EVALUATION OF THE ENZYME INHIBITORY EFFECT OF CARBOXYMETHYLATED CHITOSAN

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2003

EVALUATION OF THE ENZYME INHIBITORY EFFECT OF CARBOXYMETHYLATED CHITOSAN

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(B.Pharm)

Dissertation for partial fulfilment of the
requirements for the degree

MAGISTER SCIENTIAE (PHARMACEUTICS)

in the
School of Pharmacy
at the

POTCHEFSTROOMSE UNIVERSITEIT VIR
CHRISTELIKE HÖER ONDERWYS

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POTCHEFSTROOM
2003

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Introduction and aim of study

A high degree of bioavailability has been difficult to achieve for many drugs such as protein or peptide drugs that degrade in the acid gastric environment or those that are poorly absorbed. Much has been discovered through research in drug delivery of peroral administrated drugs. Chitosan has proved to aid the bioavailability of several drugs such as prednisolone, ketoprofen and theophylline by sustaining constant plasma levels of these drugs. The effects of chitosan are largely due to the bioadhesive properties it possesses which extends the contact with the absorbing epithelia (Paul & Sharma, 2000:6).

Co-administrating bioadhesive polymers with peptide drugs to improve bioavailability of these drugs is but one way of creating such an effect. Peroral administrated peptide drugs are broken down by intestinal protease enzymes. Inhibiting these enzymes may slow down the enzymatic degeneration of these drugs in the gastrointestinal tract and may therefore increase the absorption and hence, the bioavailability of the drugs. α-Chymotrypsin, a luminal protease enzyme, often initiates the degradation of peptide drugs administered perorally. Exopeptidase enzymes such as carboxypeptidases, aminopeptidases and oligopeptidases then further digest the resulting fragments (Lueßen *et al.,* 1996:117). Inhibiting a luminal protease enzyme such as α-chymotrypsin may hinder the initiation of the digestion process for these drugs. Further digestion will also be hampered as it will be harder to manage the digestion of larger fragments by exopeptidase enzymes.

Chitosan, having exceptional mucoadhesive and permeation enhancing properties as well as the capability to control the release of incorporated drugs, does not present any sufficient enzyme inhibitory properties. Various chemical modifications has been made to chitosan to improve its enzyme inhibitory properties through conjugating chitosan with

known enzyme inhibitors such as NTA, EDTA and Bowman-Birk inhibitor (Bernkop-Schnürch & Kast, 2001:131). Enzyme inhibitors administrated orally must have certain intrinsic properties that will render it suitable for human consumption. In other words it must be safe and effective.

The aim of this study was to determine whether another group of chitosan derivatives i.e. *N,N*-dicarboxymethylated chitosans possess the ability to inhibit the protease enzyme α-chymotrypsin. *N,N*-dicarboxymethylated chitosans have proven to be safe for human consumption and have several uses in the world of medicine (Muzzarelli *et* al., 1993:34). Various *N,N*-dicarboxymethylated chitosan polymers were tested and compared with the well known enzyme inhibitor Carbopol 934P® by monitoring the formation of the product *N*-Acetyl-L-Tyrosine or AT, through an enzyme-substrate reaction over a period of time.

The specific objectives of this study can be ordered as follows:

Chapter 1: A literature study on

- enzymes function and the importance of enzymes,
- the transition state complex and
- specific mechanisms of enzyme action.

Chapter 2: A literature study on

- the types of enzyme inhibition and
- enzyme inhibitors.

Chapter 3: The synthesis of *N,N*-dicarboxymethyl chitosans and their characterisation through

- ^{1}H-NMR spectrometry,
- infrared (IR) spectrometry and
- differential scanning calorimetry (DSC).

Chapter 4: The validation of the High Performance Liquid Chromatography method used for analysis by validating the

- specificity,
- linearity,
- accuracy and precision of the method and
- the stability of the sample.

Chapter 5: The evaluation of the enzyme inhibiting properties of

- Carbopol 934P®,
- *N,N*-dicarboxymethyl chitosan,
- *N,O*-dicarboxymethyl chitosan,
- *N,N*-dicarboxymethyl chitosan oligomer and
- *N*-trimethyl chitosan chloride.

Chapter 6: Final conclusions, summary and future prospects.

Abstract

Degradation of peroral administered drugs by various enzymes in the gastrointestinal tract has proven to be troublesome for the absorption and bioavailability of protein and peptide drugs. Mucoadhesive polymers such as poly(acrylates) have proven to inhibit protease enzymes responsible for initiating digestion of peptide drugs. Enzyme inhibitors have unique chemical properties enabling it to interact with enzymes to form complexes with such enzymes prohibiting it from functioning properly. Anionic carboxymethylated chitosan derivatives such as *N,N*-dicarboxymethyl chitosan and *N,O*-carboxymethyl chitosan display unique structural similarities to enzyme inhibitors being anionic polymers that may interact with bi-valent cations.

N,N-dicarboxymethyl chitosan and *N,N*-dicarboxymethyl chitosan oligomer were synthesised and were tested together with *N,O*-carboxymethyl chitosan for inhibition of α-chymotrypsin, a luminal protease enzyme. The synthesised polymers were characterise by ^{1}H-NMR, IR and DSC. Inhibition of α-chymotrypsin by Carbopol 934P®, a known enzyme inhibitor, was also investigated to establish whether the method used was adequate and to compare the inhibition capabilities of the anionic chitosan derivatives with that of Carbopol 934P®. *N*-trimethyl chitosan chloride or TMC, was also tested to illustrate and compare the enzyme inhibition capability of a cationic chitosan derivative with that of the anionic polymers.

Carbopol 934P® showed very good inhibition of α-chymotrypsin whereas the anionic chitosan polymers, though showing inhibition, had a far less inhibitory effect on the enzyme. The low molecular weight *N,N*-dicarboxymethyl chitosan oligomer and high molecular weight *N,O*-carboxymethyl chitosan showed better inhibition than the high molecular weight *N,N*-dicarboxymethyl chitosan. This might be because of stereochemistry allowing *N,N*-dicarboxymethyl chitosan oligomer and high molecular

weight *N,O*-carboxymethyl chitosan to interact more completely with bi-valent cations. TMC did not show any inhibition of α-chymotrypsin.

It was concluded that anionic chitosan derivatives does inhibit proteolytic α-chymotrypsin to some extend but it cannot be concluded if the inhibition would be sufficient to promote the bioavailability of suitable drugs in formulated dosage forms.

Key words: Mucoadhesive polymers, protease enzymes, enzyme inhibitors, *N,N*-dicarboxymethyl chitosan, *N,O*-carboxymethyl chitosan, proteolytic α-chymotrypsin.

Uittreksel

Die afbraak of degradasie van geneesmiddels wat oraal toegedien word, deur verskeie ensieme in die gastrointestinale kanaal, kan problematies wees vir die absorpsie en biobeskikbaarheid van veral proteïen en peptiedgeneesmiddels. Dit is egter bekend dat mukoklewende polimere, soos poli(akrilate) sekere protease ensieme wat verantwoordelik is vir die afbraak van peptiedgeneesmiddels, kan inhibeer. Ensieminhibeerders het unieke chemiese eienskappe wat hulle in staat stel om met ensieme te reageer op so 'n wyse dat dit komplekse vorm met die ensieme en daardeur ensieme verhinder om volkome te funksioneer. Derivate van anioniese karboksiemetiel kitosaan, soos byvoorbeeld *N,N*-dikarboksiemetiel kitosaan en *N,O*-karboksiemetiel kitosaan, het unieke struktuurverwantskappe wat ooreenkom met bekende ensieminhibeerders. Heelwat ensieminhibeerders is anioniese polimere wat reageer met bivalente katione.

N,N-dikarboksiemetiel kitosaan en *N,N*-dikarboksiemetiel kitosaan oligomeer is gesintetiseer en saam met *N,O*-karboksiemetiel kitosaan, wat kommersiëel beskikbaar is, geëvalueer vir inhibisie van α-chemotripsien, wat 'n luminale protease ensiem is. Die gesintetiseerde polimere is gekarakteriseer met behulp van ^{1}H-KMR, IR en DSC. Die inhibisie van α-chemotripsien deur Carbopol 934P®, 'n bekende ensieminhibeerder, is ook ondersoek en vergelyk met die anioniese polimere ten einde 'n sinvolle vergelyking te kan tref tussen die inhibisie effekte van die verskillende polimere. 'n Kationiese kitosaan derivaat naamlik *N*-trimetiel kitosaan chloried, of TMC, is ook geëvalueer om die ensieminhiberende eienskappe van 'n kationiese kitosaan polimeer te ondersoek.

Al die anioniese kitosaan polimere het ensieminhiberende eienskappe op α-chemotripsien getoon maar dit was nie in die selfde orde as die inhibisie wat met Carbopol 934P®

verkry is nie. *N,N*-dikarboksiemetiel kitosaan oligomeer, wat 'n lae molekulêre gewig polimeer is, en *N,O*-karboksiemetiel kitosaan, wat 'n hoë molekulêre gewig polimeer is, het beter inhiberende eienskappe as hoë molekulêre gewig *N,N*-dikarboksiemetiel kitosaan vertoon. Die rede hiervoor mag wees as gevolg van die meer gunstige stereochemie van *N,N*-dikarboksiemetiel kitosaan oligomeer en *N,O*-karboksiemetiel kitosaan wat toelaat dat hierdie polimere meer volledig reageer met bivalente katione. TMC het geen inhibisie van α-chemotripsien getoon nie.

Hierdie studie het aangetoon dat anioniese kitosaan derivate nie proteolitiese ensieme soos α-chemotripsien tot dieselfde mate as Carbopol 934P® inhibeer nie. Dit is egter nog nie vasgestel of die ensieminhiberende eienskappe van hierdie anioniese polimere voldoende is om die biobeskikbaarheid van geneesmiddels in doseervorme te bevorder nie.

Kernwoorde: Mukoklewende polimere, protease ensieme, ensieminhibeerders, *N,N*-dikarboksiemetiel kitosaan, *N,O*-karboksiemetiel kitosaan, α-chemotripsien.

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Chapter 1

Enzymes

1.1 Introduction

Since the origin of drug discovery and development it has been found that various biochemical processes in the human body has an influenced on the absorption, distribution, metabolism and elimination of drugs. Many of these biochemical processes or pathways are influenced by enzyme activity.

As biocatalysts that regulate the rates at which all physiological processes take place, enzymes play a central role in health and disease. Life as we know it would not be possible without the existence of enzymes. When one is healthy all physiologic processes occur in an ordered regulated manner and homeostasis is maintained, which can be profoundly disturbed in pathologic states. The severe tissue injury in liver cirrhosis, for example, can profoundly impair the ability of cells to form enzymes, which catalyse a key metabolic process such as urea synthesis. Thus toxic ammonia cannot be converted to nontoxic urea and can then lead to ammonia intoxication, which can ultimately result in a hepatic coma. There exists a spectrum of rare but frequently debilitating and fatal genetic diseases that provide additional dramatic examples of the drastic physiological consequences that can follow impairment of the activity of but a single enzyme (Rodwell, 1993:60).

A century ago only a few enzymes were known. These, most of which catalysed the hydrolysis of covalent bonds, were identified by adding the suffix *–ase* to the name of the substance, or substrate which they hydrolysed. Thus it is seen that lipases hydrolysed fat

(lipos), amylases hydrolysed starch (amylon) and proteases hydrolysed proteins (Rodwell, 1993:60).

Protease enzymes in the gastrointestinal tract break down peroral administered peptide drugs. It is here where the various enzymes inactivate a great part of peptide drugs. Inhibiting these enzymes may lead to the enhancement of absorption of drugs broken down by intestinal enzymes. Various ways of inhibiting enzymes exist of which the use of enzyme inhibitors is the most common (Lueßen *et al.*, 1996:23).

1.2 What are enzymes?

According to Nelson and Cox (2000:244), enzymes are proteins, with the exception of a small group catalytic RNA molecules. Enzymes act as catalysts in biochemical reactions where the catalytic activity depends on the integrity of their native protein conformation. Enzymes are relatively large molecules with molecular weights ranging from ± 12000 to over 1 million g/mole. Silverman (1988:98) defines enzymes as special types of receptors, but also refers to it as proteins with high molecular weights that catalyse reactions in a biological system.

There are some enzymes that require no chemical groups other than their amino acid residues for activity. Other enzymes require an additional chemical compound called a co-factor or co-enzyme (Rodwell, 1993:61). This co-factor can be either a) inorganic ions such as Fe^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+}, or b) a complex organic or metal organic molecule. Some enzymes require both a) and b) for activity (Nelson & Cox, 2000:244). Enzymes that require co-enzymes include those which catalyse redox reactions that form covalent bonds and perform group transfer- and isomerisation reactions. Lytic reactions, including hydrolic reactions catalysed by digestive enzymes, do not require co-enzymes (Rodwell, 1993:61).

1.3 The basic working mechanism of enzymes

Enzymes serve as distinctive substances that are essential in the digestion of food, the sending of nerve impulses and the contracting of muscles. The distinguishing feature of an enzyme-catalysed reaction is that it occurs within the enzyme surface called the *active site* (Nelson & Cox, 2000:245).

The initial concept of an active site is largely attributed to the proposal of Emil Fishers' "lock and key" theory (Figure 1.1) first put forward in the 1890's and is still appealing in some respects. In his theory the active site of the enzyme is conceived as being a rigid structure with a well-defined geometry, similar to a lock.

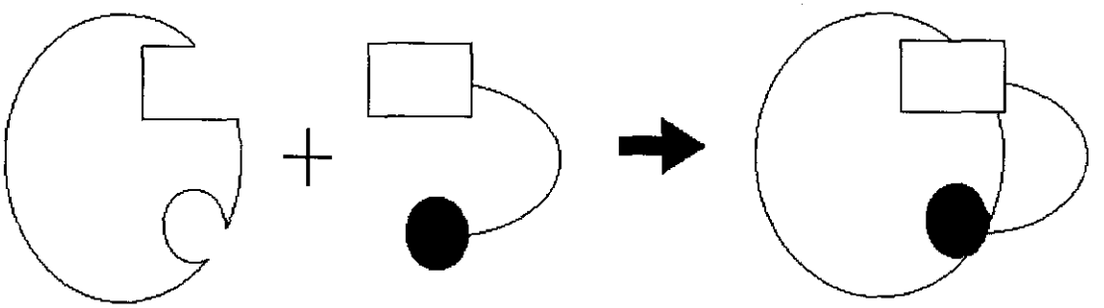

Figure 1.1 Schematical representation of the formation of an enzyme-substrate complex according to the Fisher template hypothesis (Rodwell, 1993:75).

A molecule with a complementary geometry, which is known as the "key", may only fit this so called "lock". More recently researchers such as Koshland and Gutfreund have presented theories of enzymes as being more flexible and the enzymatic catalysis as being a more dynamic process involving co-operative interactions between an enzyme and its substrate (Zeffren & Hall, 1973:12). This more general model is known as the "induced fit" model and has considerable experimental support. Fishers' model presumes the active site to be preshaped to fit the substrate and in the "induced fit" model, the substrate induces a conformational change in the enzyme (Rodwell, 1993:75).

A key property of enzymes is their ability to bind one or (more frequently) both reactants in a bimolecular reaction with an accompanying increase in local reactant concentration and reaction rate. Enzymes are both extremely efficient and serve as highly selective catalysts of various biochemical reactions in human physiology due to the "lock and key" working mechanism (Rodwell, 1993:75).

The substrate is the molecule that binds to the enzyme on the active site. The surface of the active site is lined with residues of amino acids which substituent groups bind the substrate and catalyses its chemical transformation. This enzyme-substrate complex was first proposed by Wurtz in 1880 and is central to the action of enzymes (Nelson & Cox, 2000:248):

$$E + S \leftrightarrow ES \leftrightarrow EP \leftrightarrow E + P \qquad \text{(Equation 1.1)}$$

The overall catalytic efficiency of enzymes is usually attributed to a combination of 3 general aspects of the enzymatic process: a) The formation of a non-covalent complex with the substrate binding; b) the manifestation of the substrate "specificity" by the enzyme which is the relative preference of an enzyme for one specific substrate over another, or the ability of the enzyme to distinguish between optical isomers of a material, interacting with one but not the other and c) the catalytic process itself (Zeffren & Hall, 1973:10).

