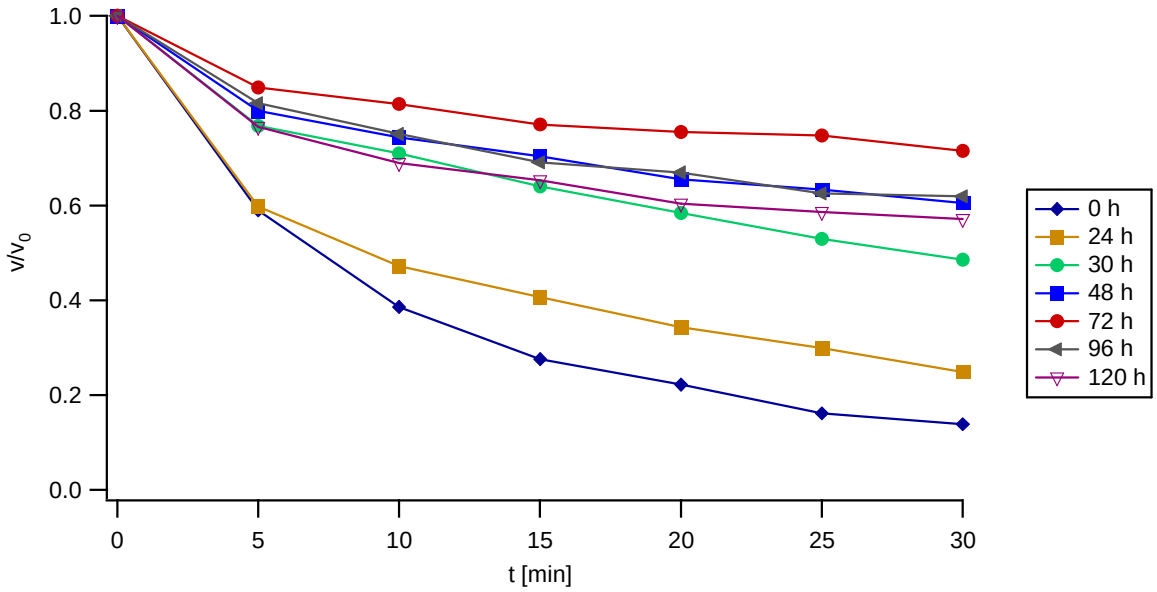


Figure 3.2. *Relative activity of HRP in 10 mM MES incubation buffer, pH 6.5, at different times after preparation of the incubation buffer at 60°C.*

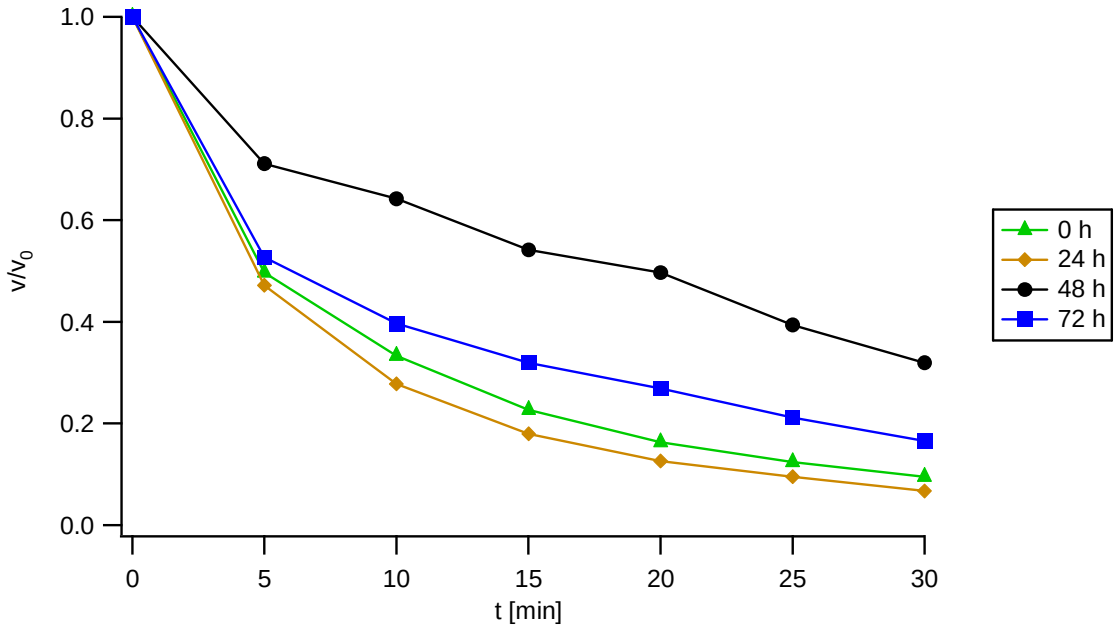


Figure 3.3. *Relative activity of HRP in 10 mM phosphate incubation buffer, pH 6.5, at different times after preparation of the incubation buffer at 60 °C.*

The results shown in Figure 3.2 and Figure 3.3 are only examples of how these buffers behaved, in some cases maximum stability was obtained sooner, later or not at all. Figure 3.3 shows what was very common, the phosphate buffer was very unreliable and the MES incubation buffer was preferred in all the thermal stability measurements. To measure

increased thermal stability of the HRP-chitosan conjugate it was very important to exclude any uncertainty caused by instability due to the incubation buffer. Therefore a sample of free HRP was measured in parallel with the HRP conjugate and the results were discarded if the free HRP sample did not show maximum stability.

When thermal stability of HRP in chitosan solution was measured this incubation buffer behaviour was reduced and generally took about one day to reach its maximum stability and gave very consistent results.

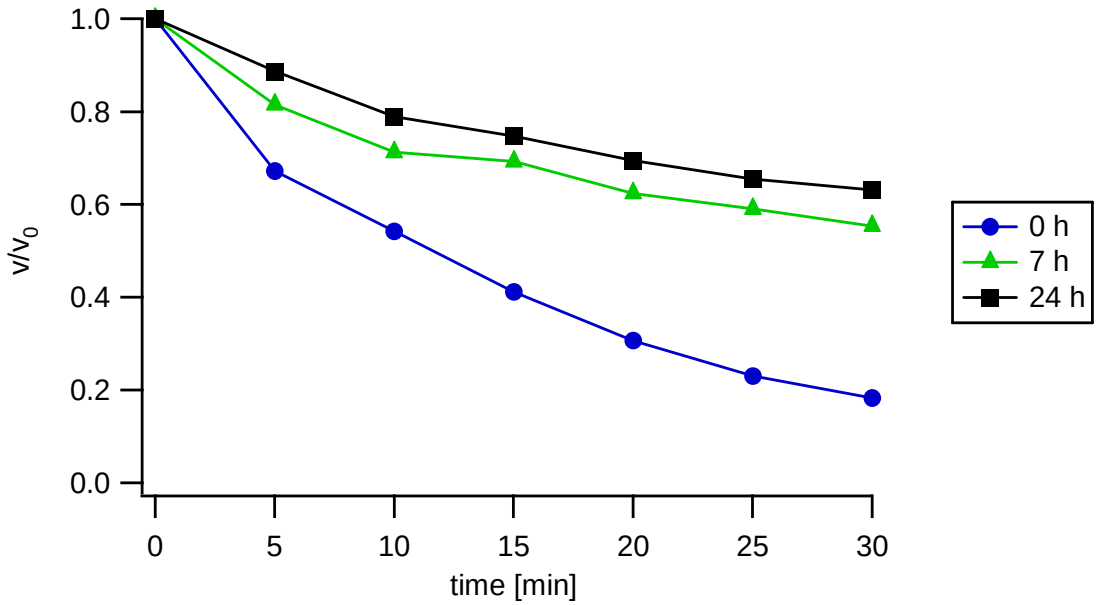


Figure 3.4. *Relative activity of HRP in 10 mM MES incubation buffer, 8 mg/mL chitosan (DP 4-8), pH 6.5, at different times after preparation of the incubation solution at 65°C.*

The phosphate buffer was made by dissolving potassium dihydrogen phosphate in distilled water and the pH adjusted to the desired value with sodium hydroxide. Phosphate has pK_{a2} of 7.21 and HRP is very active in the range from pH 6-8 therefore three different pH values (6.5, 7.0 and 7.5) were tried but all three gave inconsistent results. When the same incubation buffer was used over a period of a few days the pH was measured regularly to make sure that the instability due to the buffer was not caused by a change in pH. Any change in pH would indicate that the equilibrium of the phosphate ions was changing but no change in pH was ever observed. Other forms of phosphate salts were tested, as well as phosphoric acid titrated to the desired pH.

Since the incubation buffer solutions showed increased thermal stability for HRP after a few days it could indicate that some sort of slowly obtained equilibrium was needed. Dissolved gases from the atmosphere that needed to reach equilibrium was another factor that was investigated but degassed buffer turned out to give both types of results, i.e. good and poor thermal stability. The effect of ionic concentration was also investigated by adding different concentrations of sodium chloride to the incubation buffer solution but that turned out to have little effect.

3.3 Thermal stability of horseradish peroxidase in 10 mM MES buffer

Results for thermal stability of HRP in MES buffer, in chitosan solutions and conjugated to chitosan are divided into sections 3.3, 3.4 and 3.5 for free HRP, HRP in chitosan solutions and HRP-chitosan conjugates respectively.

First of all the stability of free HRP at 60, 65 and 70°C was determined in 10 mM MES buffer at pH 6.5. Since all further results are compared to the stability of free HRP at least six consistent deactivation curves were gathered for each temperature and their average is presented in the following graphs. All chitosan solutions are in 10 mM MES buffer pH 6.5.

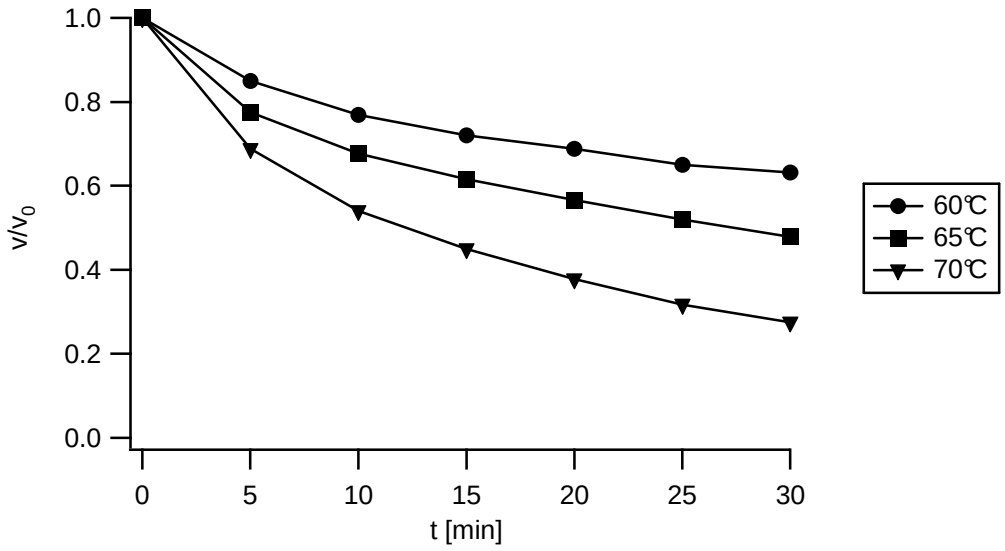


Figure 3.5. *Relative activity of HRP at 60°C (circles), 65°C (squares) and 70°C (triangles).*

When the natural logarithm is applied to each deactivation curve the rate constant of deactivation (k) can be found for each temperature. Then the natural logarithm of the rate constant is plotted against 1/T (temperature in Kelvin; Figure 3.7) to reveal the energy of deactivation, E_{da} (see Table 3.4).

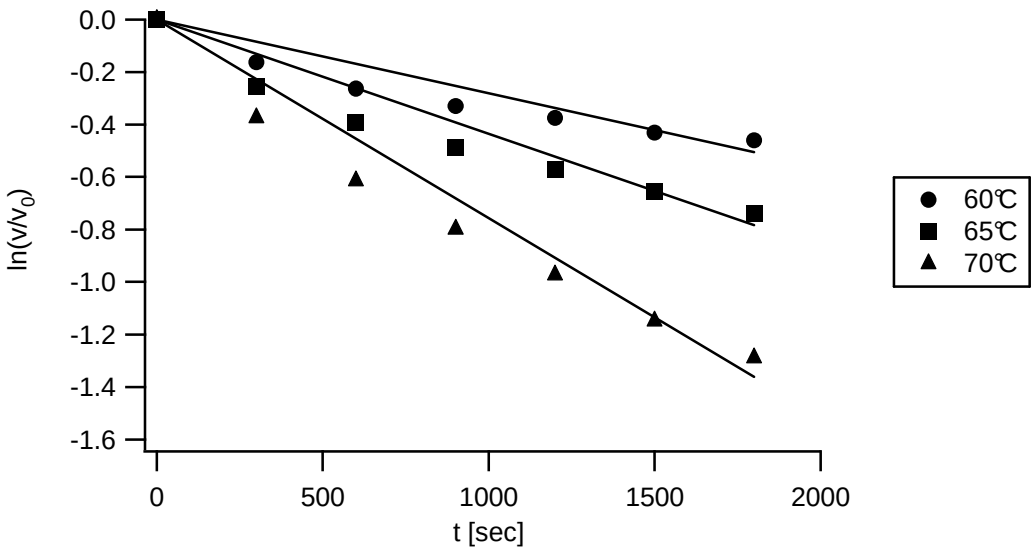


Figure 3.6. *Natural logarithm of relative activity versus time in seconds at 60°C (circles), 65°C (squares) and 70°C (triangles). The slope of the line at each temperature equals the rate of deactivation constant (k) in the Arrhenius equation.*

The results obtained from Figure 3.6 are in Table 3.1 and are used to produce Figure 3.7.