The concept of enzyme-substrate combination as the first step in enzyme catalysis gradually gained acceptance and was formulated concisely by Michaelis and Mentin (1913) and is as follows:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

(Equation 1.2)

$$k_m = \frac{k_{-1} + k_2}{k_1} = \frac{[E][S]}{[ES]} \qquad \text{(Equation 1.3)}$$

where E is the enzyme, S is the substrate, ES the enzyme-substrate complex, P the product and k_m the Michaelis constant as the apparent constant of the complex. The constant, k_m, is directly proportional to the affinity of the enzyme (E) for the substrate (S). It is found that the actual physical significance of k_m is dependant on the ratio of k_2/k_{-1}. As this ratio approaches zero one might be justified to interpret the Michaelis constant as a dissociation constant. It is also possible to calculate the binding energies to differentiate between the specificities of the different substrates.

Quite a lot of effort has been applied to the development of different methods by which one can evaluate the equilibrium constant k_{-1}/k_1. The reason for this is so that the various affinities of an enzyme's binding site for a range of substrates can be measured and compared.

The next important step after the formulation of the Michaelis- Mentin equation (1.2) was the steady-state treatment of enzyme reactions, done by Briggs and Haldane (1925). They pointed out that although the equilibrium assumption is not necessarily justified, the steady-state treatment gives the same result as the Michaelis-Mentin assumption of the reaction velocity in terms of $[E]_0$, [S], k_m and k_0 where $[E]_0$ is the concentration of the total enzyme present while [E] is the concentration of the free enzyme. If [E] is substituted with $[E]_0 - [ES]$ then one finds that;

$$k_m = \frac{[S]([E]_0 - [ES])}{[ES]} \qquad \text{(Equation 1.4)}$$

It can then be derived that the concentration of the enzyme substrate complex is given by:

$$[ES] = \frac{[S][E]_0}{k_m + [S]} \qquad \text{(Equation 1.5)}$$

Since k_0 is defined as the rate constant for the rate determining step, the rate of the reaction is given by $V = d(P)/dt = k_0[ES]$ and combining this with Equation 1.5 states that:

$$V = \frac{k_0[S][E]_0}{k_m + [S]} \qquad \text{(Equation 1.6)}$$

Many of the basic simple equations of enzyme kinetics used to interpret reactions of enzymes with their substrates and inhibitors are derived from these simple assumptions (Gutfreund, 1965:4).

Substances that bind but do not chemically react with the enzyme often affect the overall rate of the enzymatic reaction in either a positive or negative way. The reason is because in the presence of a bond, unreactive species can alter the catalytic properties of an enzyme. If such an effect is positive on the enzymatic reaction, then the substance is known as an activator and if it has a negative effect, then it is known as an inhibitor. Generally inhibition is more common than activation.

The inhibition process is characterised by a dissociation constant, k_i, known as the inhibition constant and is derived from the reaction of the enzyme with its inhibitor

$$E + I \underset{k_{-i'}}{\overset{k_{i'}}{\rightleftharpoons}} EI \qquad \text{(Equation 1.7)}$$

to give

$$K_i = \frac{k-1'}{k_{1'}} = \frac{[E][I]}{[EI]} \qquad \text{(Equation 1.8)}$$

Many enzymes are strictly specific and react mainly with one substrate only (Zeffren & Hall, 1973:11). Enzymes are also specific for the type of reaction catalysed. Lytic

enzymes, for example, act on specific chemical groupings, e.g., glycosidase on glycosides, pepsin and trypsin on peptide bonds, and esterase on esters. Different peptide substrates may be attacked, lessening the number of digestive enzymes required. Proteases may also catalyse the hydrolysis of esters (Rodwell, 1993:61).

1.4 The transition state complex

When Equation 1.1 is expressed as $A + B \leftrightarrow C + D$ it is evident that it must now include the formation of the transition state complex, often also referred to as the activated complex, wherein the formation of a certain complex occurs during the reaction. It is therefore derived that $A + B \leftrightarrow X^+ \leftrightarrow C + D$ where X^+ is the transition state complex. The energy acquired by the molecules in order to form the transition state complex in the reaction is defined as the activation energy. This is the difference in the energy between the complex X^+ and the reactants and can be regarded as a barrier, which prevents the reaction of A and B until sufficient energy is acquired.

It is therefore evident that energy changes occur during enzymatic reactions. Figure 1.2 is a diagrammed example of these energy changes in the reaction $A + B \leftrightarrow C + D$. It is now possible to determine, from classical thermodynamic calculations, the free energies of the reactants A and B in the initial state and of the products C and D in the final state and hence, to determine the overall free energy change for the reaction. Through the reaction classical or equilibrium thermodynamic measurements would indicate that there was an overall decrease in energy, ΔE, which suggests that the reaction should occur spontaneously.

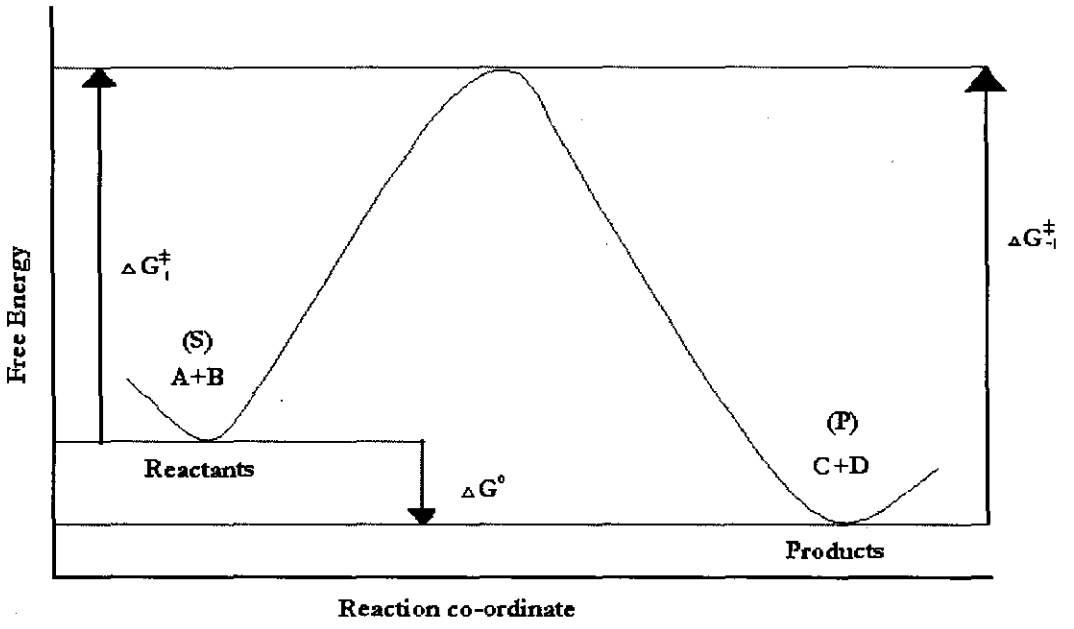

Figure 1.2 Schematic representation of the free energy changes in the chemical reaction $A + B \leftrightarrow C + D$ (Roberts, 1977:22).

These classical thermodynamic measurements, however, do not give any indication of the size of the activation energy barrier that must be overcome before the reaction between A and B can occur (Roberts, 1977:23).

1.5 The specific mechanisms of enzyme action

The mechanism of enzyme action is described by the molecular events that accompany the conversion of substrates to products. These molecular events in catalysis are termed "intermediary enzymology". High resolution structural information obtained by x-ray crystallography, combined with mechanistic information now facilitates the design of drugs to inhibit specific enzymes such as HMG-CoA reductase, the pacemaker enzyme in cholesterol biosynthesis. These studies lead to a rational approach to therapy and drug

design, which are areas of great potential for development in the immediate future (Rodwell, 1993:86).

1.5.1 The "intermediary enzymology" of chymotrypsin

Chymotrypsin specifically catalyses hydrolysis of peptide bonds in which the carboxyl group is attributed by an aromatic amino acid such as phenylalanine, tyrosine and tryptophan or by one with a bulky non-polar group such as a methyl group. Chymotrypsin also catalyse the hydrolysis of certain esters (Fersht, 1977:18).

Even though the ability of chymotrypsin to hydrolyse esters is of no physiological significance, it facilitates the study of the catalytic mechanism. It is possible to study the kinetics of chymotrypsin hydrolysis by the detection of intermediates by the use of "stop-flow" spectrophotometry. Stop-flow experiments use substrate quantities of enzymes and measure the events that occur in the first few milliseconds after the enzyme and substrate are mixed. The synthetic substrate *p*-nitrophenylacetate facilitates the colorimetric analysis of chymotrypsin activity because the hydrolysis of *p*-nitrophenylacetate produces *p*-nitrophenol. In an alkaline medium it converts to the yellow *p*-nitrophenylate anion (Rodwell, 1993:86).

The original work on *p*-nitrophenylacetate has been extended by synthesising *p*-nitrophenylesters of specific acyl groups, such as acetyl-L-phenylalanine, -tyrosine, and -tryptophan. The rate of acylation of the enzyme is determined from the rate of appearance of the nitrophenol or nitrophenolate ion, which absorbs at a different wavelength from the apparent ester (Fersht, 1977:176).

The release of *p*-nitrophenylate anion occurs in 2 distinct phases illustrated in Figure 1.3. Phase 1 is known as the "burst" phase, characterised by a rapid liberation of the *p*-nitrophenylate anion and the second phase is a subsequent, slower release of additional *p*-nitrophenylate anion.

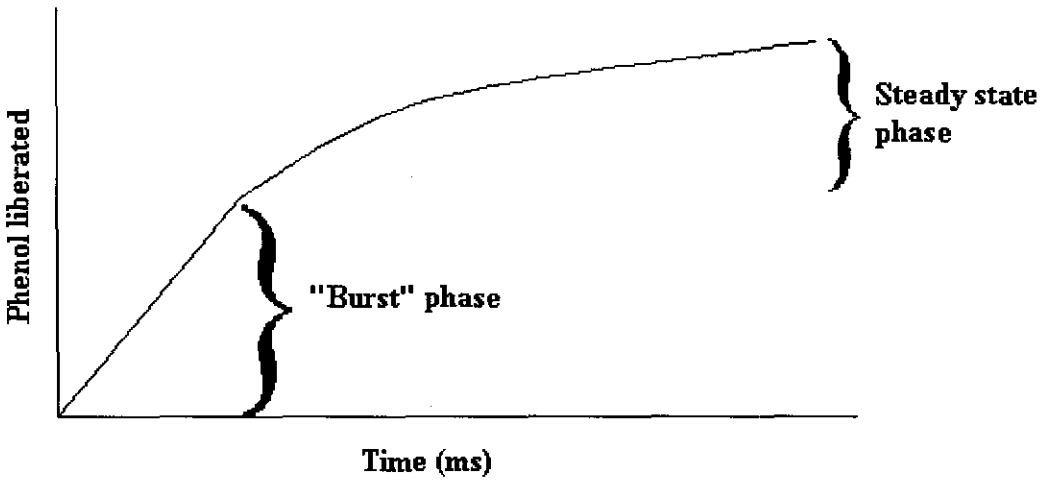

Figure 1.3 The biphasic kinetics of the release of *p*-nitrophenylate anion when chymotrypsin hydrolise *p*-nitrophenylacetate in a stop-flow apparatus. "Phenol liberated" was calculated from optical density (Rodwell, 1993:87).

The biphasic character of the release of the *p*-nitrophenylate anion is described in terms of the successive steps in catalysis shown in Figure 1.4. The formation of the CT-PNPA and CT-Ac complexes is fast relative to the hydrolysis of the CT-Ac complex (Rodwell, 1993:86).

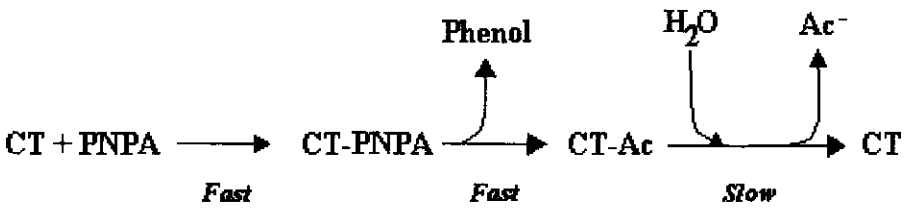

Figure 1.4 Intermediate steps in the catalysis of the hydrolysis of *p*-nitrophenylacetate by chymotrypsin (Rodwell, 1993:87). CT is chymotrypsin, PNPA is *p*-nitrophenylacetate, CT-PNPA the complex of

the two, CT-Ac is the chymotrypsin-acetate complex, phenol is the *p*-nitrophenylate anion and Ac^- is the acetate anion (Rodwell, 1993:87).

Hydrolyses of chymotrypsin-acetate is the slow step in the intermediate catalysis process. When all of the available chymotrypsin has been converted to CT-Ac, then no further release of *p*-nitrophenylate anion can occur until more free chymotrypsin is liberated by the slow, hydrolytic removal of acetate anion from the CT-Ac complex (Figure 1.4). The "burst" phase of the *p*-nitrophenylate anion release corresponds to the conversion of all of the available free chymotrypsin to the CT-Ac complex with the simultaneous release of the *p*-nitrophenylate anion. This subsequent release of *p*-nitrophenylate anion (phenol:PNPA) that follows the burst phase results from the slow formation of free chymotrypsin by hydrolysis of the CT-Ac complex. This free chymotrypsin is then available for further formation of the CT-PNPA and CT-Ac complexes with a further release of PNPA. The magnitude of the "burst" phase is directly proportionate to the number of moles of chymotrypsin initially present (Rodwell, 1993:87).

1.6 Conclusion

Enzyme studies have come a long way in the process of science and of understanding the human body. There is no doubt that life would not be possible without the existence of enzymes. It completes an essential part in the biochemical processes of the human body and has an important role to play in the metabolism of peptide drugs. Understanding the mechanism of enzyme action can offer a variety of answers to how biochemical processes affect the pharmacokinetics of various drugs. By altering these metabolic pathways through enzyme control (inhibition or activation) it is possible to optimise drug action- and therapy. An example is the inhibition of α-chymotrypsin to enhance the absorption of orally administered peptide drugs.

Chapter 2

Enzyme inhibition and enzyme inhibitors

2.1 Introduction

In observing the pathology of diseases it is found that most of the diseases known to man or at least symptoms of it, are caused from a deficiency or excess of a specific metabolite in the body, from an infestation of a foreign organism or from aberrant cell growth. To treat or cure these diseases it is then obvious to base the mechanisms of treatment on these findings and therefore aim to normalise the deficiency or excess of metabolites or destroy the foreign organism or aberrant cell growth. All of these factors can be affected by specific enzyme inhibition (Silverman, 1992:147).

An enzyme *inhibitor* can be defined as any compound that slows down or blocks enzyme catalysis. If the interaction with the target enzyme is irreversible (usually covalent), then the compound is referred to as an enzyme *inactivator*. Many of the drugs used today function on the mechanism of enzyme inhibition or inactivation and therefore work in on major metabolic pathways in the body. Consider what happens when an enzyme is blocked. The specific substrates for that enzyme cannot be metabolised, as there exists no enzyme in that pathway to do so, so the metabolic products in that metabolic pathway cannot be produced.

Enzyme inhibition therefore plays an important role in the modification of metabolic pathways and is widely used in the treatment of diseases in various ways. It is important that an enzyme inhibitor should be totally specific for the one target enzyme. Since this

is rare, if attained at all, highly selective inhibition is a more realistic objective (Silverman, 1992:147).

There has been only limited success in delivering peptides through the oral route, though being the most convenient route for drug administration. The reason for this is the existence of two major barriers for successful oral peptide delivery. The one is the enzymatic degradation of peptide drugs and the other is the absorption through the gastrointestinal epithelium. In the aim to reduce this metabolic barrier in the gut various approaches have been taken such as the co-administration of peptides with protease inhibitors and absorption enhancers, structural modification of the peptide to prevent proteolytic attack and carrier systems to protect the peptide from luminal proteolytic degradation and release of the drug at the site of the gut most favorable for absorption (Walker *et al.*, 1999:1). Inhibiting enzymes that break down or metabolise peptide drugs in the gastrointestinal tract may lead to an increase in peptide drug absorption (Lee *et al.*, 1986:87).

2.2 Types of enzyme inhibition

Enzyme inhibition can be either reversible or irreversible. If inhibition is reversible then the activity of the enzyme can be regained by the removal of the inhibitor by physical or chemical processes. There exists a chemical equilibrium between the enzyme and the inhibitor which can be displaced in the favour of the enzyme by the removal of the inhibitor through several means, e.g. dialysis or gel filtration. Removal of the inhibitor restores the enzyme to its original value.