Table 3.1.*Rate of deactivation constants (k) and half lives ($t_{1/2}$) of HRP at 60, 65 and 70°C for horseradish peroxidase in 10 mM MES pH 6.5.*

Temperature [°C]	**k x 10^{-4} [sec^{-1}]**	**$t_{1/2}$ [min]**
60	2.9620	39.002
65	4.5627	25.319
70	7.8306	14.753

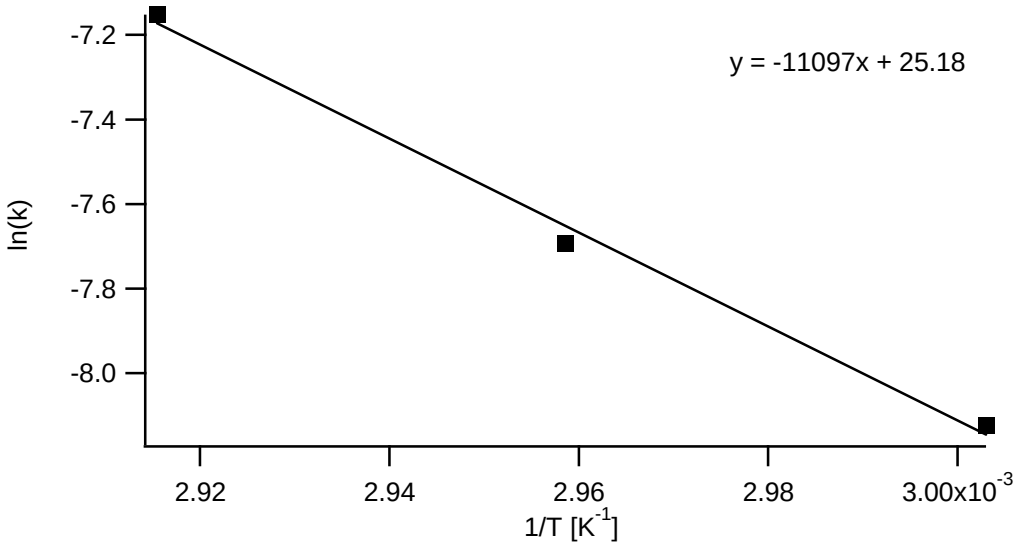


Figure 3.7. *Arrhenius plot of rate of deactivation constants (k) versus 1/T. The slope of the line equals* E_{da}/R.

Results obtained from the Arrhenius plots are summarised in Table 3.4.

3.4 Thermal stability of horseradish peroxidase in chitosan solutions

When the stability of free HRP had been established the effect of chitosan oligomer with degree of polymerisation of 4-8 was investigated. The oligomer showed good solubility in water and initially a solution of 10 mg/mL was prepared at pH 6.5 and the enzyme was incubated for 30 minutes and samples were taken every five minutes. Free HRP retained about 60% of its initial activity after 30 minutes at 60°C while HRP in the DP 4-8 solution only lost about 5% (Figure 3.8) of its initial activity. At 70°C the relative activity of the two differently incubated solutions after 30 minutes were 28% and 34% for the free HRP and the DP 4-8 incubated HRP respectively.

When the concentration of the DP 4-8 chitosan oligomer was increased to 50 mg/mL the thermal stability was increased further (Figure 3.11). After incubation at 65°C for 30 minutes the free HRP retained 48% of its initial activity, the 10 mg/mL solution HRP retained 74% and the 50 mg/mL solution HRP 82%.

3.4.1 Thermal stability of horseradish peroxidase in 10 mg/mL chitosan DP 4-8 solution

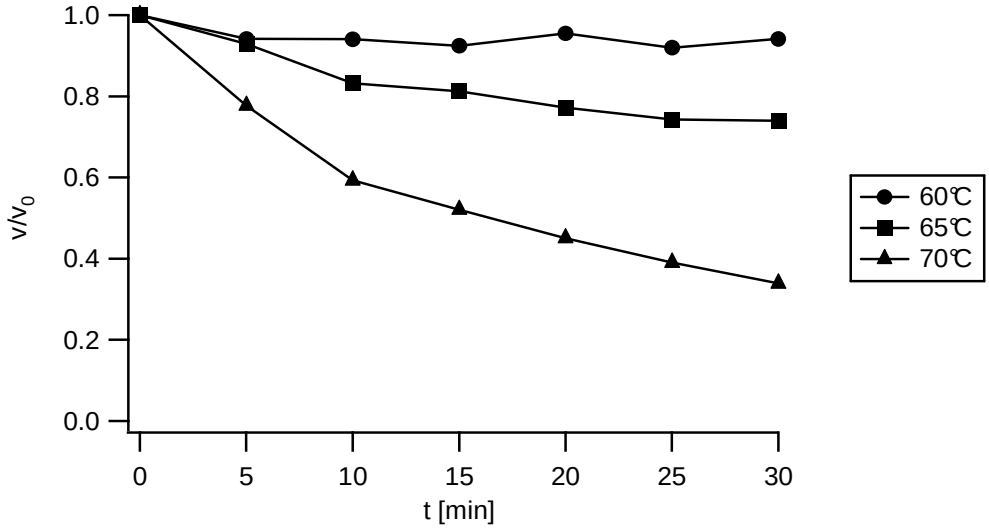


Figure 3.8. *Relative activity of HRP in 10 mg/mL chitosan DP 4-8 solution at 60°C (circles), 65°C (squares) and 70°C (triangles).*

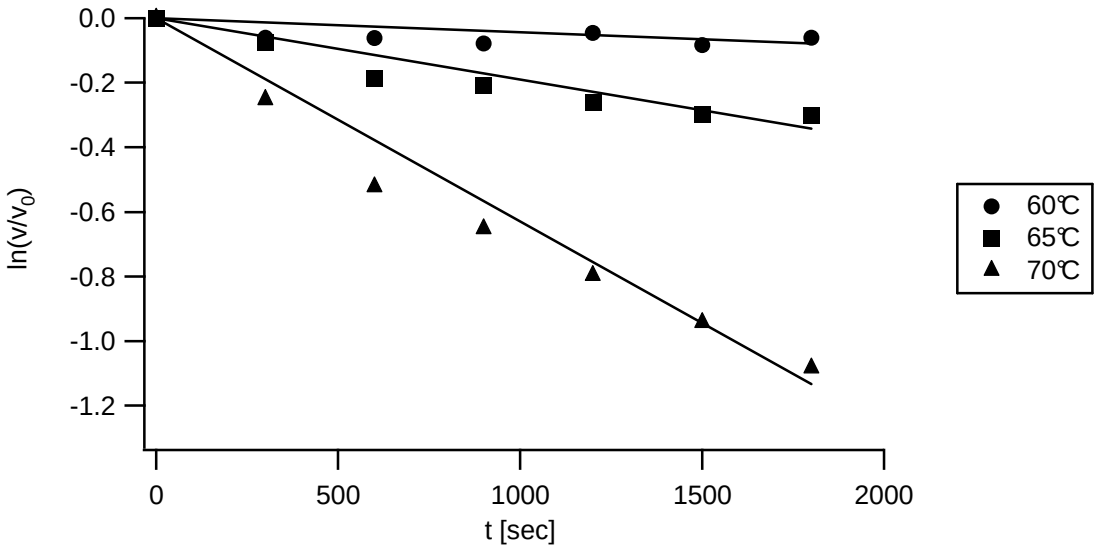


Figure 3.9. *Natural logarithm of relative activity of HRP in 10 mg/mL chitosan DP 4-8 solution versus time in seconds at 60°C (circles), 65°C (squares) and 70°C (triangles). The slope at each temperature equals the rate of deactivation constant (k) in the Arrhenius equation.*

Table 3.2. *Rate of deactivation constants (k) and half lives ($t_{1/2}$) of HRP in 10 mg/mL solution of DP 4-8 chitosan at 60, 65 and 70°C for horseradish peroxidase in 10 mM MES pH 6.5.*

Temperature [°C]	**k x 10^{-4} [sec^{-1}]**	**$t_{1/2}$ [min]**
60	0.50731	227.72
65	1.9787	58.384
70	6.4686	17.859

At 60°C the increase in enzyme half life is almost six fold compared to the free HRP and these data form a straight line in an Arrhenius plot. The energy of deactivation is given in Table 3.4.

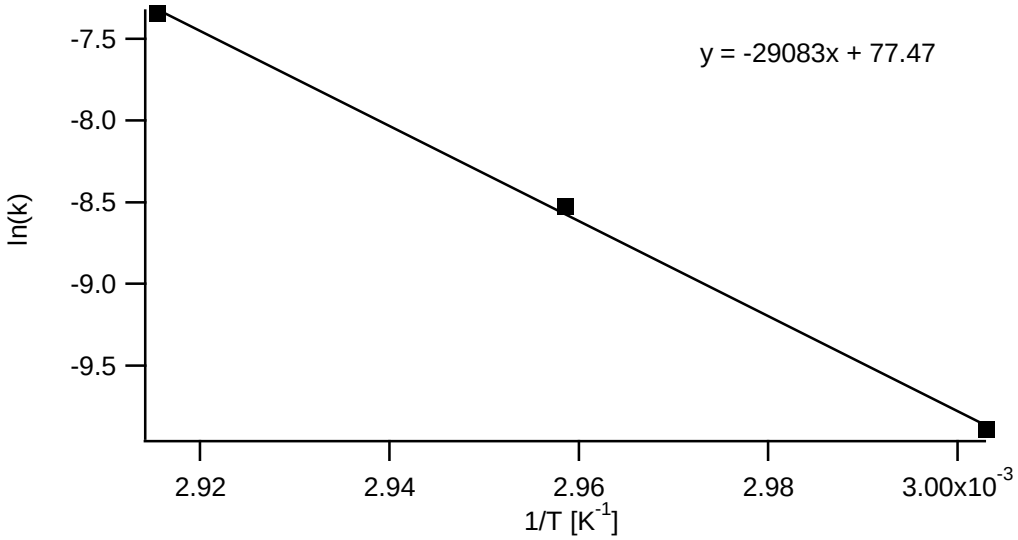


Figure 3.10. *Arrhenius plot of rate of deactivation constants (k) versus 1/T for HRP in 10 mg/mL chitosan DP 4-8 solution. The slope of the line equals E_{da}/R.*

3.4.2 Thermal stability of horseradish peroxidase in 50 mg/mL chitosan DP 4-8 solution

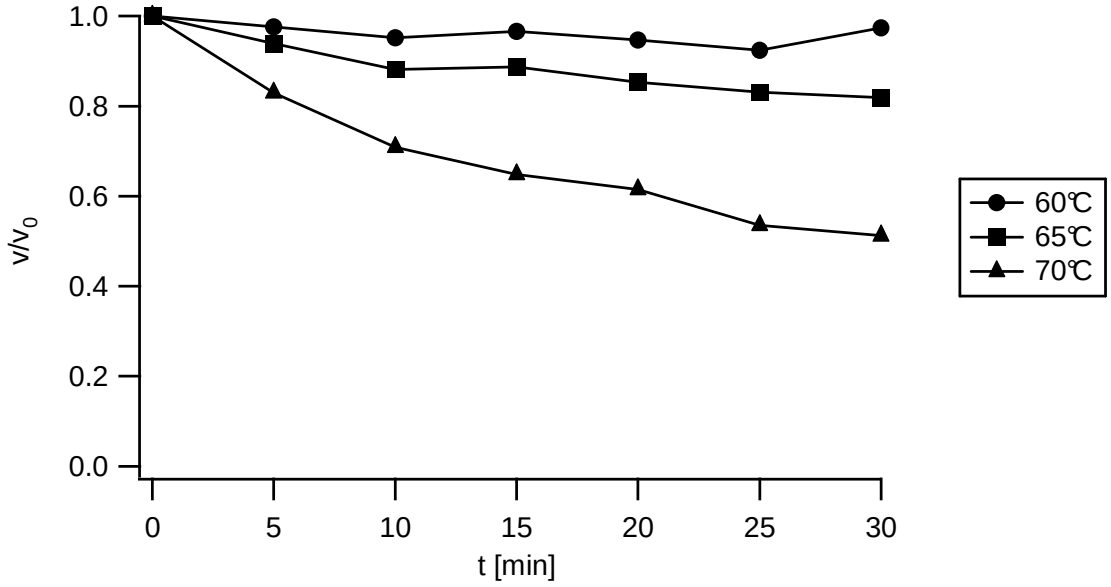


Figure 3.11. *Relative activity of HRP in 50 mg/mL chitosan DP 4-8 solution at 60°C (circles), 65°C (squares) and 70°C (triangles).*

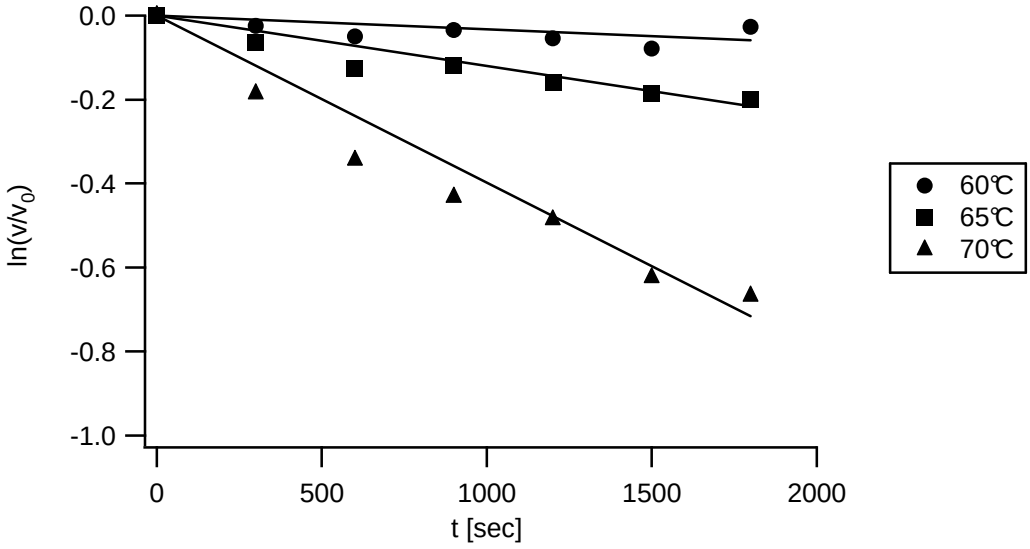


Figure 3.12. *Natural logarithm of relative activity of HRP in 50 mg/mL chitosan DP 4-8 solution versus time in seconds at 60°C (circles), 65°C (squares) and 70°C (triangles). The slope at each temperature equals the rate of deactivation constant (k) in the Arrhenius equation.*

Table 3.3. *Rate of deactivation constants (k) and half lives ($t_{1/2}$) of HRP in 50 mg/mL solution of DP 4-8 chitosan at 60, 65 and 70°C for horseradish peroxidase in 10 mM MES pH 6.5.*

Temperature	**k x 10^{-4} [sec^{-1}]**	**$t_{1/2}$ [min]**
60	0.36470	316.77
65	1.2581	91.825
70	4.1225	28.023

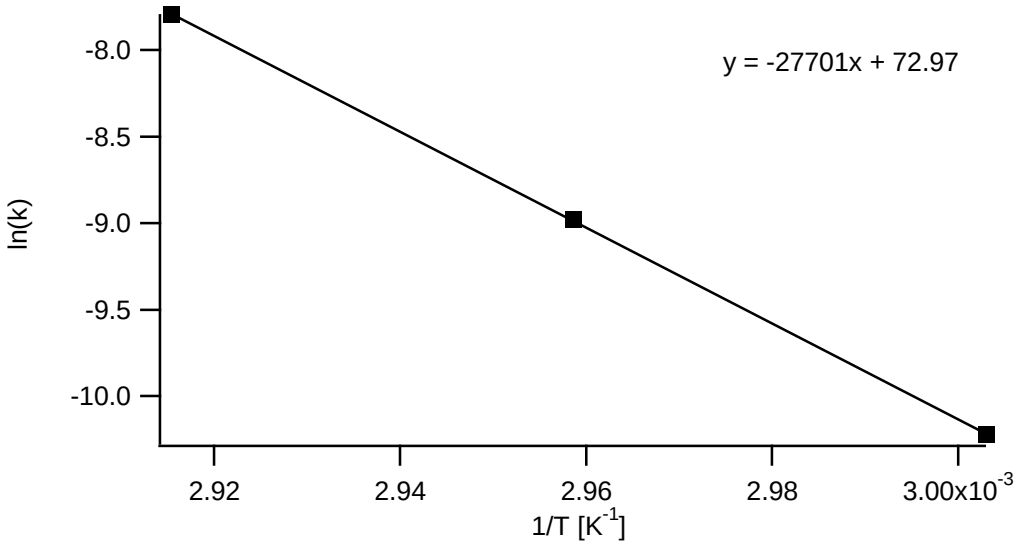


Figure 3.13. *Arrhenius plot of rate of deactivation constants (k) versus 1/T for HRP in 50 mg/mL chitosan DP 4-8 solution. The slope of the line equals E_{da}/R.*

Increasing the concentration of the DP 4-8 chitosan oligomer greatly improves the already improved thermal stability of HRP. To evaluate the effect it has the energy of deactivation is given in Table 3.4 for comparison of the three different thermal stability assays presented here.

Table 3.4. *Comparison of E_{da} values for free HRP and HRP in chitosan DP 4-8 solutions and HRP conjugated to chitosan DP 4-8.*

Sample	**Slope**	**R^2**	**E_{da} [kJ/mol]**	**% increase**
Free HRP	-11097	0.995	92.26	-
10 mg/mL DP 4-8	-29083	0.999	241.8	362
50 mg/mL DP 4-8	-27701	1.000	230.3	249
DP 4-8 conjugate*	-19116	0.998	158.9	72

*DP 4-8 conjugated HRP results are in section 3.5.1.

3.4.3 Thermal stability of horseradish peroxidase in 5 mg/mL chitosan polymer solution

The chitosan polymer solution had much higher viscosity than the oligomer solution and was not easily transferred by pipette in concentrations above 5 mg/mL. The polymer was very effective at preventing loss of catalytic activity at 60°C where the enzyme retained 97% of its initial activity after 30 minutes of incubation. At 70°C the polymer however failed to protect the enzyme and increased the rate of deactivation over the first minutes although the relative activity after 30 minutes was identical for the HRP in MES incubation buffer and the polymer solution.

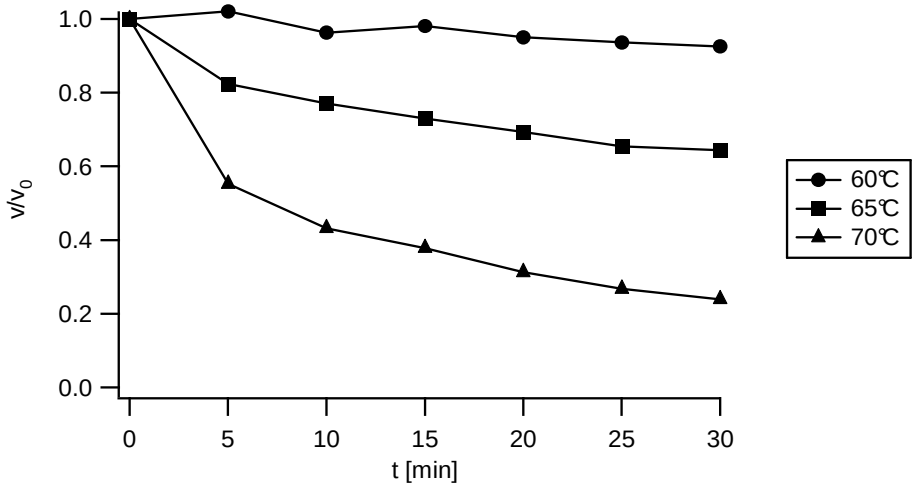


Figure 3.14. *Relative activity of HRP in 5 mg/mL chitosan polymer solution at 60°C (circles), 65°C (squares) and 70°C (triangles).*

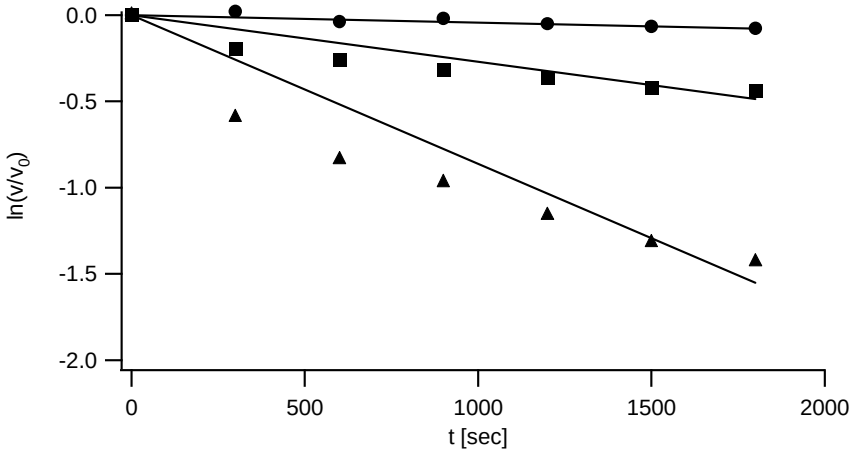


Figure 3.15. *Natural logarithm of relative activity of HRP in 5 mg/mL chitosan polymer solution versus time in seconds at 60°C (circles), 65°C (squares) and 70°C (triangles). The slope of the line at each temperature equals the rate of deactivation constant (k) in the Arrhenius equation.*

The deactivation curve at 70°C shows poor linearity which affects the Arrhenius plot and no E_{da} value can be calculated.

3.5 Thermal stability of horseradish peroxidase chitosan conjugates

Chitosan with degree of polymerisation of 4-8, Tchos and a polymer was conjugated to horseradish peroxidase and the stability of the conjugates was measured in 10 mM MES buffer pH 6.5.

3.5.1 Thermal stability of horseradish peroxidase chitosan DP 4-8 conjugate

As before the relative activity of HRP was measured over 30 minutes at different temperatures.

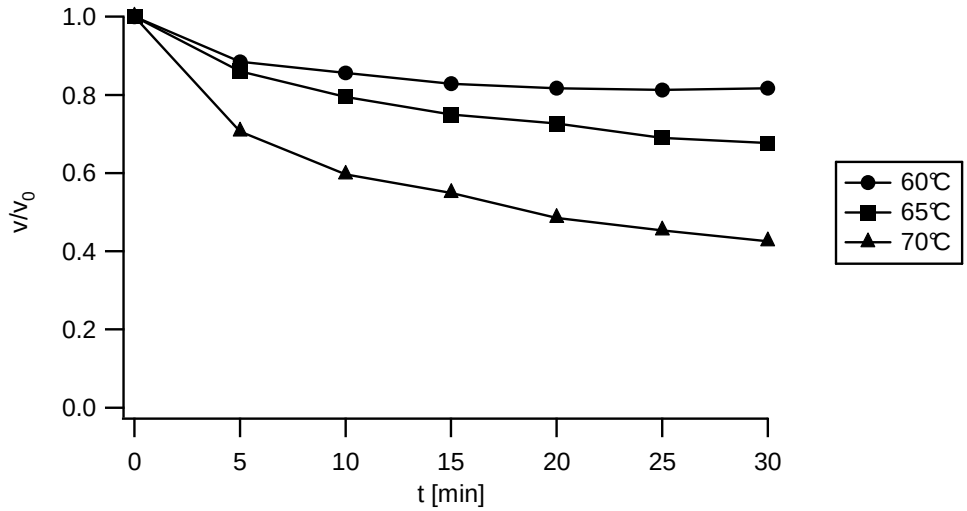


Figure 3.16. *Relative activity of HRP conjugated to chitosan DP 4-8 at 60°C (circles), 65°C (squares) and 70°C (triangles).*

When compared with Figure 3.5 the increase in thermal stability is clear. When these data are used to produce an Arrhenius plot it shows 72% increase in energy of deactivation compared to the free enzyme.

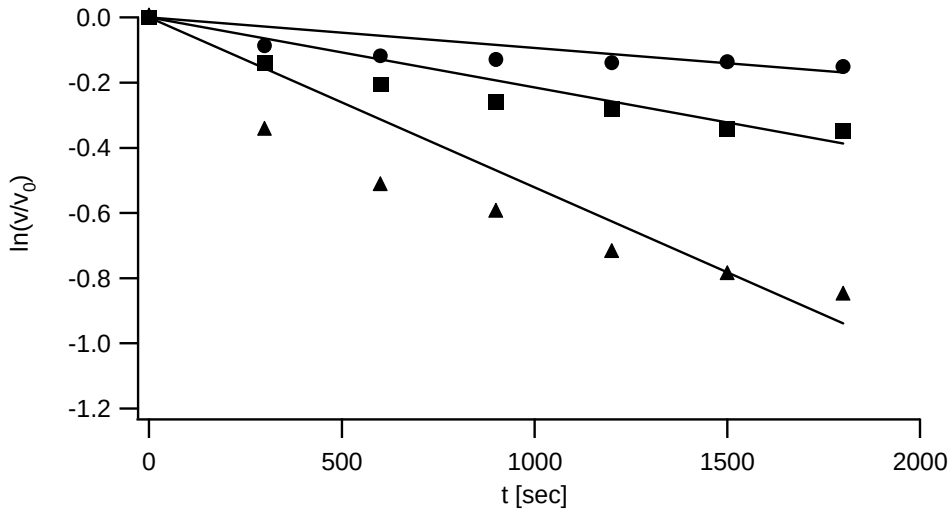


Figure 3.17. *Natural logarithm of relative activity of HRP conjugated to chitosan DP 4-8 versus time in seconds at 60°C (circles), 65°C (squares) and 70°C (triangles). The slope at each temperature equals the rate of deactivation constant (k) in the Arrhenius equation.*

Table 3.5. *Rate of deactivation constants (k) and half lives ($t_{1/2}$) of HRP conjugated to DP 4-8 chitosan at 60, 65 and 70°C for horseradish peroxidase in 10 mM MES pH 6.5.*

Temperature [°C]	**k x 10^{-4} [sec^{-1}]**	**$t_{1/2}$ [min]**
60	1.0396	111.12
65	2.2842	50.575
70	5,5462	20.829

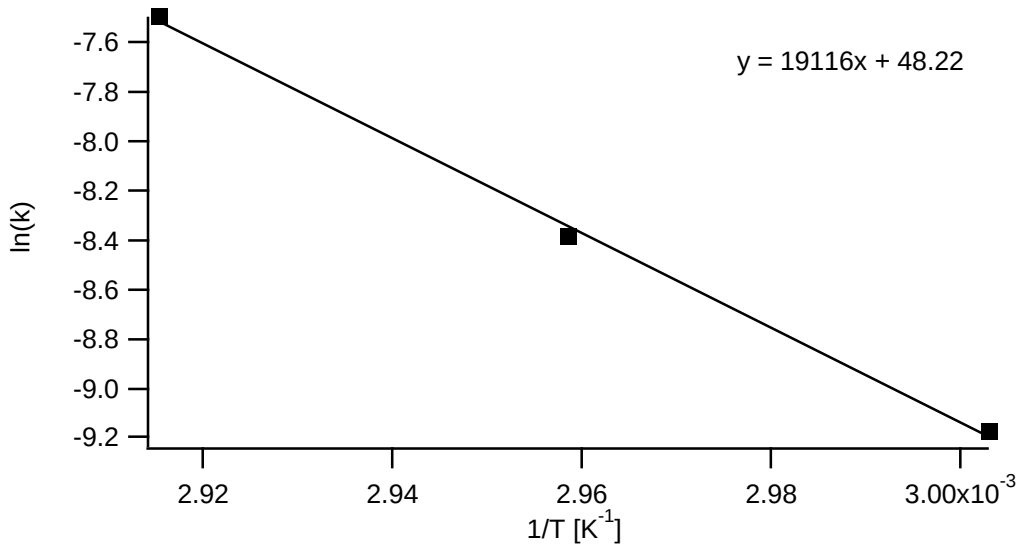


Figure 3.18. *Arrhenius plot of rate of deactivation constants (k) versus 1/T for HRP conjugated to chitosan DP 4-8. The slope of the line equals E_{da}/R.*