When there is a complete loss of enzyme activity after a period of time even in the presence of a low concentration of inhibitor (provided it is in excess of the enzyme) then the process is known as irreversible enzyme inhibition. Enzyme activity cannot be regained by physical treatment, e.g. dialysis, but it may be possible to regenerate the enzyme by chemical means. The inhibition of acetyl cholinesterase by "nerve gases" is an example of irreversible inhibition.

Irreversible inhibition is usually quantified in terms of the velocity of inhibition whereas reversible inhibition is expressed in terms of the equilibrium constant k_i, for the enzyme and its inhibitor. Reversible inhibition is discussed below (Roberts, 1977:48).

2.2.1 Reversible inhibition

As discussed earlier, reversible inhibition implies that removing the inhibitor that act on the enzyme can restore the enzyme's activity. The different means of removal (dialysis, chromatography, electrophoresis and other methods) must not harm or damage the native enzyme. An inhibitor may inhibit an enzyme through various ways thus creating different types of reversible inhibition (Zeffren & Hall, 1973:89). The various types of reversible inhibition are discussed in the next sections.

2.2.1.1 Fully competitive inhibition

It has been discussed that the steady-state treatment of Briggs and Haldane (1925) leads to rate Equation 1.6, which was derived from Equation 1.2. in chapter 1.

When looking at fully competitive inhibition, consider an inhibitor molecule, I, which can also combine with E in such a way that EI can no longer bind S.

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EI$$

(Equation 2.1)

If it is further stipulated that ES cannot bind I, then Equations 1.6 and 2.1 describe the conditions of fully competitive inhibition, E will only bind I or only S, but not both. Only ES will break down to products. In this case $[E]_0$ is given by

$$[E]_0 = [E] + [ES] + [EI] \qquad \text{(Equation 2.2)}$$

and K_i is defined as

$$K_i = \frac{[E][I]}{[EI]} = \frac{k_{-i}}{k_i} \qquad \text{(Equation 2.3)}$$

Thus

$$[EI] = \frac{[E][I]}{K_i} \qquad \text{(Equation 2.4)a}$$

$$[ES] = \frac{[E][S]}{K_m} \qquad \text{(Equation 2.4)b}$$

Substituting these in Equation 2.2 gives

$$[E]_0 = [E]\left(1 + \frac{[S]}{K_m} + \frac{[I]}{K_i}\right)$$

$$[E] = \frac{[E]_0}{1 + [S]/K_m + [I]/K_i} \qquad \text{(Equation 2.5)}$$

In Equation 2.4b we can substitute for [E] and get

$$[ES] = \frac{[E]_0 S}{K_m(1 + [S]/K_m + [I]/K_i} = \frac{[E]_0 S}{[S] + K_m(1 + [I]/K_i)} \qquad \text{(Equation 2.6)}$$

From the equation

$$\upsilon = k_2[ES] \qquad \text{(Equation 2.7)}$$

we get

$$\upsilon = \frac{k_2[E]_0[S]}{K_m(1+[I]/K_i)+[S]} \qquad \text{(Equation 2.8)}$$

This equation is the expression that describes fully competitive inhibition, no matter what the kinetic significance of K_m might be. It is observed that this expression has a similar form than that of Equation 1.6 except that now the apparent K_m has a different meaning. It is evident from Equation 2.8 that only K_m will change, and it will change by the factor $(1+[I]/K_i)$ and V_{max} will be unaffected. Before this approach to determine K_i can be taken it is important to be sure that inhibition is indeed competitive (Zeffren & Hall, 1973:89).

2.2.1.2 Partially competitive inhibition

This type of inhibition is similar to fully competitive inhibition where E may combine with S or I but now also EI may combine with S and ES may combine with I to give the same EIS complex. Both EIS and ES are broken down to products at the same rate. Thus the following equations:

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad \text{(Equation 2.9)}$$

$$k_m = \frac{k_{-1}+k_2}{k_1} = \frac{[E][S]}{[ES]} \qquad \text{(Equation 2.10)}$$

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EI \qquad K_i = \frac{[E][I]}{[EI]}$$ (Equation 2.11a)

$$E + S \underset{k'_{-i}}{\overset{k'_i}{\rightleftharpoons}} EI \qquad K'_i = \frac{[EI][S]}{[EIS]}$$ (Equation 2.11b)

$$E + S \underset{k'_{-i}}{\overset{k'_i}{\rightleftharpoons}} EIS \qquad K'_i = \frac{[ES][I]}{[EIS]}$$ (Equation 2.11c)

$$EIS \xrightarrow{k_2} EI + P$$ (Equation 2.11d)

$$[E]_0 = [E] + [ES] + [EI] + [EIS]$$ (Equation 2.12)

The velocity of the reaction is then given by

$$\upsilon = k([ES] + [EIS])$$ (Equation 2.13)

From the definitions of the constants K_m, K_i and K'_i it may be written that

$$[ES] = \frac{[E][S]}{K_m}$$ (Equation 2.14a)

$$[EI] = \frac{[E][I]}{K_i}$$ (Equation 2.14)b

$$[EIS] = \frac{[EI][S]}{K'_m}$$ (Equation 2.14)c

For this reason it can be configured that

$$[E]+[EIS]=E\left(\frac{[S]}{K_m}+\frac{[I][S]}{K_iK_i'}\right) \qquad \text{(Equation 2.15)}$$

It can now be derived from Equations 2.16 and 2.12 that

$$[E]=\frac{[E]_0}{1+[I]/K_i+[S]/K_m+[I][S]/K_iK_i'} \qquad \text{(Equation 2.16)}$$

Substituting for [E] in Equation 2.16 gives

$$[ES]+[EIS]=\frac{[E]_0[S](1/K_m+I/K_iK_i')}{1+[I]/K_i+[S]/K_m+[I][S]/K_iK_i'}$$

$$=\frac{[E]_0[S](1/K_m+[I]/K_iK_i')K_m}{K_m(1+[I]/K_i)+[S](1+[I]K_m/K_iK_i')} \qquad \text{(Equation 2.17)}$$

This leads to Equation 2.18 known as the rate expression

$$\upsilon=\frac{k_2[E]_0[S]}{\frac{K_m(1+[I]/K_i)}{K_m(1/K_m)(1+[I]K_m/K_iK_i)}+\frac{[S](1+[I]K_m/K_iK_i')}{1/K_m(1+[I]K_m/K_iK_i')K_m}}$$

$$= \frac{k_2[E]_0[S]}{K_m\left[\frac{1+[I]/K_i}{1+[I]K_m/K_iK_i'}\right]+[S]}$$

(Equation 2.18)

It should be obvious that by varying the substrate concentration in a series of experiments at a constant inhibitor concentration you would be able to distinguish this form of inhibition from the purely competitive type. To do so will require a series of experiments by making use of a variable inhibitor concentration at a fixed substrate concentration. At very large values of [I], one gets that

$$\upsilon = \frac{k_2[E]_0[S]}{K_i'+[S]}$$

(Equation 2.19)

If it is known that one is dealing with partial competitive inhibition and working at a level of [I] such that equation 2.15 holds, then K_i' may be determined. If K_m has been determined previously from experiments in the absence of the inhibitor, K_i may be determined from experiments in the range of inhibitor concentrations where equation 2.24 holds. This behavior is complex and requires a fair amount of effort to elucidate completely (Zeffren & Hall, 1973:90).

2.2.1.3 Fully non-competitive inhibition

In this type of inhibition the affinity of the enzyme for the substrate is not affected by complexation with the inhibitor, and vice versa. It is also found that pure non-competitive inhibition has only ES, not EIS, break down to products. In partially non-competitive inhibition EIS can break down to products and does so at a rate different from that of ES. The equations describing purely non-competitive behavior are

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad K_m = \frac{[E][S]}{[ES]} = \frac{[EI][S]}{[EIS]} \qquad \text{(Equation 2.20a)}$$

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EI \qquad K_i = \frac{[E][I]}{[EI]} = \frac{[ES][I]}{[EIS]} \qquad \text{(Equation 2.20b)}$$

$$EI + S \xleftrightarrow{K_m} EIS \qquad \text{(Equation 2.20c)}$$

$$ES + I \xleftrightarrow{K_i} EIS \qquad \text{(Equation 2.20d)}$$

The conservation equation here is

$$[E]_0 = [E] + [ES] + [EI] + [EIS] \qquad \text{(Equation 2.21)}$$

From Equation 2.20a it is derived that

$$[ES] = \frac{[E][S]}{K_m} \qquad \text{(Equation 2.22a)}$$

$$[EI] = \frac{[E][I]}{K_i} \qquad \text{(Equation 2.22b)}$$

$$[EIS] = \frac{[ES][I]}{K_i} = \frac{[E][S][I]}{K_m K_i} \qquad \text{(Equation 2.22c)}$$

To solve [E] Equation 2.21 is re-written as

$$[E] = \frac{[E]_0}{1 + [S]/K_M + [I]/K_i + [S][I]/K_m K_i} \qquad \text{(Equation 2.23)}$$

Since it is known through the initial restrictions that

$$\upsilon = k_S[ES] = k_2 \frac{[E][S]}{K_m} \qquad \text{(Equation 2.24)}$$

it is possible to write

$$\upsilon = \frac{k_2[E]_0[S]}{K_m\left(1+[S]/K_m+[I]/K_i+[S][I]/K_mK_i\right)}$$

$$= \frac{k_2[E]_0[S]}{K_m\left(1+[I]/K_i\right)+[S]\left(1+[I]/K_i\right)} \qquad \text{(Equation 2.25)}$$

Dividing both the numerator and the denominator by factor $(1+[I]/K_i)$ gives

$$\upsilon = \frac{\left(k_2[E]_0/(1+[I]/K_i)\right)[S]}{K_m+[S]} \qquad \text{(Equation 2.26)}$$

Thus it is seen that in purely non-competitive inhibition K_m is unaffected while V_{max} is changed. It seems to be just the opposite of what happens in purely competitive inhibition. The Lineweaver-Burk plot (section 2.3) of purely non-competitive behavior shows a changing ordinate intercept for a series of reactions at fixed inhibitor concentrations and variable substrate concentrations (Zeffren & Hall, 1973:92).

2.2.1.4 Partially non-competitive inhibition

For this type of inhibition Equations 2.20a and 2.20d still apply, but they must be supplemented by Equation 2.20e:

$$EIS \xrightarrow{k_2'} EI + P \qquad \text{(Equation 2.20e)}$$

The overall velocity of the enzymatic reaction in this situation is then given by

$$\upsilon = k_2[ES] + k_2'[EIS] \qquad \text{(Equation 2.27)}$$

Through a derivation analogous to that leading to Equation 2.26 it is possible to derive

$$\upsilon = \frac{k_2[E]_0[S]/(1+[I]/K_i)}{K_m+[S]} + \frac{k_2'[E]_0[S]([I]/K_i)/(1+[I]/K_i)}{K_m+[S]} \qquad \text{(Equation 2.28)}$$

which may be written as

$$\upsilon = \frac{\dfrac{k_2[E]_0[S]+k_2'[E]_0[S][I]/K_i}{1+[I]/K_i}}{K_m+[S]} \qquad \text{(Equation 2.29)}$$

If [I] is very large then $V_{max} = k_2'[E]_0$, which allows determination of k_2'. Determination of K_i is somewhat involved, although less so than for the partially competitive case. Here, K_i can be calculated from the ordinate intercept as long as k_2' has been determined (Zeffren & Hall, 1973:94).

2.2.1.5 Uncompetitive inhibition

Uncompetitive inhibition is characterised by the complex formation of the inhibitor with ES only. The inhibitor will not form a complex with the native enzyme, E. This type of inhibition is fairly rare in monomeric, single substrate enzymes but occurs more frequently in more complex systems. This scheme is described by the following equations:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad \text{(Equation 1.2)}$$

$$k_m = \frac{k_{-1} + k_2}{k_1} = \frac{[E][S]}{[ES]} \qquad \text{(Equation 1.3)}$$

$$ES + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EIS \qquad K_i = \frac{[ES][I]}{[EIS]} \qquad \text{(Equation 2.30)}$$

The conservation equation is then

$$[E]_0 = [E] + [ES] + [EIS] \qquad \text{(Equation 2.31)}$$

The rate of the reaction is given by

$$\upsilon = k_2[ES] = \frac{k_2[E][S]}{K_m} \qquad \text{(Equation 2.32)}$$

We can now solve for E if we use the definitions of K_m and K_i and Equation 2.31:

$$[E] = \frac{[E]_0}{1 + [S]/K_m + [S][I]/K_m K_i} \qquad \text{(Equation 2.33)}$$

Thus we get the rate expression as

$$\upsilon = \frac{k_2[E]_0[S]}{K_m + S(1+[I]/K_i)} \qquad \text{(Equation 2.34)}$$

The Lineweaver-Burk plot of this treatment (section 2.3) shows an unchanged slope and intercepts that enlarge as [I] becomes larger. Thus if V_{max} is known from experiments in the absence of inhibitor then K_i is easily calculated. According to Dixon and Webb a noncompetitive inhibitor of an enzyme acting on a substrate for which $k_2 >> k_{-1}$, the Lineweaver-Burk plots will be indistinguishable from those of uncompetitive inhibition, even though E can combine with I (Zeffren & Hall 1973:94).

2.3 Determination of inhibitor constants

Perhaps the most widely used approach in the determination of inhibitor constants is the Lineweaver-Burk double reciprocal plot. From the slope and intercepts of these plots drawn from experiments done in the presence and absence of an inhibitor, it is often possible, but not always, to determine k_{cat} (or V_{max}), K_m and K_i. This can best be seen by representing the reciprocal relations, as it is done in Table 2.1.

The Lineweaver-Burk plots for these types of inhibition exemplified in Table 2.1 are represented in Figure 2.1. It is apparent from the table and the figure that K_i is readily determined for the various cases *a, c,* and *e*. One simply studies the enzymatic reaction at a series of substrate concentrations at a fixed inhibitor concentration and draws the appropriate double reciprocal plot. A straightforward calculation of K_i can then be done. More involved studies are necessary to determine K_i values for cases *b* and *d*.

One other method is that of Dixon which is a simple graphical method that requires only the determination of v at two or three different substrate concentrations by using a series

of inhibitor concentrations at each substrate level. A plot of (1/v) versus [I] for each set of reactions will give a series of lines that will intersect at a value of [I] equal to $-K_i$, moreover, the non-competitive inhibition intersection point will be on the abscissa while the competitive inhibition intersection point will be above it. This is shown as follows.

Table 2.1 Lineweaver-Burk relations and intercept and slope definitions (Zeffren & Hall, 1973:96).