3.5.2 Thermal stability of horseradish peroxidase chitosan polymer conjugate

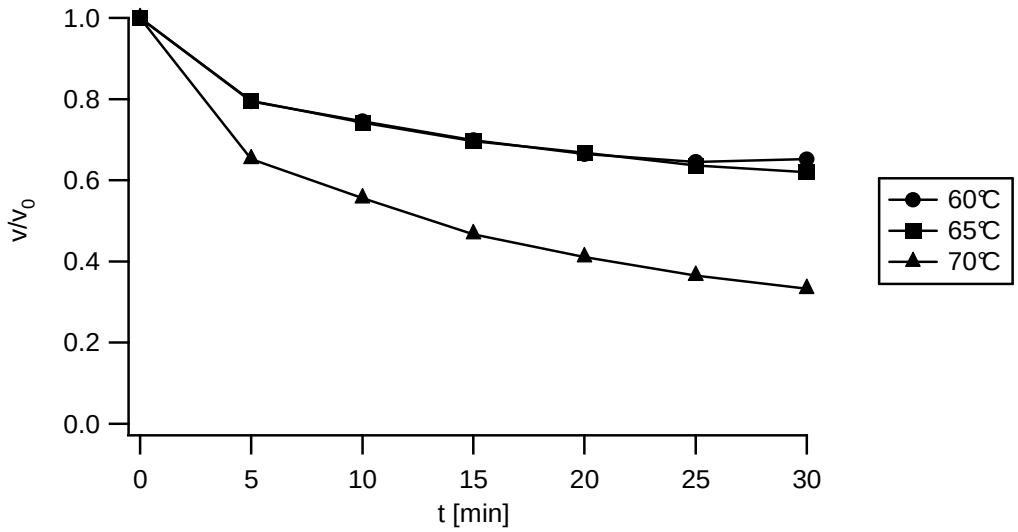


Figure 3.19. *Relative activity of HRP conjugated to chitosan DP 4-8 at 60°C (circles), 65°C (squares) and 70°C (triangles).*

As Figure 3.19 shows, the conjugated polymer was not very effective in increasing the thermal stability of HRP. The order of the denaturation reaction is clearly not one as can be seen in Figure 3.22. Different presentations are needed to describe the effect the polymer has on the thermal stability (section 3.6).

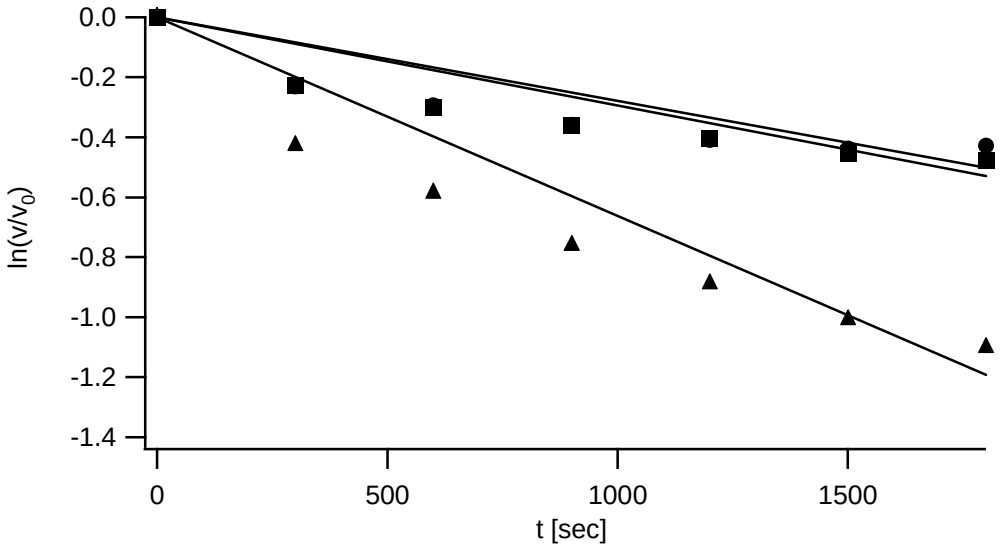


Figure 3.20. *Natural logarithm of relative activity of HRP conjugated to chitosan DP 4-8 versus time in seconds at 60°C (circles), 65°C (squares) and 70°C (triangles). The slope of the line at each temperature equals the rate of deactivation constant (k) in the Arrhenius equation.*

3.5.3 Thermal stability of horseradish peroxidase Tchos chitosan conjugate

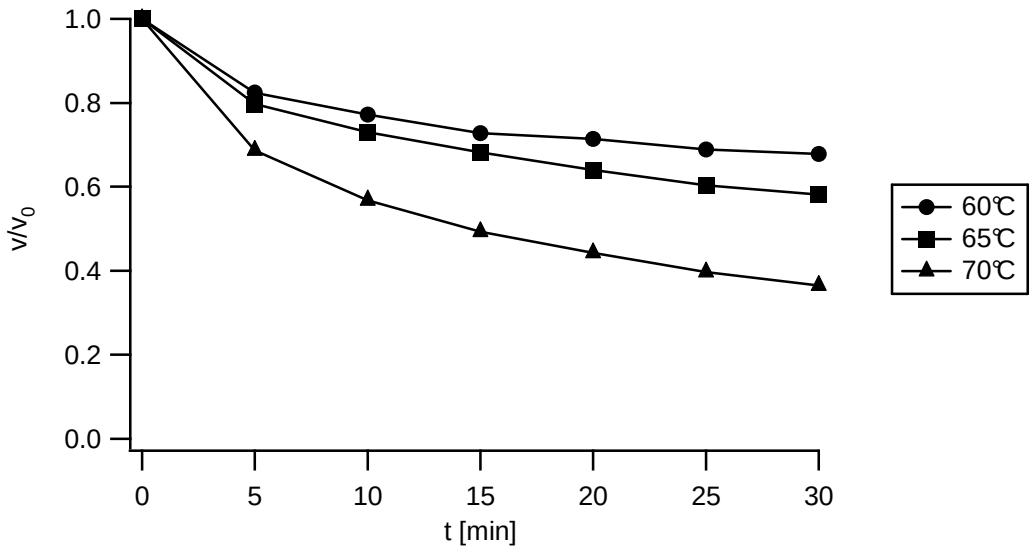


Figure 3.21. *Relative activity of HRP conjugated to chitosan DP 4-8 at 60°C (circles), 65°C (squares) and 70°C (triangles).*

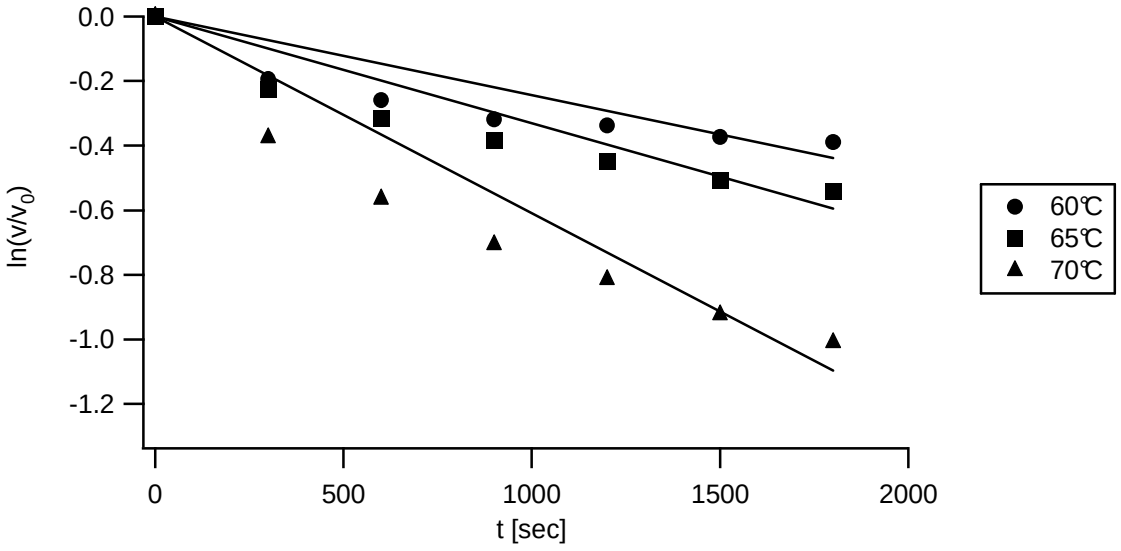


Figure 3.22. *Natural logarithm of relative activity of HRP conjugated to Tchos chitosan versus time in seconds at 60°C (circles), 65°C (squares) and 70°C (triangles). The slope of the line at each temperature equals the rate of deactivation constant (k) in the Arrhenius equation.*

3.6 Comparison of effects on thermal stability

The order of the deactivation reaction is not always one as can be seen in the poor linearity of some of the natural logarithm of relative activity versus time graphs. This prevents accurate half lives from being obtained from the graphs and other representations are needed. However most of the data at 65°C gives reasonable linearity and Figure 3.23 shows the half lives at 65°C for each modification of HRP.

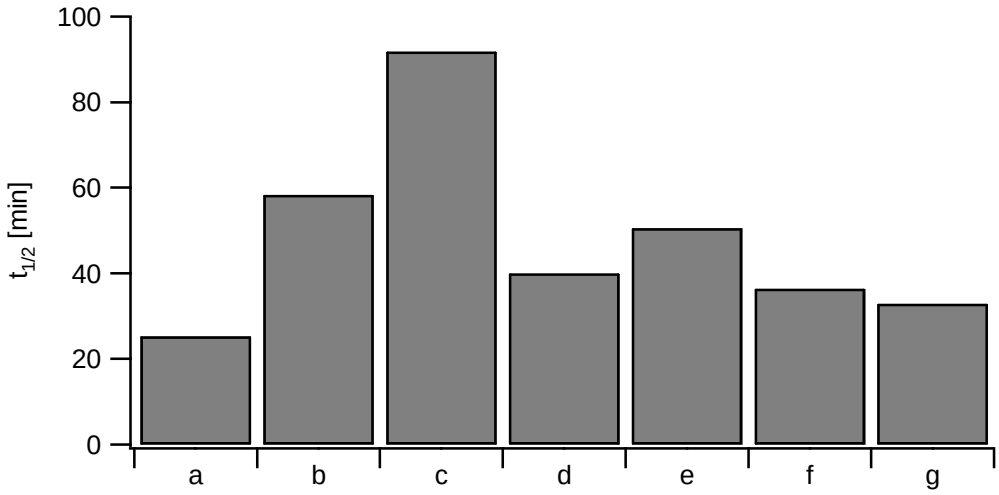


Figure 3.23. *Half lives of HRP at 65°C in MES buffer (a), 10 mg/mL chitosan DP 4-8 solution (b), 50 mg/mL chitosan DP 4-8 solution (c), 5 mg/mL polymer solution (d), DP 4-8 conjugate (e), polymer conjugate (f) and Tchos conjugate (g).*

Greatest stability increase was in the 50 mg/mL chitosan DP 4-8 solution where the half life was increased from around 25 minutes for HRP in MES buffer to 92 minutes in the chitosan solution. All the enzyme-chitosan conjugates showed increase in half life and the DP 4-8 chitosan was most effective with half life of 51 minutes.

3.6.1 One hour thermal deactivation of DP 4-8 conjugate

From the data presented it was obvious that the chitosan DP 4-8 oligosaccharide was most effective at increasing HRP's thermal stability. When the incubation time was doubled similar results were obtained (Figure 3.26) and after 60 minutes of incubation at 65°C the free HRP and the conjugated HRP had relative activities of 32% and 52% respectively. After 60 minutes of incubation at 70°C the free HRP was down to 8% of its initial activity while the conjugated enzyme was still at about 30% of its initial activity.