Type	Equation	Ordinate Intercept	Slope	Abscissa Intercept
No inhibitor	$\frac{1}{\upsilon}=\frac{K_m}{k_2[E]_0}\left(\frac{1}{[S]}\right)+\frac{1}{k_2[E]_0}$	$\frac{1}{k_2[E]_0}$	$\frac{K_m}{k_2[E]_0}$	$\frac{-1}{K_m}$
a	$\frac{1}{\upsilon}=\frac{K_m\left(1+\frac{[I]}{K_i}\right)}{k_2[E]_0}+\frac{1}{k_2[E]_0}$	$\frac{1}{k_2[E]_0}$	$\frac{K_m\left(1+\frac{[I]}{K_i}\right)}{k_2[E]_0}$	$-\frac{1}{K_m\left(1+\frac{[I]}{K_m}\right)}$
b	$\frac{1}{\upsilon}=\frac{K_m\left[\left(1+\frac{[I]}{K_i}\right)/\left(1+\frac{[I]K_m}{K_iK_i'}\right)\right]}{k_2[E]_0}\left(\frac{1}{[S]}\right)+\frac{1}{k_2[E]_0}$	$\frac{1}{k_2[E]_0}$	$\frac{K_m}{k_2[E]_0}\left[\frac{1+\frac{[I]}{K_i}}{1+\frac{[I]K_m}{K_iK_i'}}\right]$	$-\left[\frac{1+\frac{[I]K_m}{K_iK_i'}}{K_m\left(1+\frac{[I]}{K_i}\right)}\right]$
c	$\frac{1}{\upsilon}=\frac{K_m\left(1+\frac{[I]}{K_i}\right)}{k_2[E]_0}+\frac{1+\frac{[I]}{K_i}}{k_2[E]_0}$	$\frac{1+\frac{[I]}{K_i}}{k_2[E]_0}$	$\frac{K_m\left(1+\frac{[I]}{K_i}\right)}{k_2[E]_0}$	$\frac{-1}{K_m}$
d	$\frac{1}{\upsilon}=\frac{K_m\left(1+\frac{[I]}{K_i}\right)}{k_2[E]_0+\frac{k_2'[E]_0[I]}{K_i}}\left(\frac{1}{[S]}\right)+\frac{1+\frac{[I]}{K_i}}{k_2[E]_0+\frac{k_2'[E]_0[I]}{K_m}}$	$\frac{1+\frac{[I]}{K_i}}{k_2[E]_0+\frac{k_2'[E]_0[I]}{K_m}}$	$\frac{K_m\left(1+\frac{[I]}{K_i}\right)}{k_2[E]_0+\frac{k_2'[E]_0[I]}{K_i}}$	
e	$\frac{1}{\upsilon}=\frac{K_m}{k_2[E]_0}\left(\frac{1}{[S]}\right)+\frac{1+\frac{[I]}{K_i}}{k_2[E]_0}$	$\frac{1+\frac{[I]}{K_i}}{k_2[E]_0}$	$\frac{K_m}{k_2[E]_0}$	$-\frac{1+\frac{[I]}{K_i}}{K_m}$

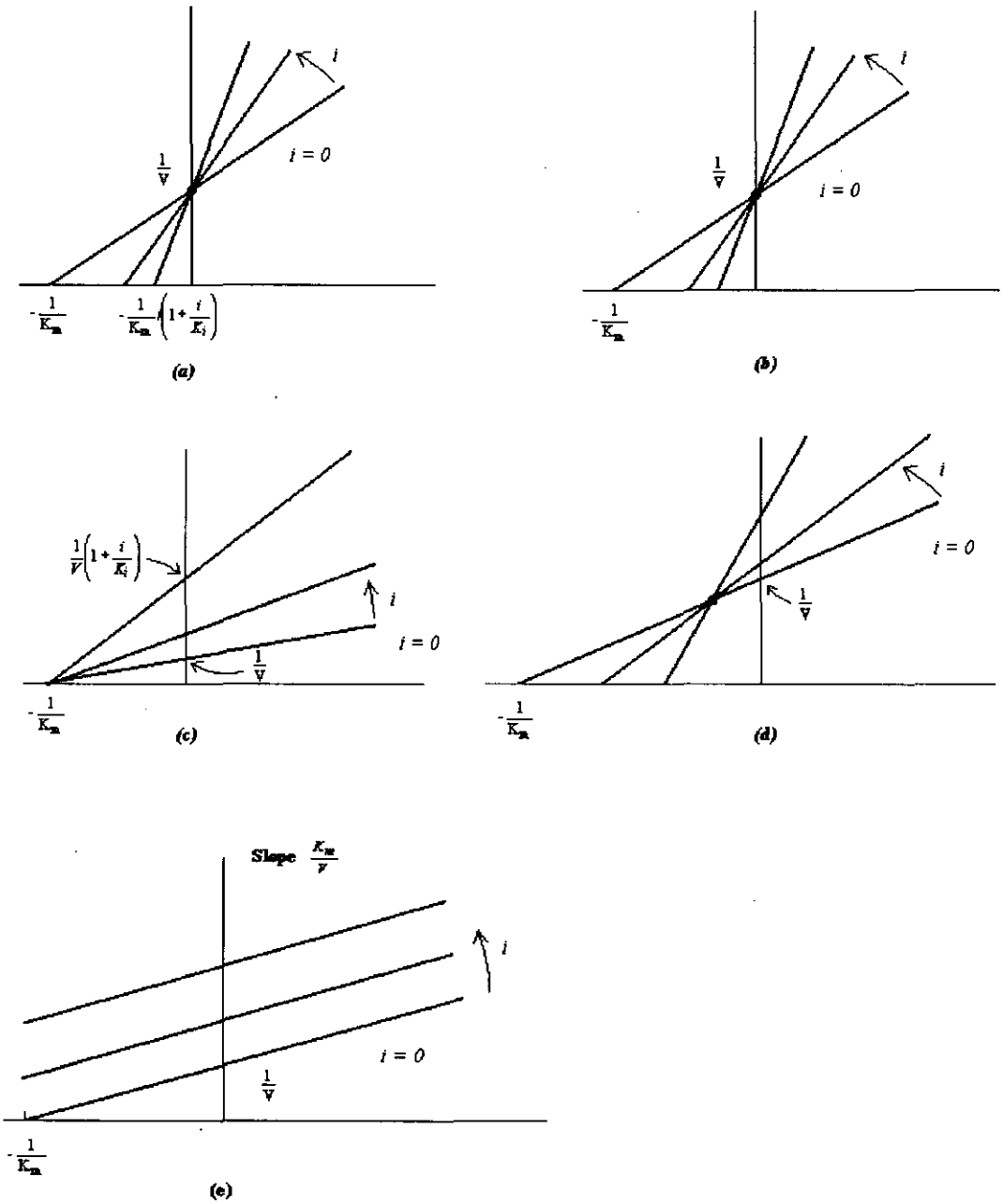

Figure 2.1 Lineweaver-Burk plots of the various types of inhibition. (a) Fully competitive. (b) Partially competitive. (c) Fully non-competitive. (d) Partially non-competitive. (e) Uncompetitive. The plots are given as 1/v vs 1/s (Zeffren & Hall, 1973:97).

For competitive inhibition:

$$\frac{1}{\upsilon} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{K_m}{V_{max}}\frac{[I]}{K_i}\frac{1}{[S]} + \frac{1}{V_{max}} \qquad \text{(Equation 2.35)}$$

The aim is to plot (1/v) versus [I] at a series of [I] values at each of two substrate levels. Where the lines cross, the values of 1/v and [I] are the same for both substrate levels. Since it is competitive inhibition, for which V_{max} is dependent of [I], it is possible to write the following expression, which holds only at the intersection point:

$$K_m\frac{1}{[S]_1} + K_m\frac{1}{[S]_1}\frac{[I]}{K_i} = K_m\frac{1}{[S]_2} + K_m\frac{1}{[S]_2}\frac{[I]}{K_i} \qquad \text{(Equation 2.36)}$$

which may be written as

$$\frac{1}{[S]_1}\left(1+\frac{[I]}{K_i}\right) = \frac{1}{[S]_2}\left(1+\frac{[I]}{K_i}\right) \qquad \text{(Equation 2.37)}$$

Because $[S]_1 \neq [S]_2$, equation 2.37 can hold only if $[I] = -K_i$. If a horizontal line is drawn above the [I] axis at the value $1/V_{max}$, then it should intersect at this same point as for competitive inhibition (see Figures 2.2a and 2.2c).

From the reciprocal expression for case *c*, it can be seen that this method is also applicable to non-competitive inhibition with one important difference; the intersection point is on the abscissa, in other words it occurs when 1/v = 0. This can be seen by setting the Lineweaver-Burk equation for *c* equal to zero and solving for [I] (Figure 2.2b). This method offers one of the clearest differentiations available between competitive and non-competitive inhibition, but for the data to be reliable, one needs to ensure that the range of inhibition concentrations used incorporates the (assumed) value of K_i, or at least

comes within 50% of it. Otherwise an excessive graphical extrapolation will be necessary (Zeffren & Hall, 1973:95).

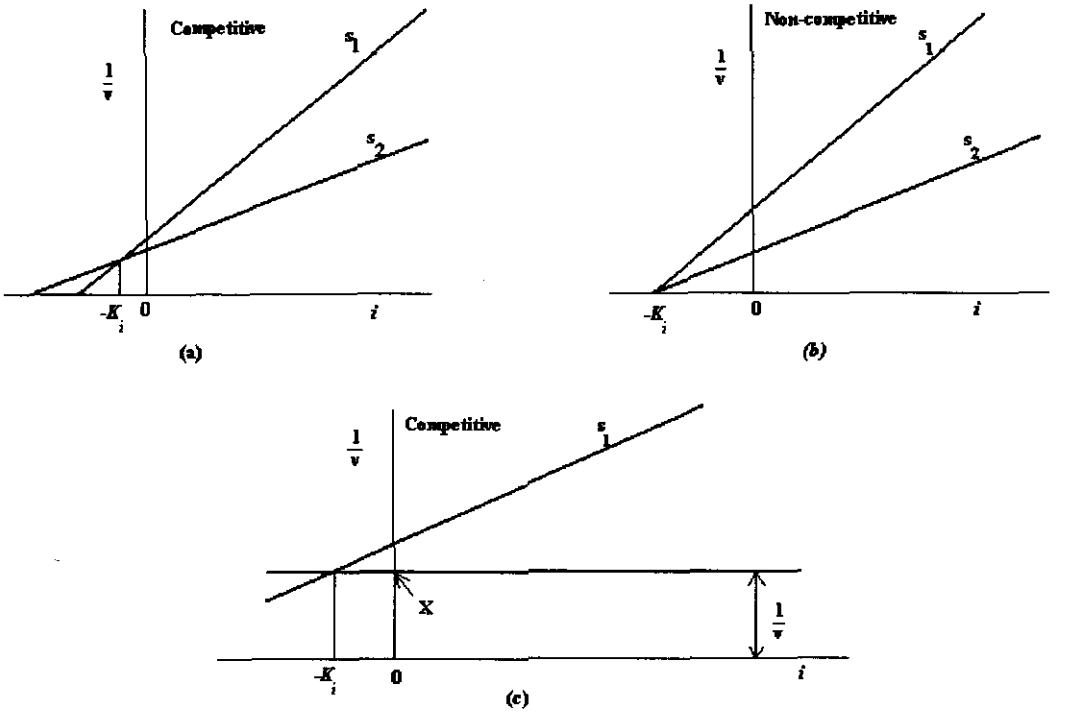

Figure 2.2 The graphical determination of inhibitor constants by the method of Dixon (Zeffren & Hall, 1973:98).

2.4 The enzymatic barrier of peroral application for peptide drugs

As mentioned earlier, proteolytic enzymes, or proteases, break down peptide drugs in the gastrointestinal tract. Three different groups of proteases are present in the gastrointestinal tract namely a) luminal, b) membrane bound and c) cytosolic enzymes. Trypsin, α chymotrypsin, carboxypeptidase A & B and aminopeptidase are luminal enzymes, which degrade perorally administered peptides and are therefore more important enzymes when focusing on enzyme inhibition in drug kinetics (Lee *et al.*, 1986:87).

2.5 Enzyme inhibitors

Enzyme inhibitors exists such a vast quantity in the world of medicine and are widely used to alter a metabolic or biochemical pathway in order to treat and cure several diseases and pathological conditions. HMG-CoA inhibitors that lower cholesterol, angiotensin converting enzymes (ACE) inhibitors that lower blood pressure, cyclo-oxygenase (COX) inhibitors that is used to stop the process of pain and inflammation, asetylcholine esterase inhibitors used in Myasthenia Gravis and mono-amine oxidase (MAO) inhibitors used as antidepressants are just a few among thousands of enzyme inhibitors used in the world of medicine today.

As mentioned earlier, there exist many different types of enzyme inhibition. Enzyme inhibition may be used in various ways to alter or modify biochemical processes in the human body. An enzyme inhibitor may act directly on a biochemical pathway, by direct or indirect inhibition, and through a reduction in products and substrates produce the desired effect. It also may produce the desired effect by acting indirectly on the specific process. Enzyme inhibitors that function indirectly may alter one biochemical process or pathway, which in turn automatically alters another, and then produce the desired effect. Inhibiting protease enzymes, which break down peptide drugs, and thus increase the absorption of these drugs, can thus be considered a direct effect on increased drug absorption and an indirect effect on the decreased dosage and increased efficiency in drug therapy. Increased peptide drug absorption may be facilitated by the co-administration of penetration enhancers to alter membrane permeability and protein inhibitors to restrain the activity of proteolytic enzymes (Lee, 1986:87).

Carbomers which are polymers of acrylic acid, have been shown to enhance the bioavailability of several peptides. Polycarbophil, which is a weakly cross-linked polyacrylate, dispersed in saline was shown to enhance intestinal absorption of the peptide [Arg^8, des-Gly-$NH^9{}_2$]-vasopressin (DGAVP) both *in vitro* and *in vivo* in rats. More recently, Carbomer 934P (Carbopol®) was shown to significantly improve the intestinal absorption of buserelin, a LHRH superagonist, in rats (Walker *et al.*, 1999:1).

In recent studies it was shown that buffered polyacrylic acid polymer dispersions at pH 6.7 inhibit the activity of trypsin, α chymotrypsin and carboxypeptidase A and the cytosolic leucine aminopeptidase (Lueßen *et al.*, 1996:126).

It was then suggested that polyacrylic acid polymers bind the essential co-factors, calcium and zinc, from the enzyme causing a change in enzyme conformation, resulting in enzyme autolysis and loss of enzyme activity. Interactions of polymers with proteins have been shown to cause structural changes and aggregation of the protein which resulted in the inactivation of the protein. Peptides also react with polymers, which makes it conceivable that polyacrylic acid polymers may reduce proteolytic activity by interaction with the enzyme or the substrate. It has also been proven that Carbomer 934P (Carbopol®) reduces the proteolytic activity of trypsin *in vitro.* It was concluded that the inhibition of trypsin by Carbomer 934P is the result of an *enzyme-polymer interaction* reducing the free concentration of trypsin and in part denaturing the enzyme (Walker *et al.*, 1999:6). Walker and co-workers also found that Carbomer 934P strongly inhibits chymotrypsin and that it supports the mechanism determined for the inhibition of trypsin. However it was found that chymotrypsin is far more susceptible to enzyme inactivation than trypsin and therefore Carbomer 934P will be more effective in protecting orally administered peptide drugs that are susceptible to chymotrypsin hydrolysis (Walker *et al.*, submitted for publication).

Some of the major problems of inhibitors are their high toxicity, especially in chronic drug therapy, and their limited activity, which is mainly for luminal enzymes with preference to endopeptidases. Since it is difficult to achieve a direct interaction between the enzyme and inhibitor, protease enzymes imbedded in the mucus layer or located in the apical membrane of the epithelial cells are not easily affected. This particularly holds for high molecular weight structures for which diffusion is hampered by the mucus layer such as soybean trypsin inhibitor, aprotinin and Bowman-Birk inhibitor (Lueßen *et al.*, 1996:118).

2.6 Conclusion

Enzyme inhibition has been studied for decades and has grasped the attention of many scientists all over the world. Both the importance and scientific interest of this subject has lead to vast research in the field, which lead to a thorough understanding of the topic that proved to be very technical and complicated.

After describing the mechanism of enzyme action it is evident that there exist different types of enzyme inhibition. It proves to be an advantage if the type of inhibition of such inhibitor is known. It makes it much easier to predict the outcome of enzyme-inhibitor reactions if the type of inhibition is known, because the equilibrium constants, reaction velocities and the overall nature of such an inhibition is characteristic to that specific inhibition. By studying such characteristics it is possible to determine the type of inhibition of an inhibitor with great accuracy.

In this chapter the importance of enzyme inhibition has become evident because of its influence on human biochemistry. Enzymes serves as a very important component in human physiology and makes life possible by controlling and regulating numerous biochemical processes in the human body. Controlling enzyme activity in the human body to promote health or treat disease has become the basis of many drugs in use today. It was also mentioned that inhibiting enzymes that metabolise or inactivate perorally administered peptide drugs in the stomach or intestines may lead to an increase in absorption and hence the bioavailability of such a drug.

Chapter 3

Synthesis and characterisation of *N,N*-dicarboxymethylated chitosan derivatives

3.1 Introduction

By inhibiting protease enzymes it is possible to increase the bioavailability of several perorally administered peptide drugs. Chitosan in itself does not have any enzyme inhibiting properties, but it has been widely used as a carrier for enzyme immobilisation agents. This is made possible by the amino and hydroxyl groups on the polymer which provide areas for physical and chemical modifications or linkages. The immobilisation process usually involved a cross-linking step by making use of glutaraldehyde. Enzymes immobilised by chitosan gels include α-chymotrypsin, α-glycosidase, invertase, β-galactosidase, α-amylase, lactase and cyclodextrin glucanotransferase (Li *et al.,* 1997:15). If chitosan can be modified in such a way that it may act as an enzyme inhibitor and retain its unique and desired properties it may take chitosan derivatives, as drug carriers, to a next step.

Bernkop-Schnürch and Kast (2001:1) has proven that chemically modified chitosans does exhibit enzyme inhibitory properties. This has been done by covalently attaching protease inhibitors to chitosan via the primary amino groups or the hydroxyl groups of chitosan and to monitor their effect on the bioavailability of perorally administered peptide drugs. When studying several of these chitosan derivatives (Figure 3.1) one can see the striking resemblance between the chemical structure of the enzyme inhibitor attached to chitosan and that of *N,N*-dicarboxymethyl chitosan (Figure 3.3) when in its

anionic form. It is therefore theoretically possible that *N,N*-dicarboxymethyl chitosan may inhibit protease enzymes *in vitro*.

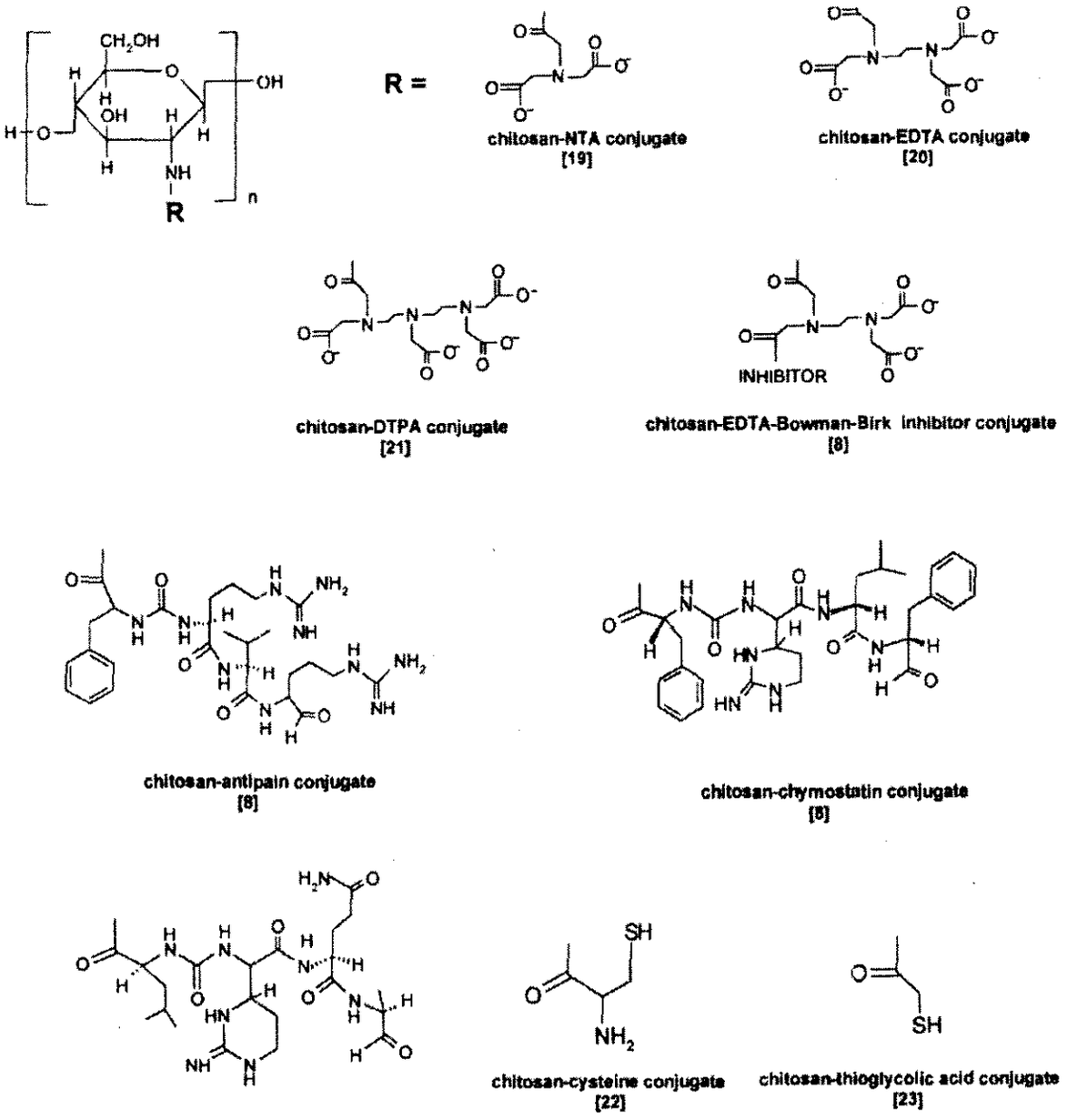

Figure 3.1 Chemical structures of several chitosan derivatives that display enzyme inhibitory properties on protease enzymes (Bernkop-Schnürch & Kast, 2001:131).

3.2 Chitosan

Much has been said about chitosan but what exactly is chitosan? To answer this question one must go back to the origin of this substance. Chitin, a structural polysaccharide, is a major constituent of the exoskeleton of crustaceous water animals and in the hyphae or spores of lower plants. In 1811 it was described by Braconnot as a distinct substance identified in plants and that it occurs naturally in three polymorphic forms: a) α-chitin, b) β-chitin and c) γ-chitin. In 1859 Rouget boiled chitin in a concentrated potassium hydroxide solution and found that the product dissolved in dilute iodine and acid solutions. It was only in 1894 that this substance was named chitosan by Hoppe-Seyler. β(1→4) 2-amino 2-deoxy β-D glucan, or chitosan, is the deacetylated form of chitin and a mucopolysaccharide which has similar structural characteristics than that of glycosaminoglycans with the chemical formula $(C_6H_{11}O_4N)_n$ (Paul & Sharma, 2000:5).

3.2.1 Synthesis and physicochemical properties of chitosan

Synthesising chitosan incorporates the deacetylation of chitin and is described in Figure 3.2. Crab and shrimp shells are decalcified in dilute hydrochloric acid and deproteinated in a dilute sodium hydroxide solution. Chitin is then boiled in an aqueous sodium hydroxide solution and thus deacetylated to form chitosan (Paul & Sharma. 2000:5).

This process of deacetylation of chitin in an alkaline solution is never completed, even under harsh conditions, and therefore different degrees of deacetylation occurs which ranges between 70 and 95 % (Li *et al.* 1997:5). Chitosan used as pharmaceutical grades has a degree of deacetylation between 90 and 95 % and the food grade is between 75 and 80 % deacetylated (Paul & Sharma. 2000:5). The degree of deacetylation is determined through various analytical techniques such as UV spectrophotometry, dye adsorption, IR spectrometry, metachromatic titration and gas chromatography.

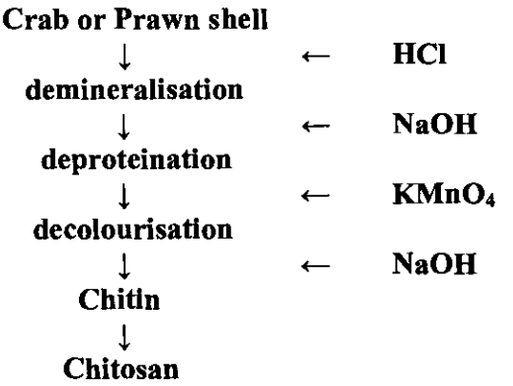

Figure 3.2 The basic steps in the synthesis of chitosan from chitin (Paul & Sharma, 2000:5).

It is suggested that UV-spectrophotometry at 199 nm is probably the best non-destructive and accurate method when determining the degree of deacetylation of chitosan samples. Determining the molecular weight of chitosan can be done by either chromatography, light scattering or viscometry. The latter is found to be the most simple and fastest method but is influenced by the ionic strength of the solution. It has been found that the molecular weight of native chitosan is usually larger than 1 000 000 g/mole while commercial chitosan products have molecular weights between 100 000 and 1 200 000 g/mole. This may be because of the degradation of the chitosan product during the manufacturing process (Li *et al.*, 1997:6).

Like many polymers, chitosan has a relatively high viscosity. Chitosan's viscosity, while in solution, is influenced by factors such as molecular weight, polymer deacetylation, concentration, temperature, pH and ionic strength. Chitosan is insoluble in water, alkali and organic solvents but it is, however, soluble in organic acids if the pH of the solution is less than 6. Acetic and formic acids are most widely used for dissolving chitosan.

It was also found that chitosan is a fairly good coagulating agent and flocculant. This is due to the high density of amino groups which can interact with negatively charged substances such as proteins, dyes, solids and polymers. Chitosan also has the ability to adsorb metal ions and the chitosan-glucan complex is able to recover several metal ions such as Cr, Co, Ni, Cu, Cd and Pb from water (Li *et al.*, 1997:6).

3.2.2 Applications of chitosan

Because of al its unique properties, chitosan has become a precious commodity in the world of medicine, pharmaceutics, cosmetics, the food industry, water purification, papermaking, biotechnology and agriculture. In Japan the production of chitosan showed an increase of 37 % each year from 1978 up to 1983. Production reached a total annual amount of 311 tons by 1983 and 1270 tons in 1986 worldwide. In pharmaceutics and pharmaceutics related subjects, chitosan has proven to fulfil quite a unique and important role (Li *et al.*, 1997:5).

Chitosan, in itself is haemostatic. Derivatives of chitosan such as sulphated chitosan have been used as anticoagulants in chitosan bandages, sponges, artificial blood vessels and membranes with quite an amount of success. It has also been found to lower cholesterol levels by 66 % in *in vivo* studies on mice. This hypocholesterolemic activity of chitosan was proposidly due to the inhibition of micelle formation that contains cholesterol, fatty acids and monoglycerides. Thus these "building blocks" of cholesterol were radically reduced in quantities. Studies have also shown that chitosan inhibit tumor cell growth *in vitro* as well as *in vivo* when covalently linked to 5-flouroracil and tested on mice. The chitosan bound prodrug showed an enhanced inhibition effect against tumour cells without any apparent toxicity (Li *et al.*, 1997:8).

Chitosan has also been proven to have a bacteriostatic action towards skin bacteria such as *Staphylococcus epidermis, Staphylococcus aureus* and *Pseudomonas auruginosa.* It has also been proven useful in eye preparations for surgery as well as in the production of soft contact lenses such as chitin *n*-butyrate lenses, chitosan lenses and blue chitosan lenses. Other studies described the manufacturing and testing of artificial skin on rats as well as chitosan's usefulness in treating bone disease and accelerating bone formation. Chitosan is also famous for its role in controlled-release and for its advanced drug carrier abilities. Furthermore chitosan is also used in cosmetics such as shampoos (conferring shine and strength to hair), coloured powders, nail polishes, moisturisers, hair and skin fixatives, hair conditioners, cleansers and also bath lotions. The use of chitosan as a carrier for enzyme immobilisation has been discussed earlier. Published reports have

shown chitosan to be useful as a polyligand, flocculating agent and polymer support for the separation and recovery of proteins and cells (Li *et al.*, 1997:9).

It is clear that chitosan is a useful and versatile polymer and that it plays an important role in the world of pharmaceutics and biotechnology. The rest of this chapter will describe the synthesis of a derivative of chitosan, namely *N,N*-dicarboxymethyl chitosan which may possess the ability to inhibit enzymes.

3.3 *N*-carboxymethyl- and *O,N*-carboxymethyl chitosan

N-carboxymethyl chitosan appears to be one of the most studied chitin derivatives as it is attracting much attention in various fields. This may be because of its unique properties and many uses. *N*-carboxymethyl chitosan is much more water-soluble than chitosan as well as a good chelating agent towards several transition metal ions. Chelation maxima with ions such as Co(II), Cu(II), Zn(II), Hg(II) and Pb(II) are observed at pH 6 to 7 whereas Ni(II) and Cd(II) have a maxima at pH 7.5 and 5.5 respectively. There appeared to be no chelation for calcium ions. In agricultural studies with *N*-carboxymethyl chitosan it was found that it protects certain seeds from fungal attack by reducing the aflatoxins formed by *Aspergillus flavus* and *A. parasiticus* in pre-harvest crops by more than 90 %, while fungal growth was reduced by one half during the storage of these seeds. Germination of seeds and growth of seedlings are favoured by showing an increase of chitinase activity for seeds such as rice, soybean and pine (Muzzarelli *et al.*, 1993:2).

N,O-dicarboxymethyl chitosan is also a much more water-soluble polymer than chitosan itself. The substitution may be an *O*-carboxymethylation at the C-3 and C-6 positions of the D-glucosamine unit and/or a *N*-carboxymethylation on the NH_2- groups (Rinaudo *et al.*, 1992:122).

3.4 Synthesis of *N,N*-dicarboxymethyl chitosan (DCMC) and *N,N*-dicarboxymethyl chitosan oligomer (DCMO)

Muzzarelli *et al.,* (1982:200) have developed the method by which chitosan is carboxylated to form *N,N*-dicarboxymethylated chitosan (Figure 3.3). The synthesis starts with reacting the free amino groups of chitosan with glyoxylic acid to produce a soluble, gel-forming imine. The glyoxylic acid permits to direct the substitution to the nitrogen rather than to the oxygen. The product is then reduced with the reducing agent cyanoborohydride, which is very toxic. This preparation is readily effected and does not require any warming or cooling and only requires commercially available reagents. This preparation produces a variety of *N*-carboxymethylated chitosans that contains acetyl, carboxymethyl and free amino groups. The properties of chitosan such as degree of acetylation and molecular weight, as well as the amounts of glyoxylic acid used may determine the specific characteristics of the end-product.

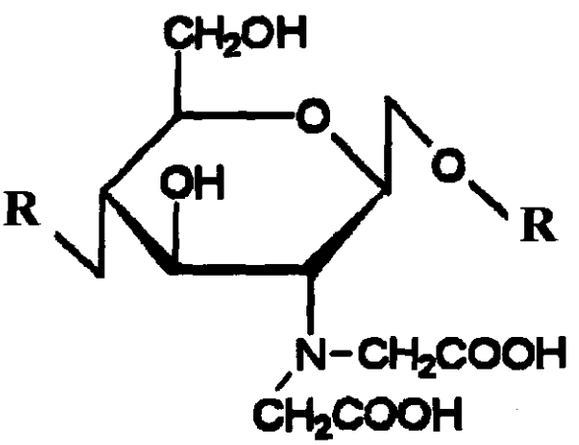

Figure 3.3 A proposed structure of *N,N*-dicarboxymethylated chitosan (DCMC) (Rinaudo *et al.*, 1992:125).

3.4.1 Materials

Chitosan and chitosan oligomer (Primex, Norway), acetic acid (Saarchem, South Africa), glyoxylic acid (Sigma, South Africa), sodium cyanoborohydride (Sigma, South Africa),

ethanol and diethylether (Saarchem, South Africa) were used to synthesise the polymers. All chemicals used were of analytical grade and were used as received.

3.4.2 Method

Chitosan (0.4 g) was dissolved in a 100 ml solution of 0.15 M acetic acid and was magnetically stirred overnight. Sufficient glyoxylic acid was then added in a ratio of glyoxylic acid / amino group 9:1 (thus 2.0 g glyoxylic acid monohydrate), that yielded a clear solution. After 20 hours the acidic pH was adjusted to 5.1 with a 2 % solution of NaOH. The NaOH was added very slowly to prevent the formation of a precipitate.

The reducing agent, sodium cyanoborohydride, was then weighed (1.37 g) and dissolved in 5 ml water and is then added in the same ratio as the glyoxylic acid (9:1). After 20 hours the pH is again adjusted with a 2.0 % (w/v) solution of NaOH to 7.5. Ethanol was then added at approximately 2 times the volume of the chitosan solution to precipitate the product. After sedimentation for 30 minutes the solution was centrifuged at 3000 rpm for 10 minutes at room temperature. The product was then washed three times with a mixture of ethanol and water of different ratios. The ethanol/water ratio was increased from 70 % / 30 %, 50 % / 50 % and 30 % / 70 % (v/v). Every washing was carried out under magnetic stirring for 30 minutes. Diethylether was also used in the washing process to eliminate any water that may stay behind in the precipitant. The final washing was made with absolute ethanol. The final product was then oven dried at 40 °C for 24 hours and a white, free-flowing powder was obtained. All the steps were done at room temperature. Two products were synthesised by using: a)long chain chitosan (263700 g/mole) and b)short chain chitosan oligomer (13500 g/mole) that yielded *N,N*-dicarboxymethylated chitosan (DCMC) and *N,N*-dicarboxymethylated chitosan oligomer (DCMO) respectively.