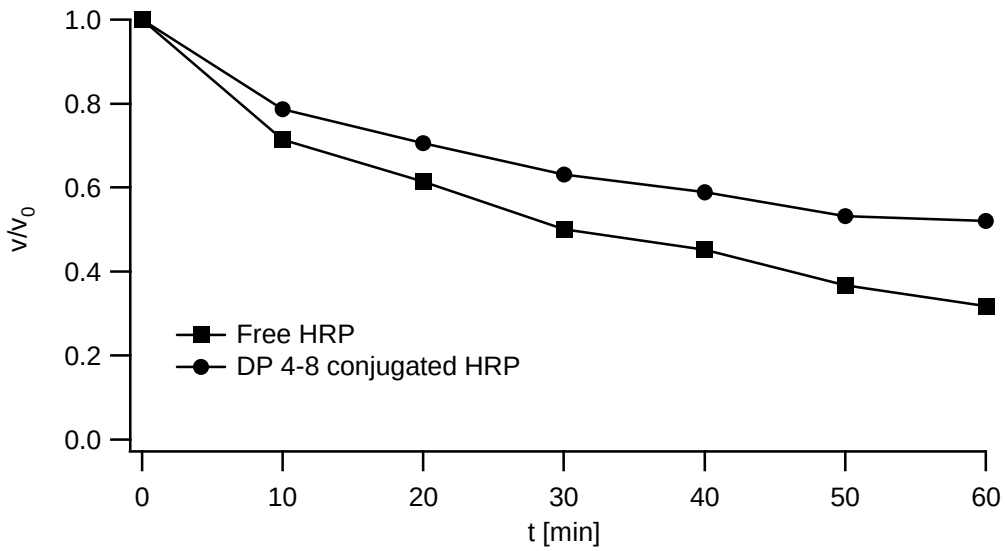


Figure 3.24. *Relative activity of HRP (squares) and DP 4-8 conjugated HRP over 60 minutes at 65°C.*

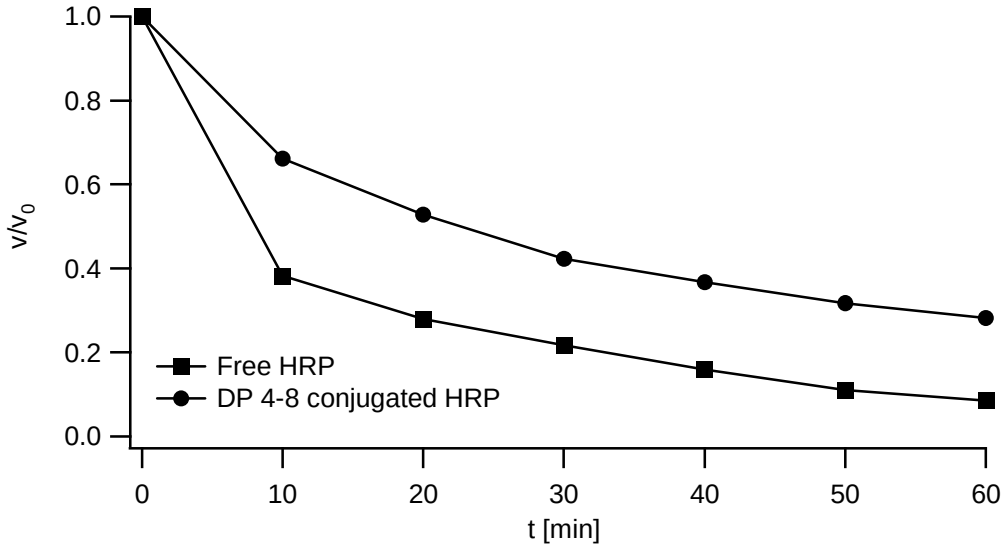


Figure 3.25. *Relative activity of HRP (squares) and DP 4-8 conjugated HRP over 60 minutes incubation at 70°C.*

3.7 Conjugation analysis

To determine if the conjugation of chitosan to horseradish peroxidase compromised the enzyme's activity the protein content of dialysed conjugate samples was measured using Bradford's method of protein quantification modified by Zaman and Verwilghen. Figure 3.26 shows the standard curve for HRP concentrations between 0 and 100 µg/mL.

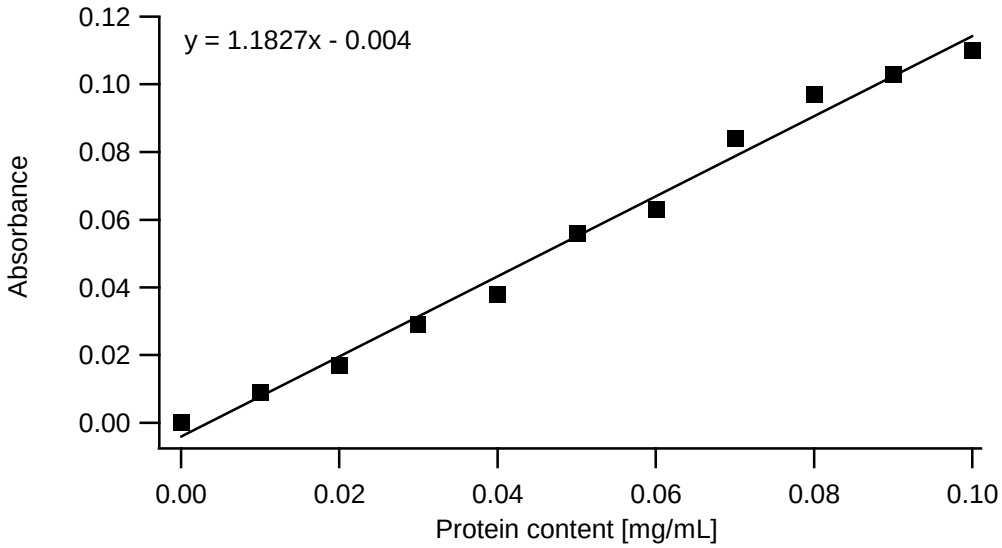


Figure 3.26. *Standard curve for HRP quantification in conjugate samples.*

The equation of the trend line in Figure 3.26 is then used to estimate the protein content of other HRP samples. Table 3.6 shows the protein content of conjugated samples after desalting followed by dialysis for 24 hours to remove as much as possible of unreacted agents.

Table 3.6. *Results of Bradford quantification of conjugate samples.*

Conjugate sample	**Absorbance**	**Dilution factor**	**Protein concentration [mg/mL]**	**Activity***
HRP	0.110	10	1.00	37.6
DP4-8	0.108	5.5	0.52	42.5
Tchos	0.073	5.5	0.36	44.2
Polymer	0.064	5.5	0.32	47.6

* Corrections were made to use the same concentration of enzyme in all activity assays.

As can be seen in Table 3.6 the activity was not compromised during conjugation. To determine the efficiency of the conjugation process carbohydrate quantification was carried out using the modified sulphuric acid-phenol assay. Standard curves were produced for each chitosan sample and are shown in Figure 3.27, Figure 3.28 and Figure 3.29.

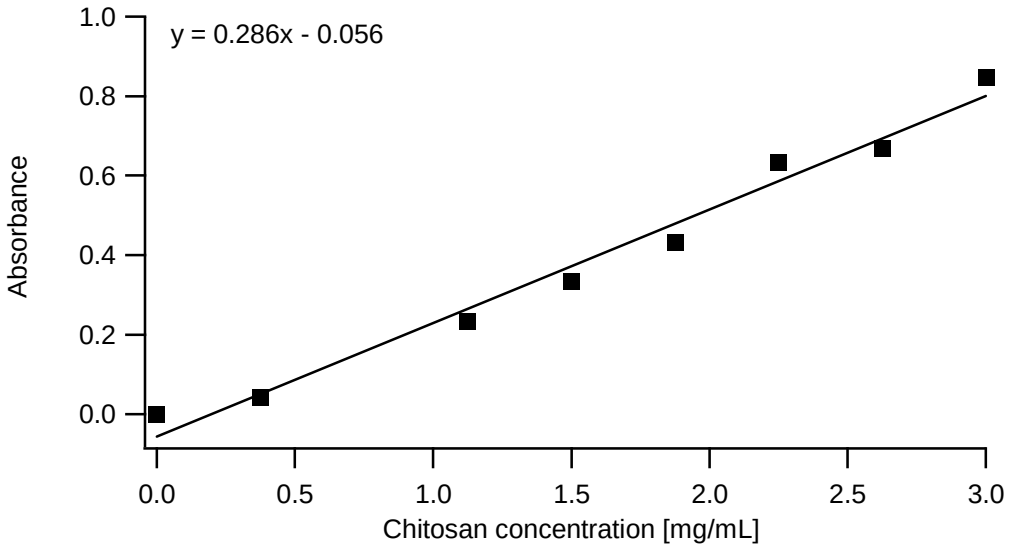


Figure 3.27. *Standard curve for quantification of chitosan DP 4-8.*

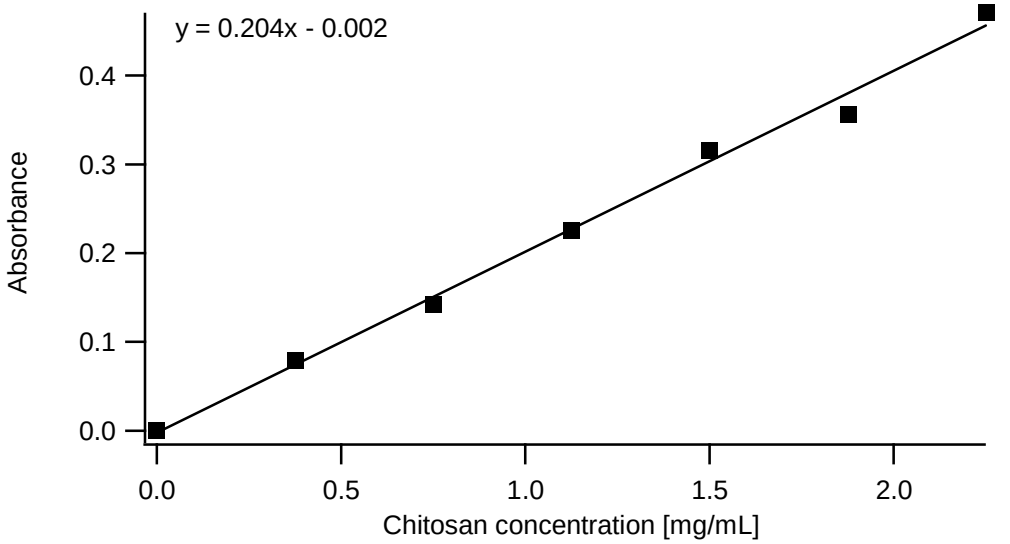


Figure 3.28. *Standard curve for quantification of chitosan Tchos.*

Table 3.7. *Properties of chitosan samples (Appendix B. Chitosan certificates)*

Chitosan sample	**Degree of polymerisation**	**Average molecular weight [kDa]**	**Degree of deacetylation**
G020701-1K	4-8	1.34	55%
G0601217K	7.95 (avg.)	1.5	34.0%
G060719P	polymer	n/a	48.4%

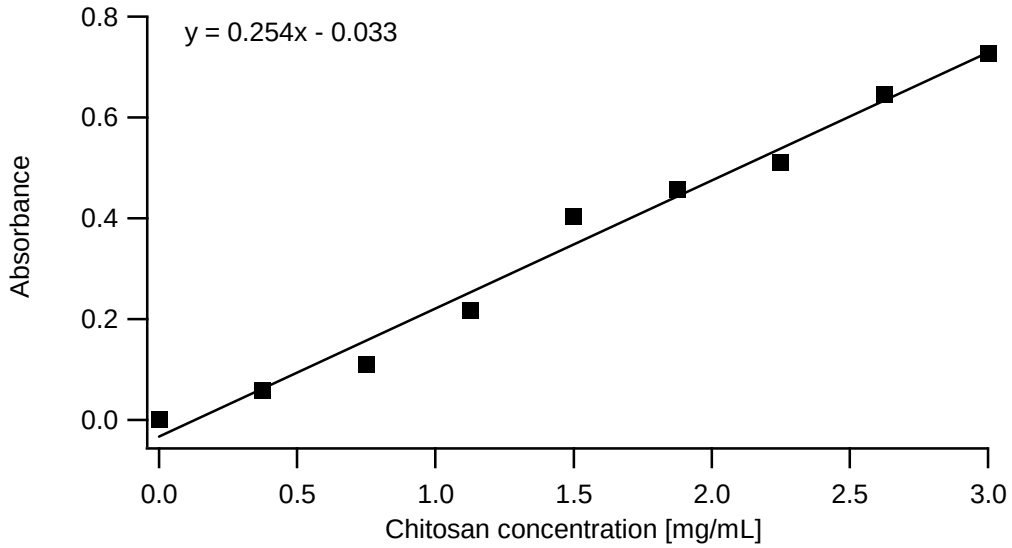


Figure 3.29. *Standard curve for quantification of chitosan polymer.*

Since horseradish peroxidase is glycosylated in its native form the effect of these carbohydrates must be accounted for when quantifying the chitosan conjugated to the enzyme. Table 3.8 shows the results of carbohydrate quantification for all three conjugates as well as for native HRP.

Table 3.8. *Results of carbohydrate quantification.*

Conjugate sample	**Absorbance**	**Protein concentration [mg/mL]**	**Original carbohydrates effect on abs.**	**Chitosan concentration [mg/mL]***
DP4-8	0.645	0.13	0.608	0.60
Tchos	0.429	0.09	0.420	0.20
Polymer	0.559	0.08	0.374	3.43

*Chitosan concentration is corrected for 4-fold dilution. Protein samples were diluted 25:100.

Table 3.8 shows that the conjugation of the DP 4-8 chitosan is most effective and also gives the most increase in thermal stability. Of the 16 mg used for conjugation these results indicate that 14 mg are conjugated to the enzyme. Further increase in chitosan concentration for the conjugation process did not result in increased thermal stability and made the reaction solution more viscous.

3.8 Effect of pH on catalytic activity of HRP and conjugates

Activity assays for horseradish peroxidase were performed to see if conjugation had any effect on the enzyme's activity at different pH. Native HRP is very active at pH 6-8 and the conjugation had no dramatic effect on its activity at different pH (Figure 3.30).