3.5 Characterisation of *N,N*-dicarboxymethylated chitosan derivatives

Different methods were used to characterise the *N,N*-dicarboxymethyl chitosan polymers that was synthesised as described in the previous section. The methods used were proton nuclear magnetic resonance (NMR) spectrometry, infrared spectrometry (IR) and differential scanning calorimetry (DSC). The degree of *N,N*-dicarboxymethylation was determined from ^{1}H-NMR spectra.

3.5.1 ^{1}H-NMR analysis

The degree of *N,N*-dicarboxymethylation was determined with ^{1}H-NMR spectrometry. Solutions were prepared in D_2O and left standing overnight and then freeze dried twice from the D_2O solutions to displace any moisture that may have been absorbed. The samples were analysed with a Bruker spectrometer (Bruker, Germany) at 300 MHz at 253 degrees Kelvin.

3.5.2 Infrared (IR) analysis

Every molecular species has a unique infrared absorption as all molecular species absorb infrared radiation. This method of analysis is applied by comparing the spectrum of a compound with a known spectrum with that of the analyte. If the compounds analysed are similar then their infrared spectra will be similar as well (Skoog *et al.*, 1996:592). The IR spectra were recorded on a Nicolet Nexus 470 FT-IR ESP spectrometer (Madison, USA). The samples were mixed with 400 mg potassium bromide (KBr) and placed in the sample holder and analysed at a range of 3800 to 400 cm^1.

3.5.3 Differential scanning calorimetry (DSC)

This form of thermal analysis is performed by raising or lowering a sample and reference pan (which is usually empty) from one temperature to another at a constant rate of change

of temperature. If the behaviour of the sample is different from that of the reference for any reason, then at some stage the signal will be out of balance. This differentiation in response is monitored and presented as a result (Lund, 1994:194). By this method it is possible to characterise a molecule or sample by comparing its behaviour to a reference or just simply to monitor its behaviour to variations in temperature. The DSC analysis of the carboxymethylated chitosan polymers was done on a Shimadzu DSC 50 (Scientific Group Analytical, Johannesburg South Africa). Approximately 3 mg of the sample was weighed in an aluminium pan which was then sealed. The temperature was then raised at 10 °C/minute and the behaviour of the sample monitored.

3.6 Results and discussion

3.6.1 ^{1}H-NMR spectrometry

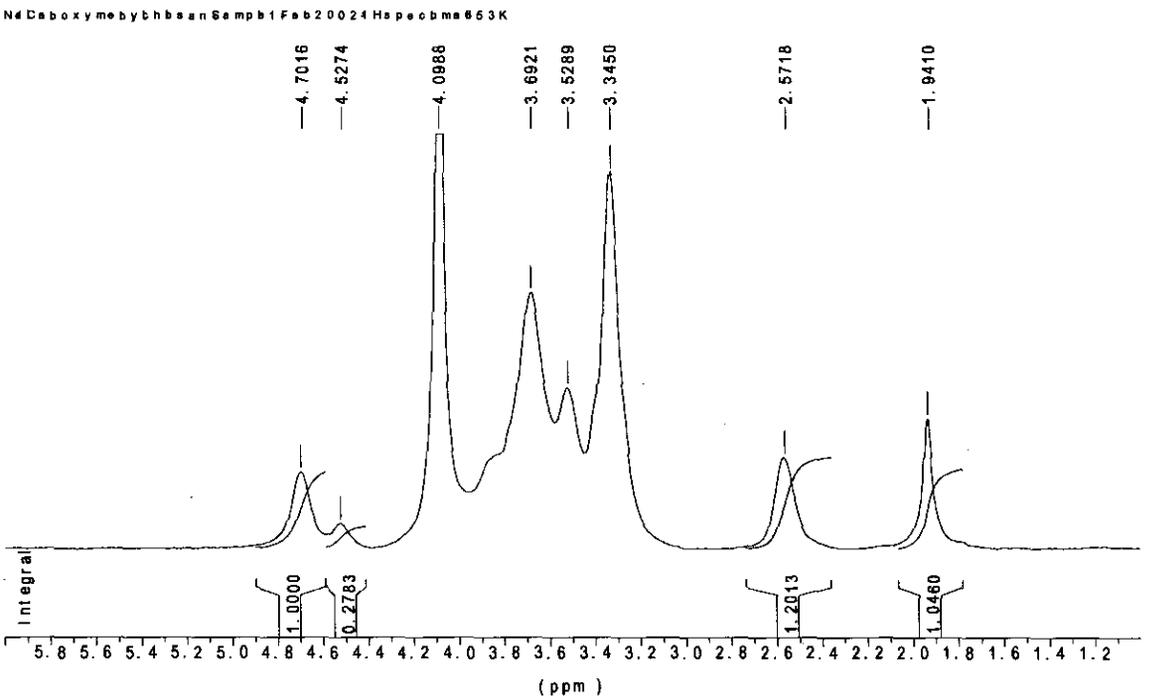

Figure 3.4 ^{1}H-NMR spectrum of the synthesised high molecular weight DCMC polymer.

The ^{1}H-NMR spectra reveal a very high degree of disubstitution with carboxylic groups on the nitrogen atom of the polymer. Both polymers appear to be almost completely substituted when observing the proton integral between 3.0 and 3.4 ppm (Figures 3.4 and 3.5) on the spectra. The degree of substitution can be determined as the fraction of carboxylic group per monomeric unit and the residual degree of acetylation from the $-CH_3$ signals in the ^{1}H-NMR spectra (Rinaudo *et al.*,1992:125) or from conductiometric titrations of the acidic form by sodium hydroxide and determined by the equation:

$$x = \frac{161Q}{2-160Q}$$

(Equation 3.1)

where x are the *N*-positions and Q the capacity of the polymer expressed in equivalents/gram of the solid material (Le Dung *et al.* 1994:213).

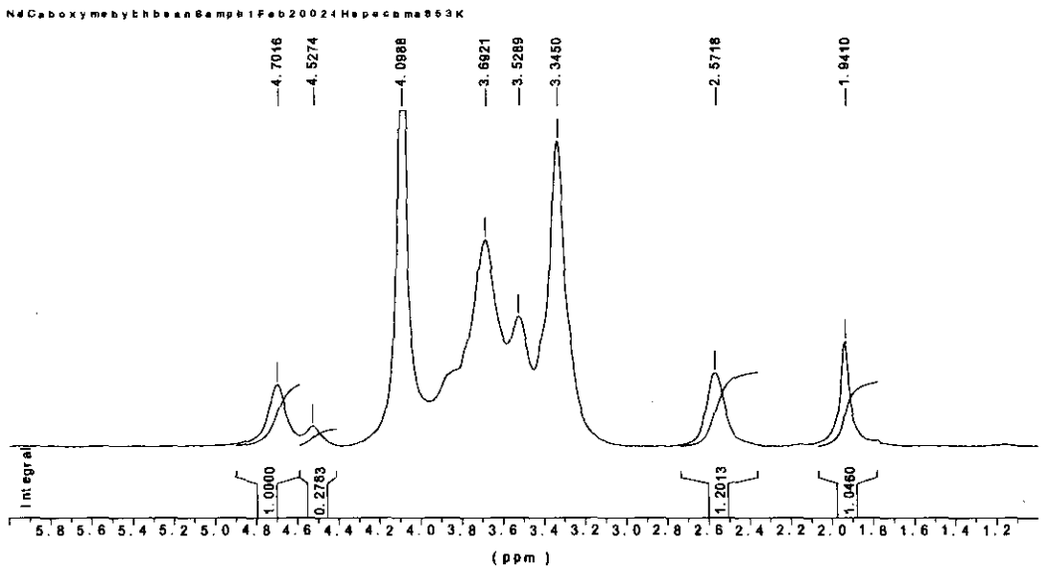

Figure 3.5 ^{1}H-NMR spectrum of the synthesised low molecular weight DCMO polymer.

3.6.2 Infrared (IR) spectrometry

Figures 3.6 and 3.7 shows the infrared spectra of fully substituted DCMC and DCMO. The 1635 cm^{-1} band suggests the presence of the carboxylic acid (COOH) group present in the carboxymethylated chitosan samples at a neutral pH (7.5) in comparison with chitosan and the chitosan oligomer. The intensity of the band at 1635 cm^{-1} would also suggest a high degree of carboxymethylation of these samples (>90 %).

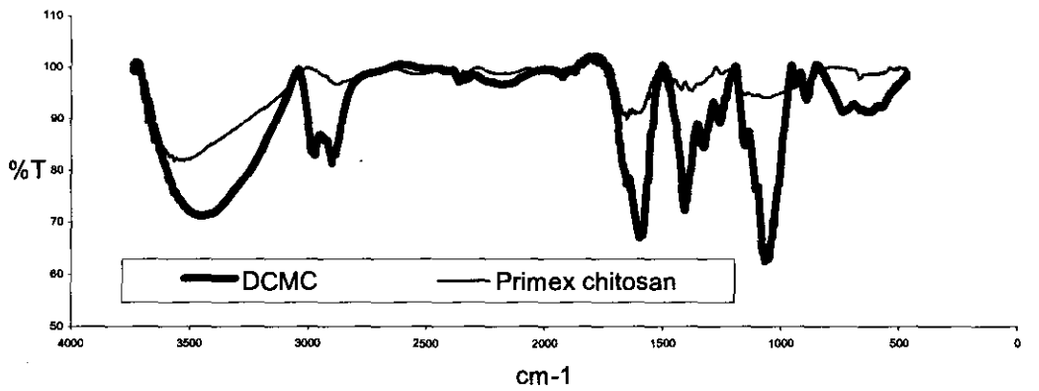

Figure 3.6 Infrared spectrum of the synthesised high molecular weight *N,N*-dicarboxymethylated chitosan (DCMC) polymer.

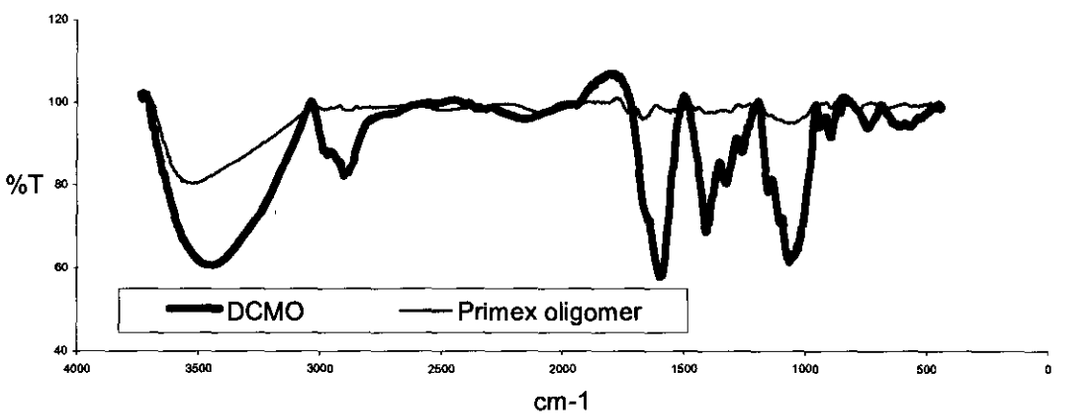

Figure 3.7 Infrared spectrum of the synthesised low molecular weight *N,N*-dicarboxymethylated chitosan oligomer (DCMO) polymer.

3.6.3 Differential scanning calorimetry (DSC)

Both carboxymethylated chitosan polymers show quite a similarity in their response to an increase in temperature. It is therefore evident that they are quite similar molecules with similar entities, even with a difference in molecular weight.

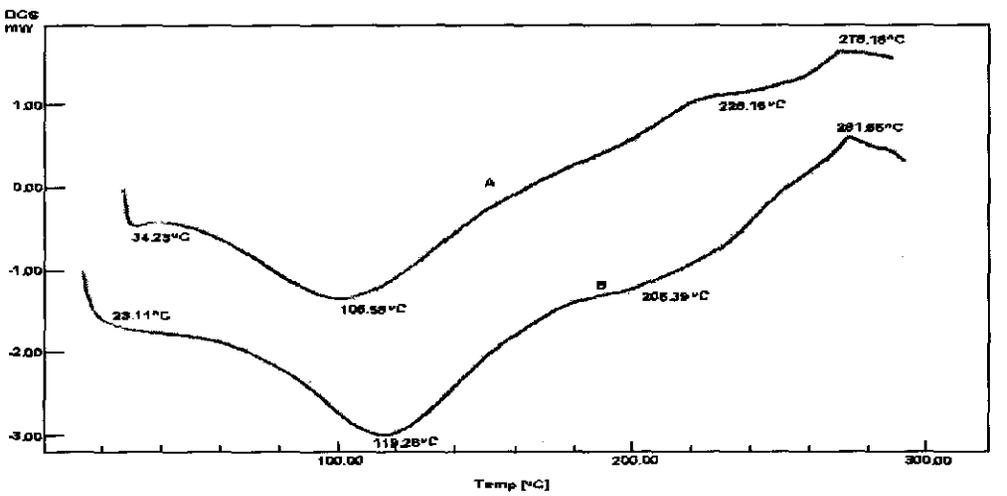

Figure 3.8 The differential scanning calorimetry spectra of the synthesised DCMC polymer (A) and the DCMO polymer (B).

3.7 Conclusion

In this chapter it was described how *N,N*-dicarboxymethylated chitosan derivatives were synthesised and characterised using different methods. *N,N*-dicarboxymethylated chitosans with high and low molecular weights were compared in terms of molecular composition and response to temperature analysis. It was confirmed by both ^{1}H-NMR spectra and IR spectra that both DCMC and DCMO have a very high degree of dicarboxymethylation at the nitrogen atoms of the polymers. The DSC spectra imply that both polymers exhibit similar characteristics in spite of their different molecular weights.

By being certain of the chemical structure of the carboxymethylated chitosan derivatives synthesised, it is assumed that it may be possible for these polymers to have enzyme inhibiting properties when comparing their chemical structure with the polymers given in Figure 3.1. Chapter 5 describes the investigation of the possible enzyme inhibiting effects of these synthesised polymers.

Chapter 4

Validation of the High Performance Liquid Chromatography (HPLC) analysis used in the enzyme inhibitory studies

4.1 Introduction

HPLC analysis was used in this study to evaluate the enzyme inhibitory effects of the synthesised *N,N*-dicarboxymethylated chitosans. In any evaluation or experimental procedure it is vital to have a valid analytical method, if such evaluation or experimental procedure requires an analytical method. There exist various aspects or characteristics when validating an analytical method together with the acceptance criteria of each validation or test. It is important that the validity of such a method falls well within the acceptance criteria of such a validation test to ensure that the analysis of such a procedure is accurate and correct. It is also important that such method is repeatable in such a way that similar results will be obtained if one wishes to use the same analytical method at any given time. The overall objective of such a validation of an analytical procedure is to demonstrate that the method is suitable for its intended purpose.

For the purpose of this study characteristics that are to be examined are specificity, linearity, accuracy, precision and repeatability of the analytical method. The analytical method used was adapted from Lueßen *et al.,* 1996:117 and modified by dr. J.L. du Preez*.

*National Institute for Industrial Pharmacy, Potchefstroomse Universiteit vir Christelike Hoër Onderwys, Potchefstroom, South Africa.

4.2 Chromatographic conditions

Analytical instrument: HP 1090 HPLC equipped with a tertiary gradient pump, autosampler, diode array UV detector and Chemstation Rev. A06.02 data acquisition analysis software or equivalent.

Column: Column L1, USP 24, 2000, p1925 (Luna 5u C18-2 column, 150 x 4.6 mm, 5.0 µm, 100 Å pores, 17.8 % carbon load, endcapped, Phenomenex, Torrance, Ca).

Mobile phase: Two eluents were used in the mobile phase:

1) Eluent A: 86.0 % (v/v) 10 mM ammonium acetate buffer pH 4.2 and 14.0 % acetonitrile.
2) Eluent B: 50.0 % (v/v) 10 mM ammonium acetate buffer pH 4.2 and 50.0 % acetonitrile.

Gradient: 0-1 min: 100 % A, isocratic; 1-5 min: 10 % A / 90 % B, linear gradient; 5-8 min: 10 % A / 90 % B, isocratic flow; 8-9 min: 100 % A, linear gradient.

Stop time: 10 minutes.

Post run: 4 minutes.

Flow rate: 1.0 ml/min.

Injection volume: 20.0 µl.

Detection: UV at 225 nm.

Retention times: Approximately 2.4 minutes for N-Acetyl-L-Tyrosine (AT) and 7.7 minutes for N-Acetyl-L-Tyrosine ethyl ester (ATEE). Only the AT was analysed for results.

4.3 Sample preparation

The sample or standard used in the validation of the analytical method was prepared as follows:

1) The buffer solution was prepared by weighing 97.6 mg of 2-[N-Morpholino]ethane-sulfonic acid (MES) and 455.5 mg of D-Mannitol and was dissolved in 10 ml of distilled water.
2) The buffer was adjusted to pH 6.7 with a 1.0 % KOH solution.
3) 10 mg N-Acetyl-L-Tyrosine (AT) was added to 10 ml of the buffer solution and stirred until it dissolved.
4) A stop solution was prepared with phosphoric acid at a pH 2.0.
5) 1.0 ml of the stop solution was injected into a HPLC vial.
6) Then a 50.0 µl sample of the AT-buffer solution was injected into the HPLC vial and sealed.

4.4 Validation of the test procedure, test results, acceptance criteria and conclusion

4.4.1 Specificity

Specificity is defined as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present in such a sample. This implicates the identification of the analyte and to ensure that all analytical procedures performed allow an accurate statement of the content of impurities of an analyte.

i) Method:

1.) A sample was prepared as described in section 4.3 but without the N-Acetyl-L-Tyrosine (AT).
2.) Then a standard sample was prepared.

3.) A standard sample was prepared but with N-Acetyl-L-Tyrosine ethyl ester (ATEE) instead of N-Acetyl-L-Tyrosine (AT).

4.) The samples was analysed in the HPLC UV 225 nm with a stop time of 10 minutes.

5.) The chromatogram was examined to see whether any other additional peaks were formed.

ii) Results:

A sample prepared without AT or ATEE is shown on the chromatogram in Figure 4.1. Figure 4.2 is the chromatogram of the standard sample and figure 4.3 is that of the sample containing ATEE. Figure 4.2 shows the retention time of AT to be 2.3 minutes and figure 4.3 shows the retention time of ATEE to be 7.7 minutes.

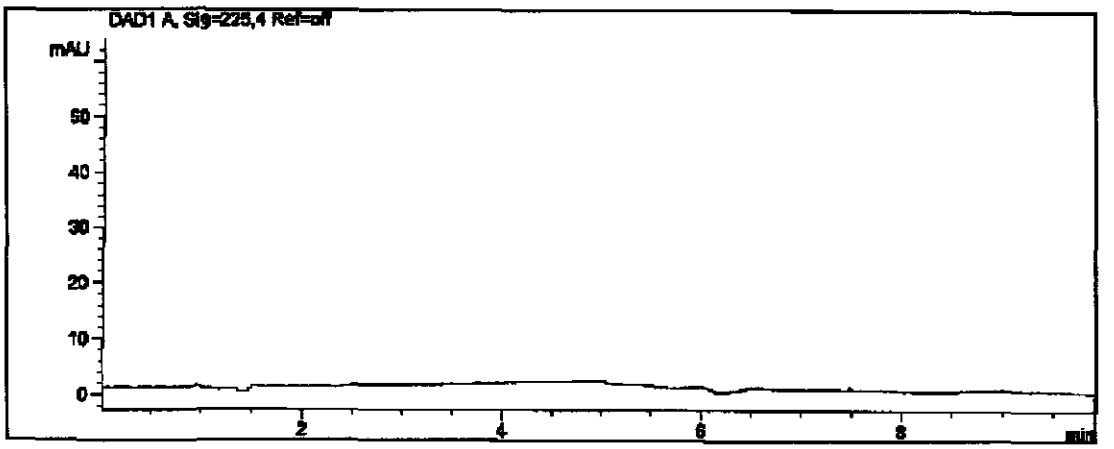

Figure 4.1 The chromatogram of the standard sample without any N-Acetyl-L-Tyrosine (AT) or N-Acetyl-L-Tyrosine ethyl ester (ATEE).

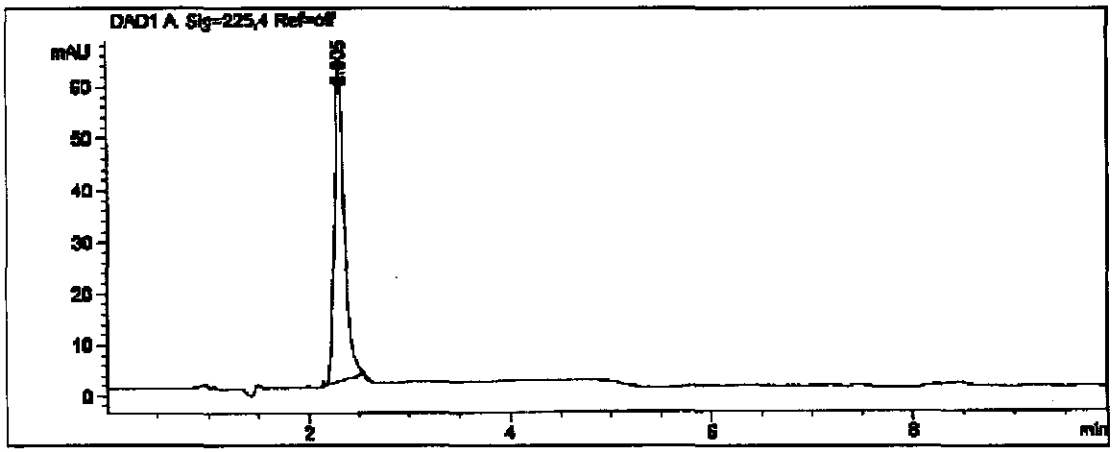

Figure 4.2 The chromatogram of the standard sample prepared as described in section 4.3.

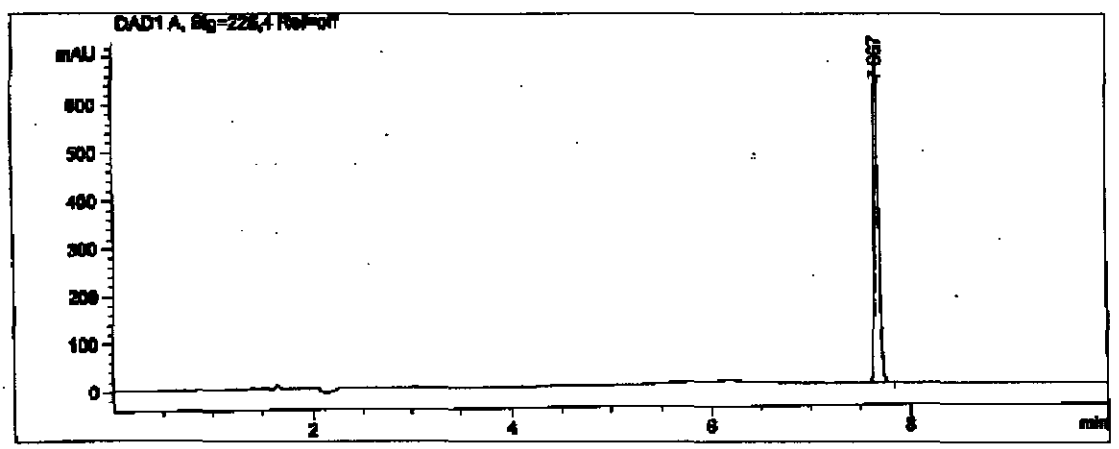

Figure 4.3 The chromatogram of the standard sample prepared with ATEE instead of AT.

iii) Acceptance criteria:

1.) The sample in figure 4.1 should not contain any peaks that will interfere with that of peak AT in figure 4.2.
2.) The sample in figure 4.3 should not contain any peaks that will interfere with that of peak AT in figure 4.2.

iv) Conclusion:

It was found that neither the blank sample nor the sample containing ATEE interfered with the analyte peak. It can be concluded that the procedure passes the acceptance criteria for this test.

4.4.2 Linearity

Linearity of an analytical procedure refers to its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of the analyte in the sample.

i) Method:

1.) A standard sample was prepared.

2.) The sample was analysed with variable injection volumes to obtain standards from 5.0 % to 120.0 % of the expected sample concentration.

ii) Results:

Table 4.1 The results obtained in the linearity study.

mg/ml	Area
0.05	58.5
0.1	126.4
0.2	234.6
0.3	341.8
0.4	470.1
0.5	566.1
0.6	661.3
0.7	776.7
0.8	886.8
0.9	991
1	1080.8
1.1	1200.5
1.2	1281.7

Table 4.2 The regression statistics for the linearity study.

R squared	0.9992
Intercept	23.864
Slope	1065.7

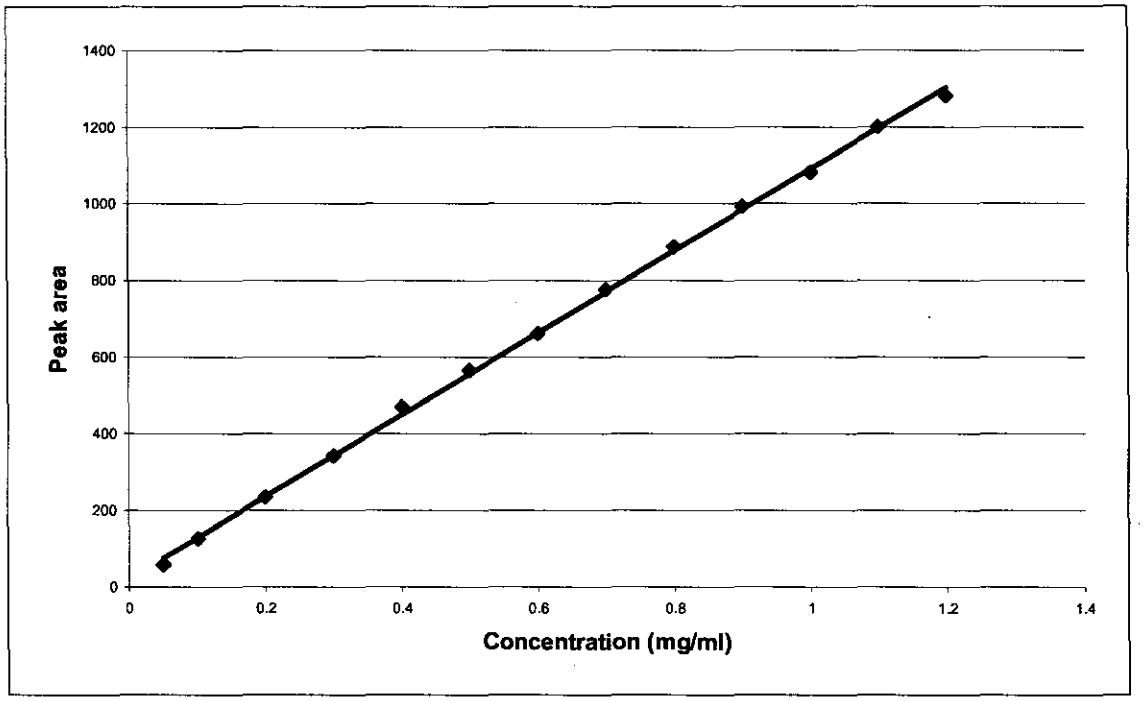

Figure 4.4 The linear regression graph for N-Acetyl-L-Tyrosine (AT).

iii) Acceptance criteria:

1.) The linear regression analysis should yield a regression coefficient (R squared) of ≥ 0.99.

iv) Conclusion:

In the linearity study it was clearly found that the method shows to be linear with a regression (R squared) of 0.9992 which makes the method acceptable. The method is linear over the concentration range 0.05 mg/ml to 1.2 mg/ml and is suitable for single point calibration.

4.4.3 Accuracy and precision

i) Method for intra-day precision (repeatability) and accuracy:

1.) A standard solution was prepared as described in section 4.3.
2.) A part of the standard solution was diluted to concentrations of 0.2 and 0.6 mg/ml.
3.) Three (3) HPLC vials were prepared for each of the concentrations namely 0.2, 0.6 and 1.0 mg/ml of the solutions.
4.) Each vial was injected twice and the peak areas of each AT curve was recorded.
5.) The recovery was then calculated.

Method for inter-day precision:

1.) A standard solution was prepared as described in section 4.3.
2.) Three concentrations of 1.0, 0.6 and 0.2 mg/ml was prepared by dilution.
3.) One (1) HPLC vial was prepared for each concentration of the solutions.
4.) Each vial was injected once, analysed and the area of peak AT was recorded.
5.) The procedure was repeated twice on different days.

ii) Results:

The results found were used for both the precision and accuracy studies. Table 4.3 and table 4.4 are the results found for the intra-day precision (repeatability) study and table 4.5 and 4.6 are the representations of the results found for the inter-day precision study.

Table 4.3 Statistical analysis of the intra-day precision study in the validation of the analytic method.

Statistical analysis	
Mean	100.9
SD	2.1
%RSD	2.1
95% confidence intervals	
Lower limit	99.2
Upper limit	106.1
Median	100.3
Confidence level	1.6

Table 4.4 The results found on the intermediate (intra-day) precision and accuracy study done with N-Acetyl-L-Tyrosine.

Conc. Spiked				Recovery	
mg/ml	Area 1	Area 2	Mean	mg/ml	%
0.2	265.7	232.1	248.9	0.21	106.1
0.2	235.2	236.1	235.7	0.2	100.4
0.2	243.8	234.7	239.3	0.2	102
0.6	649.8	662.7	656.3	0.6	99.2
0.6	651.7	661.8	656.8	0.6	99.3
0.6	670.5	668.8	669.7	0.6	101.3
1	1077.4	1077.3	1077.4	1.0	99.7
1	1082.3	1086.7	1084.5	1.0	100.3
1	1084.8	1080.6	1082.7	1.0	100.2

Table 4.5 The results found on the inter-day precision study done with N-Acetyl-L-Tyrosine.

	Day 1 %	Day 2 %	Day 3 %	Inter-day
	100.4	101.1	99.0	
	99.3	101.5	101.0	
	100.2	97.7	101.0	
Mean	99.9	100.1	100.3	100.1
SD	0.6	2.1	1.2	1.2
RSD %	0.6	2.1	1.2	1.2

Table 4.6 A summary of the inter-day precision study.

SUMMARY				
Groups	Count	Sum	Average	Variance
Day 1	3	299.9	99.97	0.3
Day 2	3	300.3	100.1	4.4
Day 3	3	301.0	100.3	1.3

iii) Acceptance criteria:

1.) For accuracy the recovery must be between 98.0 and 102.0 %
2.) For precision repeatability must be better than 2.0 % (n = 9) and for inter-day precision, precision must be better than 5.0 % (n = 9)

iv) Conclusion:

It is shown that the average recovery is 100.9 % and this falls well within the acceptance criteria and therefore passes the accuracy test. The mean values for the intra-day and inter-day studies are significantly similar. The assay should perform well, even if performed by different personnel in different laboratories.

4.4.4 Stability

i) Method:

1.) A standard sample was prepared as described in section 4.3.
2.) The sample was analysed with time intervals of one hour for 36 hours while retaining the vial in the autosampler.
3.) The area of peak AT was recorded and the amount AT remaining after each injection was determined.

ii) Results

Table 4.7 gives the results obtained in the stability study.

Table 4.7 The results obtained after testing the AT sample for stability over 36 hours.

Time (Hours)	Peak Area	% Remaining	Time (Hours)	Peak Area	% Remaining	Time (Hours)	Peak Area	% Remaining
0	884.5	99.5	13	889.0	100.0	26	874.3	98.4
1	889.1	100.0	14	887.7	99.9	27	889.1	100.0
2	887.8	99.9	15	897.1	100.9	28	888.7	100.0
3	898.7	101.1	16	891.2	100.3	29	848.6	95.5
4	888.6	100.0	17	887.2	99.8	30	894.7	100.7
5	889.4	100.1	18	895.1	100.7	31	896.6	100.9
6	886.8	99.8	19	885.5	99.6	32	849.8	95.6
7	886.7	99.8	20	893.7	100.6	33	888.6	100.0
8	888.7	100.0	21	917.3	103.2	34	885.9	99.7
9	886.5	99.7	22	886.4	99.7	35	887.4	99.8
10	884.1	99.5	23	887.7	99.8	36	888.2	99.9
11	884.4	99.5	24	890.1	100.1			
12	888.8	100.0	25	892.2	100.4			

Mean	99.8
SD	1.3
%SD	1.3

iii) Acceptance criteria:

1.) Sample solutions should not be used for a period longer than it takes to degrade by 2.0 %. In the case of degradation special precautions should be taken to compensate for the loss.

iv) Conclusion:

The sample AT showed no degradation over a period of 36 hours. The samples are suitable for analysis for periods of 36 hours or less.