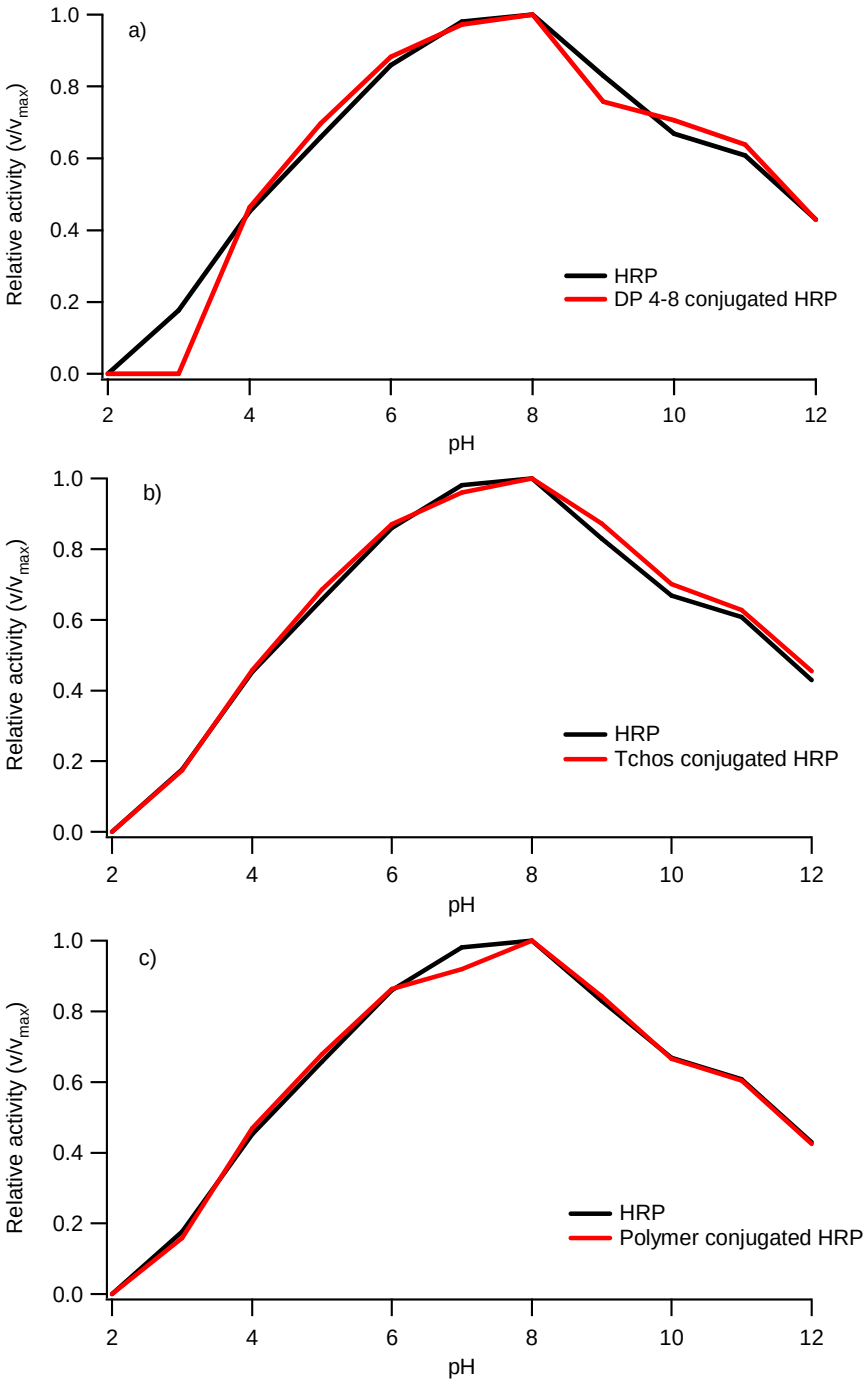


Figure 3.30. *Comparison of relative activity of HRP and a) DP 4-8 conjugated HRP, b) Tchos conjugated HRP and c) polymer conjugated HRP.*

3.9 Differential scanning calorimetry results

When proteins are denatured without aggregating the energy difference between the sample cell and the reference cell in VP-DSC is equal to the energy needed to break noncovalent bonds in the protein. Aggregation is an exothermic process that gives distorted peaks in DSC.

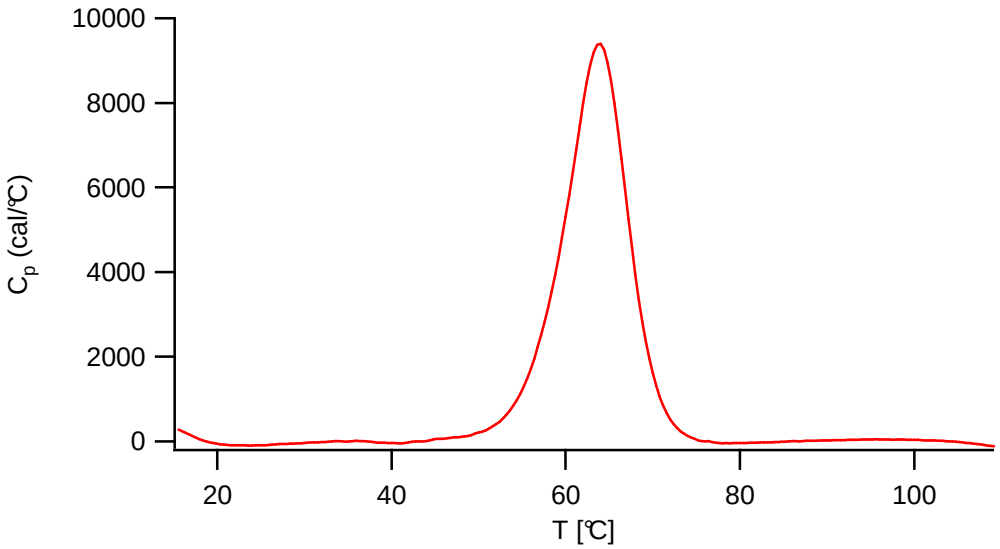


Figure 3.31. *DSC data for the denaturation of lysozyme.*

The peak obtained for the denaturation of lysozyme was very clear and no exothermic reactions distorts it. When the denaturation of horseradish peroxidase was inspected with DSC the results shown in Figure 3.32 indicates that significant aggregation occurs that gives distortion in the data and no clear peak is observed.

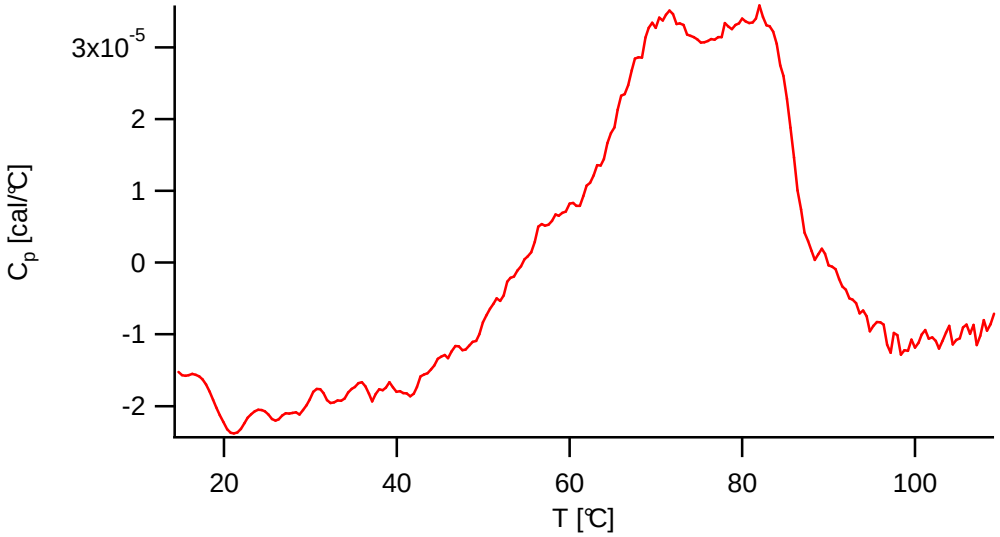


Figure 3.32. *DSC data for the denaturation of HRP at concentration of about 0.1 mg/mL.*

When the concentration was decreased further the peak disappeared into the distorted baseline. Increased concentration (Figure 3.33) did not give better results since the

aggregation of protein still distorts the peak. Even though it was not the purpose of this research to investigate the effect of conjugation of chitosan and trypsin that had already been done by Jónsson [1]. In that study the conjugation was performed without NHSS. Trypsin-chitosan conjugates were produced for the purpose of investigating if the increase in stability would show in DSC measurements (Figure 3.34). Thermal stability of the trypsin conjugate was also measured with the usual catalytic activity assay. DSC measurements showed that the T_m for the trypsin increased from 74.1°C for the free trypsin to 78.9°C for the conjugated trypsin.

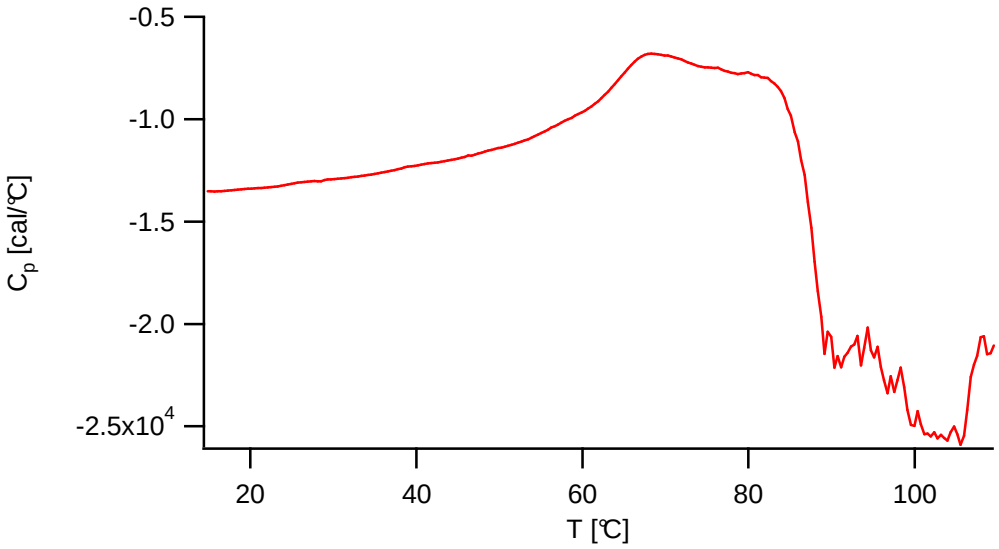


Figure 3.33. *DSC data for the denaturation of HRP at concentration of about 6.0 mg/mL.*

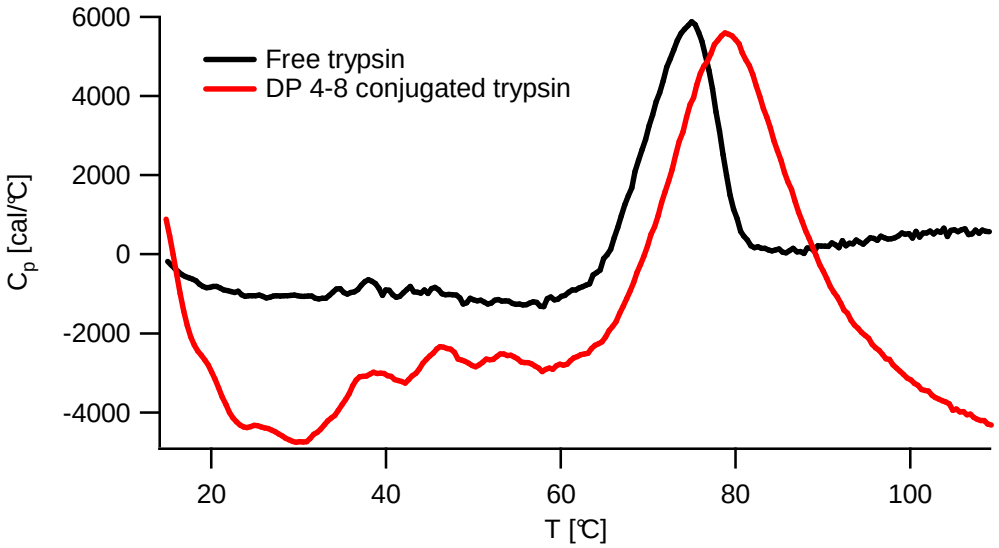


Figure 3.34. *DSC data for the denaturation of free trypsin (black line) and DP 4-8 conjugated trypsin (red line) in 0.2M acetate buffer, pH 5.0 [56], inhibited with TLCK.*

Distortions can be seen in the baseline in Figure 3.34 but not to the same degree as for HRP. DSC is therefore a very good method to detect thermal stability increases for proteins that do not aggregate.

4 Discussion

4.1 Chitosan solutions and thermal stability

Chitosan improves thermal stability of horseradish peroxidase both as a cosolute and when conjugated to the enzyme. Chitosan with a degree of polymerization of 4-8 is most effective and is also very easy to work with. The chitosan polymer was less soluble while Tchos was more soluble but not as effective. When the polymer was dissolved its concentration could not exceed about 5 mg/mL or the solution would form a gel when heated. Transferring small amounts of the polymer solution, for example to perform activity assays, proved difficult because of its viscosity. Considering that the polymer did not increase thermal stability more than the DP 4-8 chitosan its problematic application makes it inferior to the oligomers unless its texture and viscosity is of benefit, for example in cosmetics and pharmaceutical products.

Since the DP 4-8 chitosan dissolves more readily than the polymer in aqueous solutions its high concentration improves thermal stability much more than the polymer. The half life of HRP at 65°C in 10 mg/mL DP 4-8 chitosan solution was 58.4 minutes while HRP without any additives or modifications has a half life of 25.3 minutes. When the concentration of the DP 4-8 chitosan was increased to 50 mg/mL the half life was 91.8 minutes, so by increasing the chitosan concentration five-fold the half life nearly doubles.

At 60°C HRP retained about 63% of its initial activity after 30 minutes of incubation (Figure 3.5). The retention of activity increased to 94% for the 10 mg/mL solution of DP 4-8 chitosan (Figure 3.8) and the enzyme retains 97% of its initial activity in 50 mg/mL DP 4-8 chitosan solution (Figure 3.11). The polymer chitosan solution was also very effective at 60°C and the improvement in stability was similar to the DP 4-8 chitosan solution with relative activity of 93% after 30 minutes of incubation.

At 70°C the increase in stability was less than at the lower temperatures. HRP retained about 27% of its initial activity after 30 minutes of incubation while the weaker DP 4-8 chitosan solution only increased the relative activity to 34%. Relative activity of HRP in 50 mg/mL after 30 minutes of incubation was 51%, giving it half life of just over 30 minutes which is higher than the half life of free HRP at 65°C.

Water plays a big part in protein stability. Apolar sidechains of a protein's amino acid residues prefer to distance themselves from water molecules while polar sidechains are hydrophilic. Usually protein stabilisers are excluded from protein surfaces while protein denaturants bind to protein surfaces [78]. When a protein is dissolved in water every hydration site is occupied [79]. When stabilisers are in solution they affect the structure of water molecules around the surface of the protein and thereby influence the effect water has on hydrophobic interactions. Chitosan has a pK_a of 6.3 so at pH 6.5 its amine groups are mainly uncharged. Positively charged amino acids have proved to be stabilising by

interacting with negatively charged sidechains on proteins surfaces [80] and amino acids have also been used to increase hydrogen peroxide tolerance of HRP [48]. Sugars have also been used as stabilisers with good results [77, 81, 82].

4.2 Stability of enzyme-chitosan conjugates

The conjugation of chitosan to HRP resulted in considerable improvements in thermal stability. Chitosan with degree of polymerisation of 4-8 proved to be most effective in the conjugation with respect to both stability increase and efficiency of conjugation (Table 3.8). Enzyme conjugation with chitosan DP 4-8 doubled the half life of HRP at 65°C, increasing it from 25.3 minutes to 50.6 minutes. At 65°C the conjugate was less stable than HRP in 10 mg/mL solution of DP 4-8 chitosan but at 70°C the conjugate was more stable than HRP in chitosan solution with relative activities after 30 minutes of incubation of 43% and 34% respectively (Figure 3.5 and Figure 3.19). The increase in thermal stability when HRP was conjugated to the polymer was much less than for DP 4-8 chitosan. When the polymer was conjugated to the enzyme the conjugate solution was not stable over a long period and after two days precipitate was observed. The polymer was more soluble at lower pH but the conjugation conditions required pH 6.5 so the reaction solution had high viscosity that can lead to less effective conjugation. The conjugation of DP 4-8 proved to be most efficient, then the polymer conjugation and the least efficient was the Tchos conjugation (Table 3.8). This resulted in the same order with respect to increased thermal stability (Figure 3.23).

When chitosan has been conjugated to the surface of a protein its effect differs from the effect it has as a cosolute. The chitosan shields hydrophobic amino acids from the water at the surface of the protein. Chitosan also decreases flexibility of proteins thereby making denaturation more difficult [83].

Conjugation of HRP and chitosan had very little effect on the enzyme's activity at different pH (Figure 3.30). HRP is very active over a rather wide range of pH. Over the range from pH 6-9 its activity is above 80% of maximum activity (pH 7-8).

According to results in Table 3.6 and Table 3.8 about 7 mg of chitosan DP 4-8 was conjugated to 0.52 mg of HRP. In the conjugation reaction solution 2 mg of HRP were reacted with 16 mg of chitosan. The ratio of protein to chitosan was therefore 1:8 but was usually around 1:10 in the desalted and dialysed samples. During desalting the recommended volume was not collected to prevent the pollution of the collected sample with the crosslinking reagents. This could mean that the proteins with the highest degree of glycosylation were collected and the less glycosylated protein molecules were discarded. About 2.5 mL of solution were collected from the desalting column resulting in maximum yield of 1.3 mg protein (DP 4-8 conjugated). Increased concentration of chitosan did not cause increased thermal stability, possibly due to less effective conjugation due to increased viscosity of the reaction solution. Less focus was on the efficiency of the conjugation with respect to how glycosylated the protein was compared with the effect the glycosylation had on its stability. About 10 µL of the enzyme solution with protein concentration of 0.52 mg/mL and chitosan concentration of 6.93 mg/mL was incubated in 3 mL of buffer, resulting in 300 fold dilution which gives chitosan concentration of 23

µg/mL. That rules out the possibility that the increase in stability is due to chitosan in the solution, rather than conjugated to the enzyme.

4.3 Results of differential scanning calorimetry

Results for the denaturation of HRP were inconclusive due to the degree of exothermic aggregation of the protein. DSC is nevertheless very useful method in detection of increased thermal stability for proteins that undergo less aggregation during denaturation, such as lysozyme and inhibited trypsin. Appendix figure 1 and Appendix figure 2 show further results for the thermal stability measurements of trypsin. When investigating the stability of enzymes that have catalytic activity the change in

References

1. Jónsson, F., *Áhrif kítósans á stöðugleika nautatrypsíns.*
2. Kurita, K., *Controlled functionalization of the polysaccharide chitin.* Progress in Polymer Science, 2001. **26**: p. 1921-1971.
3. Lim, S.-H. and S.M. Hudson, *Synthesis and antimicrobial activity of a water-soluble chitosan derivative with a fiber-reactive group.* Carbohydrate Research, 2004. **339**(2): p. 313-319.
4. Kong, M., et al., *Antimicrobial properties of chitosan and mode of action: A state of the art review.* International Journal of Food Microbiology, 2010. **144**(1): p. 51-63.
5. Baker, R.T.M., et al., *Pigmenting efficacy of astaxanthin and canthaxanthin in fresh-water reared Atlantic salmon, Salmo salar.* Animal Food Science and Technology, 2002. **99**: p. 97-106.
6. Kumar, M.N.V.R., *A review of chitin and chitosan applications.* Reactive & Functional Polymers, 2000. **46**: p. 1-27.
7. Pillai, C.K.S., W. Paul, and C.P. Sharma, *Chitin and chitosan polymers: Chemistry, solubility and fiber formation.* Progress in Polymer Science, 2009. **34**: p. 641-678.
8. Shepherd, R., S. Reader, and A. Falshaw, *Chitosan functional properties.* Glycoconjugate Journal, 1997. **14**: p. 535-542.
9. Tsigos, I., et al., *Chitin deacetylases: new, versatile tools in biotechnology.* Trends in Biotechnology, 2000. **18**: p. 305-312.
10. Jia, Z. and D. Shen, *Effect of reaction temperature and reaction time on the preparation of low-molecular-weight chitosan using phosphoric acid.* Carbohydrate Polymers, 2002. **49**: p. 393-396.
11. He, P., S.S. Davis, and L. Illum, *In vitro evaluation of the mucoadhesive properties of chitosan microspheres.* International Journal of Pharmaceutics, 1998. **166**: p. 75-88.
12. Dodane, V. and V.D. Vilivalam, *Pharmaceutical applications of chitosan.* Pharmaceutical Science & Technology Today, 1998. **1**(6): p. 246-253.
13. Dash, M., et al., *Chitosan—A versatile semi-synthetic polymer in biomedical applications.* Progress in Polymer Science, 2011. **36**: p. 981-1014.
14. Kang, G.D. and S.-C. Song, *Effect of chitosan on the release of protein from thermosensitive poly(organophosphazene) hydrogels.* International Journal of Pharmaceutics, 2008. **349**(1-2): p. 188-195.
15. Lameiro, M.H., et al., *Incorporation of a model protein into chitosan–bile salt microparticles.* International Journal of Pharmaceutics, 2006. **312**(1-2): p. 119-130.
16. Pan, Y., et al., *Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo.* International Journal of Pharmaceutics, 2002. **249**: p. 139-147.
17. Freier, T., et al., *Controlling cell adhesion and degradation of chitosan films by N-acetylation.* Biomaterials, 2005. **26**(29): p. 5872-5878.

18. Mao, S., W. Sun, and T. Kissel, *Chitosan-based formulations for delivery of DNA and siRNA ☆.* Advanced Drug Delivery Reviews, 2010. **62**(1): p. 12-27.
19. Trapani, A., et al., *Characterization and evaluation of chitosan nanoparticles for dopamine brain delivery.* International Journal of Pharmaceutics, 2011.
20. Onishi, H. and Y. Machida, *Biodegradation and distribution of water-soluble chitosan in mice.* Biomaterials, 1999. **20**: p. 175-182.
21. Maezaki, Y., et al., *Hypocholesterolemic effect of chitosan in adult males.* Japan society for bioscience, biotechnology and agrochemistry, 1993. **57**(9): p. 1439-1444.
22. Luo, X.-L., et al., *A glucose biosensor based on chitosan–glucose oxidase–gold nanoparticles biocomposite formed by one-step electrodeposition.* Analytical Biochemistry, 2004. **334**(2): p. 284-289.
23. Krajewska, B., *Application of chitin- and chitosan-based materials for enzyme immobilizations: a review.* Enzyme and Microbial Technology, 2004. **35**(2-3): p. 126-139.
24. Boddu, V.M., et al., *Removal of Copper (II) and Nickel (II) Ions from Aqueous Solutions by a Composite Chitosan Biosorbent.* Separation Science and Technology, 2008. **43**(6): p. 1365-1381.
25. No, H.K., et al., *Antibacterial activity of chitosans and chitosan oligomers with different molecular weights.* International Journal of Food Microbiology, 2002. **74**: p. 65-72.
26. Sashiwa, H. and S.-i. Aiba, *Chemically modified chitin and chitosan as biomaterials.* Progress in Polymer Science, 2004. **29**: p. 887-908.
27. Aslam, M. and A. Dent, *Bioconjugation*. 1998: Macmillan Reference Ltd.
28. Veitch, N., *Horseradish peroxidase: a modern view of a classic enzyme.* Phytochemistry, 2004. **65**(3): p. 249-259.
29. Ryan, B.J., N. Carolan, and C. Ó'Fágáin, *Horseradish and soybean peroxidases: Comparable tools for alternative niches?* Trends in Biotechnology, 2006. **24**(8): p. 355-363.
30. Welinder, K.G., *Covalent structure of the glycoprotein horseradish peroxidase.* FEBS Letters, 1976. **72**(1): p. 19-23.
31. Welinder, K.G., *Plant peroxidases: Their primary, secondary and tertiary structures, and relation to cytochrome c peroxidase.* European Journal of Biochemistry, 1985. **151**: p. 497-504.
32. Voet, D. and J.G. Voet, *Biochemistry*. 3rd ed, ed. D. Harris and P. Fitzgerald. 2004, United States of America: Wiley.
33. Childs, R.E. and W.G. Bardsley, *The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) as chromogen.* Biochemical Journal, 1975. **145**: p. 93-103.
34. Berglund, G.I., et al., *The catalytic pathway of horseradish peroxidase at high resolution.* Letters to Nature, 2002. **417**: p. 463-468.
35. Rodríguez-López, J.N., et al., *Mechanism of reaction of hydrogen peroxide with horseradish peroxidase: Identification of intermediates in the catalytic cycle.* Journal of the American Chemical Society, 2001. **123**: p. 11838-11847.
36. Ropp, J.S.d., et al., *Influence of the distal His in Imparting imidazolate character to the proximal His in heme peroxidase: 1H NMR Spectroscopic study of cyanide-inhibited His42Ala horseradish peroxidase.* Journal of the American Chemical Society, 2002. **124**: p. 11029-11037.

37. Savenkova, M.I., S.L. Newmyer, and P.R.O.d. Montellano, *Rescue of His42Ala Horseradish Peroxidase by a Phe41His Mutation.* The journal of biological chemistry, 1996. **271**(40): p. 24598-24603.
38. Morishima, I., M. Kuro, and Y. Shiro, *Presence of endogenous calcium ion in horseradish peroxidase.* The journal of biological chemistry, 1986. **261**(20): p. 9391-9399.
39. Howes, B.D., *The Critical Role of the Proximal Calcium Ion in the Structural Properties of Horseradish Peroxidase.* Journal of Biological Chemistry, 2001. **276**(44): p. 40704-40711.
40. Miland, E., M.R. Smyth, and C.Ó. Fágáin, *Increased thermal and solvent tolerance of acetylated horseradish peroxidase.* Enzyme and Microbial Technology, 1996. **19**: p. 63-67.
41. Haifeng, L., et al., *Effects of sodium phosphate buffer on horseradish peroxidase thermal stability.* Journal of thermal analysis and calorimetry, 2008. **93**(2): p. 569-574.
42. Chang, B.S., K.H. Park, and D.B. Lund, *Thermal inactivation kinetics of horseradish peroxidase.* Journal of Food Science, 1988. **53**(3): p. 920-923.
43. Hassani, L., et al., *Horseradish peroxidase thermostabilization: The combinatorial effects of the surface modification and the polyols.* Enzyme and Microbial Technology, 2006. **38**(1-2): p. 118-125.
44. Tams, J.W. and K.G. Welinder, *Glycosylation and thermodynamic versus kinetic stability of horseradish peroxidase.* FEBS Letters, 1998. **421**: p. 234-236.
45. Liu, J.Z., et al., *Increased thermal and organic solvent tolerance of modified horseradish peroxidase.* Protein Engineering Design and Selection, 2006. **19**(4): p. 169-173.
46. Mogharrab, N., N. Ghourchian, and M. Amininasab, *Structural stabilization and functional improvement of horseradish peroxidase upon modification of accessible lysines: experiments and simulation.* Biophysical journal, 2007. **92**: p. 1192-1203.
47. Asad, S., et al., *Phosphate buffer effects on thermal stability and H_2O_2-resistance of horseradish peroxidase.* International Journal of Biological Macromolecules, 2011. **48**: p. 566-570.
48. Mahmoudi, A., et al., *Can amino acids protect horseradish peroxidase against its suicide-peroxide substrate?* Enzyme and Microbial Technology, 2008. **43**(4-5): p. 329-335.
49. Lomillo, M.A.A., J.G. Ruiz, and F.J.M. Pascual, *Biosensor based on platinum chips for glucose determination.* Analytica Chimica Acta, 2005. **547**(2): p. 209-214.
50. Azevedo, A., et al., *Ethanol biosensors based on alcohol oxidase.* Biosensors and Bioelectronics, 2005. **21**(2): p. 235-247.
51. Masuda, M., A. Sakurai, and M. Sakakibara, *Effect of temperature and pH on phenol removal using purified Coprinus cinereus peroxidase.* World Journal of Microbiology & Biotechnology, 2002. **18**: p. 739-743.
52. Caza, N., et al., *Removal of phenolic compounds from synthetic wastewater using soybean peroxidase.* Water Research, 1999. **33**(13): p. 3012-3018.
53. Hamid, M. and R. Khalil ur, *Potential applications of peroxidases.* Food Chemistry, 2009. **115**(4): p. 1177-1186.
54. Govardhan, C.P., *Crosslinking of enzymes for improved stability and performance.* Current Opinion in Biotechnology, 1999. **10**: p. 331-335.

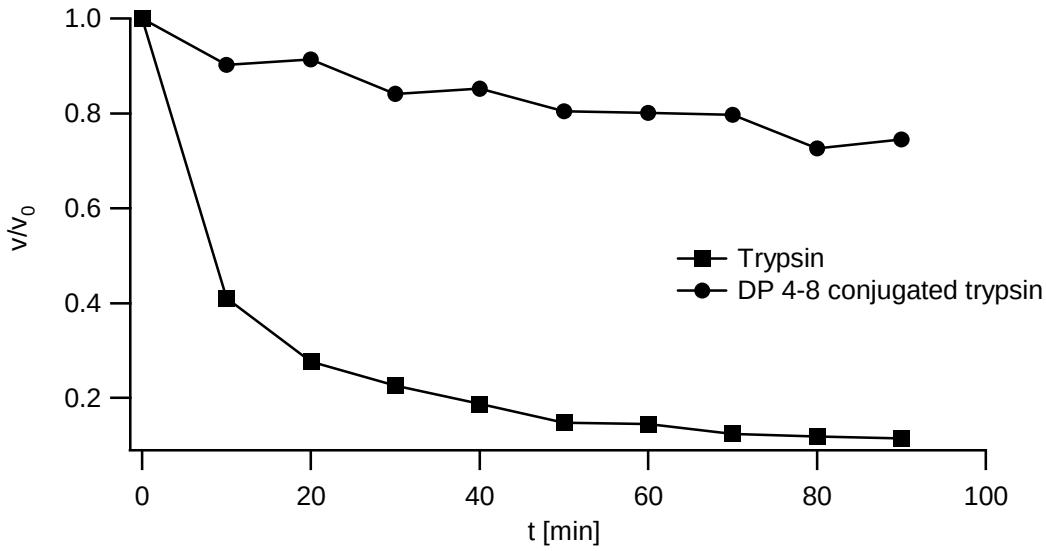
55. Jafariaghdam, J., et al., *Deglycosylation of glucoamylase from Aspergillus niger : Effects on structure, activity and stability.* Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics, 2005. **1750**: p. 61-68.
56. Pham, V.T., et al., *Glycation improves the thermostability of trypsin and chymotrypsin.* Biotechnology and bioengineering, 2008. **101**(3): p. 452-459.
57. Mateo, C., et al., *Improvement of enzyme activity, stability and selectivity via immobilization techniques.* Enzyme and Microbial Technology, 2007. **40**(6): p. 1451-1463.
58. Tardioli, P.W., et al., *Immobilization–stabilization of glucoamylase: Chemical modification of the enzyme surface followed by covalent attachment on highly activated glyoxyl-agarose supports.* Process Biochemistry, 2011. **46**(1): p. 409-412.
59. Cao, L., L.v. Langen, and R.A. Sheldon, *Immobilised enzymes: carrier-bound or carrier-free?* Current Opinion in Biotechnology, 2003. **14**(4): p. 387-394.
60. Šulek, F., et al., *Immobilization of horseradish peroxidase as crosslinked enzyme aggregates (CLEAs).* Process Biochemistry, 2011. **46**(3): p. 765-769.
61. Shah, S., A. Sharma, and M. Gupta, *Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder.* Analytical Biochemistry, 2006. **351**(2): p. 207-213.
62. Hermanson, G.T., *Bioconjugate Techniques*. 2 ed. 2008: Elsevier Inc.
63. Johnson, C.M., *Differential scanning calorimetry: Theory and practice.* GE Healthcare Life Sciences, 2010.
64. Cooper, A., *Differential scanning microcalorimetry*.
65. Cooper, A., *DSC Interpretation notes.*
66. *PD-10 Desalting Columns*, G. Healthcare, Editor.
67. Dubois, M., et al., *Colorimetric method for determination of sugars and related substances.* Analytical Chemistry, 1956. **28**(3): p. 350-356.
68. Masuko, T., et al., *Carbohydrate analysis by a phenol–sulfuric acid method in microplate format.* Analytical Biochemistry, 2005. **339**(1): p. 69-72.
69. Zaman, Z. and R.L. Verwilghen, *Quantitation of Proteins Solubilized in Sodium Dodecyl Sulfate-Mercaptoethanol-Tris Electrophoresis Buffer.* Analytical Biochemistry, 1979. **100**: p. 64-69.
70. Lífefnafræðistofa, R.H.Í.-. *Mæliaðferðir fyrir ThermoMax Plate Reader - Prótein og Ensím*, R.H. Íslands, Editor: Reykjavík.
71. Eryomin, A.N., T.V. Semashko, and R.V. Mikhailova, *Cooxidation of phenol and 4-aminoantipyrin, catalyzed by polymers and copolymers of horseradish root peroxidase and Penicillium funiculosum 46.1 glucose oxidase.* Applied Biochemistry and Microbiology, 2006. **42**(4): p. 399-408.
72. Worthington, *Peroxidase*.
73. Nicell, J.A. and H. Wright, *A model of peroxidase activity with inhibition by hydrogen peroxide.* Enzyme and Microbial Technology, 1997. **21**: p. 302-310.
74. Vojinović, V., et al., *Assay of H2O2 by HRP catalysed co-oxidation of phenol-4-sulphonic acid and 4-aminoantipyrine: characterisation and optimisation.* Journal of Molecular Catalysis B: Enzymatic, 2004. **28**(2-3): p. 129-135.
75. Fiamegos, Y., C. Stalikas, and G. Pilidis, *4-Aminoantipyrine spectrophotometric method of phenol analysis: Study of the reaction products via liquid chromatography with diode-array and mass spectrometric detection.* Analytica Chimica Acta, 2002. **467**: p. 105-114.

76. Altikatoglu, M., et al., *Stabilization of horseradish peroxidase by covalent conjugation with dextran aldehyde against temperature and pH changes.* Central European Journal of Chemistry, 2009. **7**(3): p. 423-428.
77. Altikatoglu, M. and Y. Basaran, *Additive effect of dextrans on the stability of horseradish peroxidase.* The protein journal, 2011. **30**: p. 84-90.
78. Shimizu, S. and N. Matubayasi, *Preferential hydration of proteins: A Kirkwood-Buff approach.* Chemical Physics Letters, 2006. **420**(4-6): p. 518-522.
79. Halle, B., *Protein hydration dynamics in solution: a critical survey.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2004. **359**(1448): p. 1207-1224.
80. Falconer, R.J., et al., *Stabilization of a monoclonal antibody during purification and formulation by addition of basic amino acid exipients.* Journal Of Chemical Technology & Biotechnology, 2011. **86**: p. 942-948.
81. Fernandez, L., et al., *Thermal stabilization of trypsin with glycol chitosan.* Journal of Molecular Catalysis B: Enzymatic, 2005. **34**(1-6): p. 14-17.
82. Meyer-Fernandes, M.S.-P.a.J.R., *Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: Why is trehalose more effective than other sugars?* Archives of biochemistry and biophysics, 1998. **360**(1): p. 10-14.
83. Sola, R. and K. Griebenow, *Chemical glycosylation: New insights on the interrelation between protein structural mobility, thermodynamic stability, and catalysis.* FEBS Letters, 2006. **580**(6): p. 1685-1690.

Appendix A. Thermal stability of trypsin

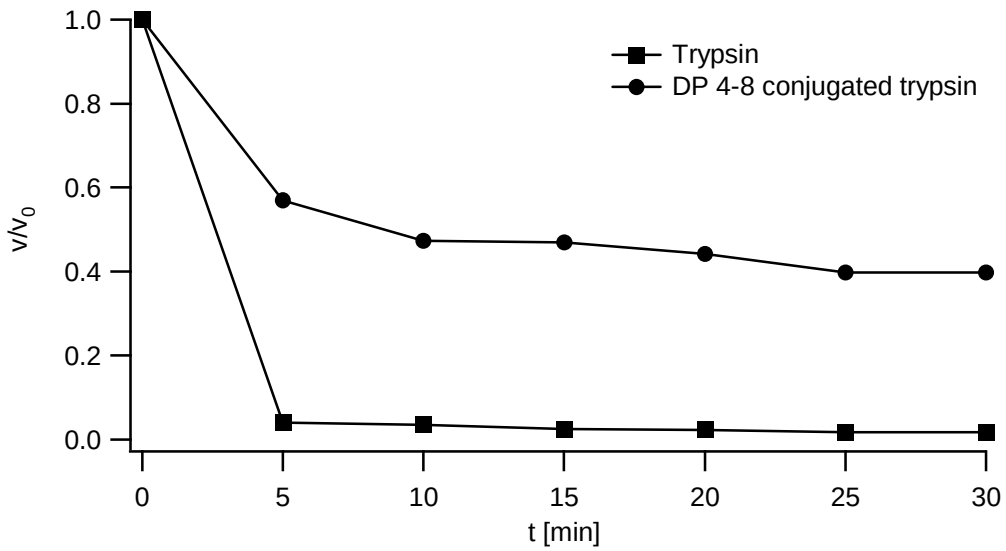
Differential scanning calorimetry indicated that the stability of trypsin from bovine pancreas was improved with conjugation to chitosan with degree of polymerisation of 4-8. To confirm these results thermal stability assay was performed similar to the method described in (2.2.4).

The reaction utilised to measure the activity of trypsin is hydrolysis of BAPNA (Nα-benzoyl-DL-arginine p-nitroanilide). The reaction is carried out in 112 mM triethanolamine, 11 mM calcium and 0.79 mM BAPNA. At 60°C free trypsin loses its activity quickly and after 10 minutes it retains only about 40% of its initial activity while the DP 4-8 chitosan conjugated trypsin was only down to 90% (Appendix figure 1). The incubation buffer for the trypsin contained 112 mM TEA and 11 mM $CaCl_2$ pH 7.80.



Appendix figure 1. *Relative activity of trypsin incubated at 60°C for up to 90 minutes.*

In Fannar Jónsson's study [1] the conjugation of chitosan to trypsin was done without NHSS and that resulted in much less increase in thermal stability.



Appendix figure 2. *Relative activity of trypsin incubated at 70°C for up to 30 minutes.*

Appendix figure 1 and Appendix figure 2 show that thermal stability of trypsin is greatly improved by conjugation to chitosan with DP of 4-8 and the activity assay is consistent with the results of the DSC measurements. The melting points (T_m) from the DSC measurements are 74.1°C and 78.9°C for free trypsin and DP 4-8 conjugated trypsin respectively. Trypsin was dissolved in 0.2 M acetate buffer, pH 5.0 [56].

Appendix B. Chitosan certificates

Certificate of analysis

PRODUCT : Oligomin®
SOURCE : *Pandalus borealis*.

GENERIC NAME : β(1→4) D-glucosamine / N-acetyl-D-glucosamine oligosaccharides

DESCRIPTION : Water soluble chitooligosaccharides

BATCH NO. : G0601217K

Prod.date : 24.7.2006
Exp.date : 23.7.2008
(Reanalysis required if used after this date).

PARAMETER			**TEST PROCEDURE**
pH	: 8.0		1% in distilled water
Dry Matter Content	: 85	%	CP-001
Ash	: 12.0	%	CP-002
Degree of Deacetylation	: 34.0	%	CP-014 (Direct titration)
HCl content (% w/w)	: 0.0	%	CP-014 (Direct titration)
Turbidity	: < 10	NTU	CP-003
Average MW	: 1500	Da	CP-017
Average DP	: 7.95	DP	CP-017
Solubility	: 100.0	%	CP-006
Feature	: Spray dried powder		
Appearance	: White powder		CP-007
Taste and odor	: No taste or smell		CP-007
Total Plate Count	: <1000	cfu/g	MB-003
Yeast and Mould	: <100	cfu/g	MB-004
Escherichia coli	: absent		MB-005
Coliform bacteria	: absent		MB-006
Salmonella sp.	: absent		MB-007

Primex ehf.
Date:

_____
Jón M. Einarsson

R&D Manager

Certificate of analysis

PRODUCT : Chitobiomer®
SOURCE : *Pandalus borealis*.

GENERIC NAME : β(1→4) D-glucosamine / N-acetyl-D-glucosamine oligosaccharides

DESCRIPTION : Water soluble polymer

BATCH NO. : G060719P Prod.date 19.7.2006
Exp.date 18.7.2008
(Reanalysis required if used after this date).

PARAMETER			**TEST PROCEDURE**
pH	: 8.0		1% in distilled water
Dry Matter Content	: 94.5	%	CP-001
Ash	: 0.5	%	CP-002
Degree of Deacetylation	: 48.4	%	CP-014 (Direct titration)
Turbidity	: <15	NTU	CP-003
Viscosity	: 300-500	mPas	
Solubility	: 100.0	%	CP-006
Feature	: Spray dried powder		
Appearance	: White powder		CP-007
Taste and odor	: No taste or smell		CP-007
Total Plate Count	: <1000	cfu/g	MB-003
Yeast and Mould	: <100	cfu/g	MB-004
Escherichia coli	: absent		MB-005
Coliform bacteria	: absent		MB-006
Salmonella sp.	: absent		MB-007

Certificate of analysis

PRODUCT : Oligomin
SOURCE : *Pandalus borealis*.

GENERIC NAME : β(1→4) D-glucosamine / N-acetyl-D-glucosamine oligosaccharides

BATCH NO. : G020701-1K
Prod.date : 1.7.2002
Exp.date : 1.7.2005
(Reanalysis required if used after this date).

PARAMETER				**TEST PROCEDURE**
Dry Matter Content	:	90.8	%	CP-001
Ash, 99% NaCl	:		%	CP-002
Degree of Deacetylation	:	55	%	Direct titration
Viscosity	:	<5	cP (mPa·s)	CP-004 (1% chitosan solution)
Solubility	:	99.0	%	CP-006
Sieve Analysis	:	100	% through 100 mesh	CP-008
Appearance	:	White powder		CP-007
Taste and odor	:	No taste or smell		CP-007
Total Plate Count	:	<100	cfu/g	MB-003
Yeast and Mould	:	<100	cfu/g	MB-004
Escherichia coli	:	absent		MB-005
Coliform bacteria	:	absent		MB-006
Salmonella sp.	:	absent		MB-007

Primex ehf.
Date:

_____
Jón M. Einarsson
R&D