4.5 Conclusion

All relevant characteristics for a proper HPLC validation such as specificity, accuracy, precision, linearity and stability were evaluated. The method performed very well and is suitable for the analysis of the formation of N-Acetyl-L-Tyrosine, or AT, over a period of 36 hours or less.

Chapter 5

Evaluation of the enzyme inhibitory properties of *N,N*-dicarboxymethyl chitosan

5.1 Introduction

Chitosan and several chitosan derivatives have been proven to be useful in the world of pharmaceutics as discussed in chapter 3. One of the uses of chitosan is that of its absorption enhancing effect for several drugs with poor absorption in the gastrointestinal tract. One of the mechanisms by which this result is achieved is by opening the tight junctions between gastrointestinal epithelial cells and thus enabling large molecules to be absorbed much easier. In addition to this, the mucoadhesive properties of chitosan and derivatives such as *N*-trimethyl chitosan chloride (TMC), enable it to exhibit such excellent absorption enhancing properties (Kotzé *et al.,* 1998.38).

Cationic methylated chitosan derivatives such as TMC have shown not to have any substantial inhibitory effect on α-chymotrypsin (Kotzé *et al.,* 1997:244). Carboxylic chitosan derivatives, being anionic and structural similar to enzyme inhibitors such as Carbopol 934P®, may have an inhibitory effect on enzymes that break down orally administered peptide drugs. In this chapter data to evaluate the enzyme inhibitory properties of *N,N*-dicarboxymethyl chitosan (DCMC) and that of *N,N*-dicarboxymethyl chitosan oligomer (DCMO) will be discussed. The synthesis of these polymers was described in chapter 3. The enzyme inhibitory properties of these polymers will also be compared with that of Carbopol 934P®, a known enzyme inhibitor, and with that of

TMC, a cationic chitosan derivative. *N,O*-carboxymethyl chitosan, which is currently commercially available, was also included in this study.

5.2 Experimental procedures for enzyme inhibition studies

5.2.1 The basic enzymatic reaction

α-Chymotrypsin often initiates the degradation of orally administered peptide drugs (Lueßen *et al.,* 1996:117) and is selected as a model enzyme for these studies for this specific reason. In this study α-chymotrypsin will react with *N*-Acetyl-L-tyrosine ethyl ester (ATEE), the substrate, to form the product *N*-Acetyl-L-Tyrosine (AT) and ethanol. The inhibition by the different polymers will be studied by monitoring the formation of the product, *N*-Acetyl-L-Tyrosine (AT) over time in the presence of the inhibitor. If a specific inhibitor inhibits α-chymotrypsin it would be likely that such an inhibitor would also inhibit other luminal endopeptidase enzymes such as trypsin or exopeptidase enzymes such as carboxypeptidases or aminopeptidase.

5.2.2 Chemicals

The following materials and reactants were used during the enzyme inhibition studies of the different polymers on α-chymotrypsin: α-Chymotrypsin (Sigma, South Africa. TLCK treated, Type VII from bovine pancreas), Potassium hydroxide pellets (Merck, South Africa), 2-[*N*-Morpholino]ethane-sulfonic acid (MES) (Sigma, South Africa), D-Mannitol (Sigma, South Africa), *N*-Acetyl-L-Tyrosine ethyl ester (ATEE) (Sigma, South Africa), *N*-Acetyl-L-Tyrosine (AT) (Sigma, South Africa), Phosphoric acid (Merck, South Africa). All chemicals used were of analytical grade.

The following polymers were used: Carbopol 934P® (BF Goodrich Speciality Chemicals, Cleveland, Ohio, USA), synthesised *N,N*-dicarboxymethyl chitosan (DCMC) and *N,N*-dicarboxymethyl chitosan oligomer (DCMO), *N*-trimethyl chitosan chloride (TMC)

(degree of quaternisation = 48.75 %, obtained from the Department of Pharmaceutics, Potchefstroom University) and *N,O*-carboxymethyl chitosan (V-Labs, Covington, Los Angeles, USA).

5.2.3 Methods

As mentioned earlier (5.2.1) the inhibition capability of every polymer is measured by monitoring the amount of product formed during the reaction in the presence of each polymer at different concentrations. This formation of *N*-Acetyl-L-Tyrosine (AT) from the degradation of *N*-Acetyl-L-Tyrosine ethyl ester (ATEE) by α-chymotrypsin is measured by a method (chapter 4) adapted from previous enzyme inhibition studies (Lueßen *et al.*, 1996:117).

5.2.3.1 The buffer system

To ensure optimum activity of α-chymotrypsin the enzyme-substrate reaction must be buffered at pH 6.7. The buffer system used consisted of a solution of 50 mM 2-[*N*-Morpholino]ethane-sulfonic acid (MES) and potassium hydroxide buffer. The buffer also contains 250 mM D-mannitol. It was prepared by dissolving the amounts of MES and D-mannitol in distilled water and adjusting the pH to 6.7 by a prepared 1.0 % potassium hydroxide solution.

5.2.3.2 Preparation of the enzyme solution

α-Chymotrypsin was dissolved in ice-cold MES/KOH buffer (pH 6.7) at a concentration of 1mg/ml and diluted 1/32.

5.2.3.3 Experimental procedure

An hour before the experiment begins a water bath was preheated to 37 °C after it has been filled with enough distilled water to cover the Eppendorf® vial holder used to house the enzyme and substrate-polymer solutions. The specific polymer was then dissolved at a specific concentration in the prepared buffer solution and the pH was then again adjusted to 6.7 with a 1.0 % potassium hydroxide solution. From this stock solution the different polymer solutions to be tested were prepared by dilution e.g. if 20.0 ml of a 1.0 % Carbopol 934P® solution was prepared, then 10.0 ml of this solution was taken and diluted to 20.0 ml to produce a 0.5 % solution.

Adding the substrate, *N*-Acetyl-L-Tyrosine ethyl ester (ATEE), has to be done very precisely to ensure that every polymer solution contains exactly the same ATEE concentration of 1 mg/ml. ATEE was weighed in it's powder form and was then added to the polymer solution to ensure that none of the substrate is lost during the transfer from container to the polymer solution and in this way increasing the accuracy of the method. The enzyme solution was then prepared as described in section 5.2.3.2.

The polymer-substrate solution (500.0 µl) was then injected into each vial in the water bath. Each polymer-substrate solution was then injected with 25.0 µl of the enzyme solution and the timer was started. Sample withdrawals of 50.0 µl were made after 10, 20, 30, 40, 50, 60, 120, 180 and 240 minutes from the relevant vials containing different concentrations of the polymer and were injected into HPLC vials containing 1.0 ml of a phosphoric acid solution at pH 2.0. Working with very small amounts of the solution not only one vial was used for all the extractions per one concentration of polymer but rather one vial for every two extractions of that specific concentration of polymer. For instance if three (3) different concentrations of the polymer were to be tested, together with a control, and extractions were made at nine (9) time intervals, then twenty (20) vials would have been used. Figure 5.1 illustrates the positioning of the vials in the water bath in all the experiments. Each experiment was repeated in triplicate.

All the samples were then analysed by High Performance Liquid Chromatography as described in chapter 4. All results were presented as a graph of the formation of the product N-Acetyl-L-Tyrosine (AT) against time.

Time intervals (minutes)	Polymer concentration (% w/v)			
	Control	X %	Y %	Z %
10 20				
30 40				
50 60				
120 180				
240				

Figure 5.1 The positioning of the vials in the water bath in all experiments. Two Sample withdrawals were made out of each vial except for those in the last row at 240 minutes from which only one sample withdrawal was made.

5.2 Results and discussion

5.3.1 Carbopol 934P®

Carbopol 934P® or Carbomer as it is also known, is a synthetic high molecular weight polymer of acrylic acid cross-linked with either allylsucrose or allylethers of pentaerythritol. It is a white, fluffy powder with a slight characteristic odour and it is hygroscopic. It also contains not less than 56.0 % or not more than 68.0 % of carboxylic

(-COOH) groups. If it is neutralised with alkali hydroxides or amines, it is soluble in water, ethanol (96.0 %) and glycerol (BP 1988:112).

Carbopol 934P® forms a viscous gel when dissolved in water and a 1.0 % dispersion in water has a pH of 3.0. Because it is hygroscopic it absorbs about 8.0 % of moisture at 25 °C at a relative humidity of 50.0 %. When dispersed in water it form an acidic colloidal solution of low viscosity but when the solution is neutralised, the solution becomes a highly viscous gel. Each gram of Carbomer requires 400 mg of sodium hydroxide to be neutralised. The Carbomer gels are most viscous between a pH of 6.0 and 11.0 and the viscosity is much reduced when the pH is reduced to 3.0 or raised higher than 12.0 (Lund, 1994:141). Carbopol 934P® was tested at concentrations of 0.1 %, 0.25 % and 0.4 % (w/v) and the results obtained are presented in table 5.1 and graphically depicted in figure 5.2.

Table 5.1 The formation of N-Acetyl-L-Tyrosine (AT) in the presence of Carbopol 934P®.

Time (min)	Area under curve AT (mAU)			
	Control	0.10 %	0.25 %	0.40 %
0	0.0	0.0	0.0	0.0
10	447.3 ± 45.9	132.1 ± 4.2	104.5 ± 28.1	76.5 ± 11.3
20	808.3 ± 28.1	255.6 ± 19.7	140.3 ± 45.9	76.9 ± 28.8
30	823.6 ± 60.8	360.7 ± 49.6	140.4 ± 19.6	80.6 ± 36.6
40	834.2 ± 25.3	408.3 ± 10.1	168.7 ± 54.2	119.8 ± 60.2
50	854.7 ± 28.3	480.0 ± 74.6	193.1 ± 2.7	120.3 ± 60.7
60	857.4 ± 139.0	487.6 ± 4.2	243.8 ± 79.6	124.0 ± 57.7
120	873.7 ± 117.5	732.8 ± 72.9	300.6 ± 83.6	164.6 ± 102.9
180	890.6 ± 97.5	776.0 ± 26.7	299.4 ± 55.4	183.7 ± 92.9
240	911.4 ± 90.5	799.9 ± 27.2	357.9 ± 55.4	228.7 ± 30.1

Each value represents the mean ± standard deviation of 3 experiments.

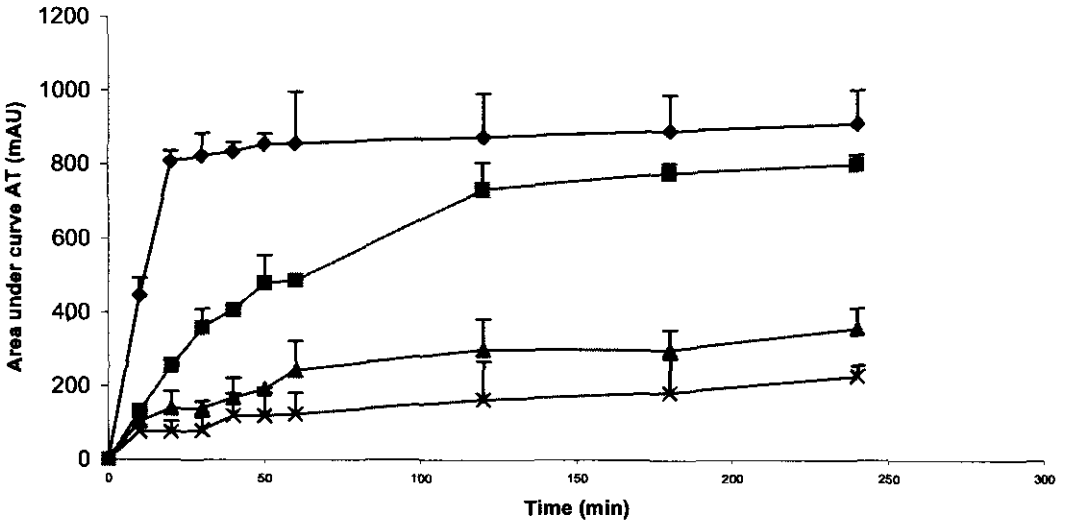

Figure 5.2 The formation of N-Acetyl-L-Tyrosine (AT) due to the degradation of N-Acetyl-L-Tyrosine ethyl ester (ATEE) by α-chymotrypsin in the presence of Carbopol 934P®. ♦, Control: ■, 0.1 % (w/v) Carbomer: ▲, 0.25 % (w/v) Carbomer and ✕ 0.4 % (w/v) Carbomer.

It is evident from the results that the inhibition of α-chymotrypsin is directly proportional to the inhibitor concentration. As the concentration of Carbopol 934P® increases, the formation of AT over time is slowed down. The formation of the product, AT, is inhibited dramatically especially in concentrations higher than 0.25 % of the polymer because of the inhibition of α-chymotrypsin. Carbopol 934P® has been proven in the past to be a very good inhibitor of α-chymotrypsin and these results are in agreement with previous results described in literature (Lueßen *et al.*, 1996:121).

5.3.2 High molecular weight *N,N*-dicarboxymethyl chitosan (DCMC)

The characteristics and properties of high molecular weight *N,N*-dicarboxymethylated chitosan (DCMC) was discussed in chapter 3. If one studies the chemical structure of the

chitosan derivatives given in Figure 3.1 (chapter 3) it is clear that there exists substantial similarities between these chitosan derivatives and that of *N,N*-dicarboxymethyl chitosan as illustrated in Figure 5.3. The enzyme inhibitors in Figure 3.1 are anionic, having carboxylic acid groups that are able to bind bi-valent organic ions essential for enzyme function. By binding these organic ions it is possible for these anionic molecules to inhibit enzyme function.

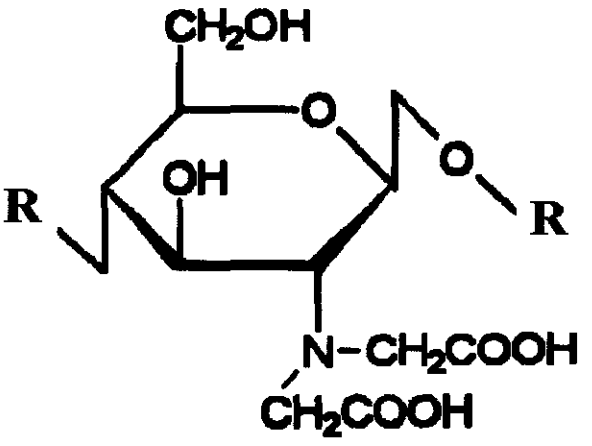

Figure 5.3 A monomer of *N,N*-dicarboxymethylated chitosan (DCMC) (Rinaudo *et al.*, 1992:125).

DCMC was tested at concentrations of 0.25 % (w/v), 0.5 % (w/v) and 1.0 % (w/v) and the results are presented in table 5.2 and Figure 5.4.

Even though some inhibition of α-chymotrypsin is evident at a polymer concentration of 1.0 % (w/v), inhibition is not so distinct when compared to that of Carbopol 934P®. It can therefore be said that high molecular weight *N,N*-dicarboxymethyl chitosan does not inhibit enzymes significantly enough to be labelled an "enzyme inhibitor".

Table 5.2 The formation of N-Acetyl-L-Tyrosine (AT) in the presence of *N,N*-dicarboxymethyl chitosan (DCMC).

Time (min)	Area under curve AT (mAU)			
	Control	0.25 %	0.50 %	1.00 %
0	0	0	0	0
10	849.1 ± 21.3	917.9 ± 67.9	802.2 ± 59.9	377.4 ± 16.7
20	1055.6 ± 24.6	1047.9 ± 8.5	1004.5 ± 62.6	682.8 ± 197.3
30	1105.4 ± 5.0	1064.7 ± 58.7	1012.4 ± 26.5	777.9 ± 21.5
40	1118.5 ± 23.5	1112.2 ± 45.3	1062.7 ± 18.4	981.5 ± 14.2
50	1135.8 ± 28.3	1115.7 ± 5.7	1068.3 ± 59.2	1005.0 ± 8.66
60	1137.6 ± 48.8	1116.6 ± 12.2	1083.1 ± 28.8	1018.1 ± 43.5
120	1148.1 ± 61.7	1131.1 ± 8.2	1118.1 ± 50.8	1023.5 ± 24.4
180	1162.4 ± 48.6	1156.4 ±25.6	1118.2 ± 56.5	1032.6 ± 10.2
240	1166.7 ±59.7	1158.6 ± 32.1	1118.4 ± 60.5	1048.9 ± 16.4

Each value represents the mean ● standard deviation of 3 experiments.

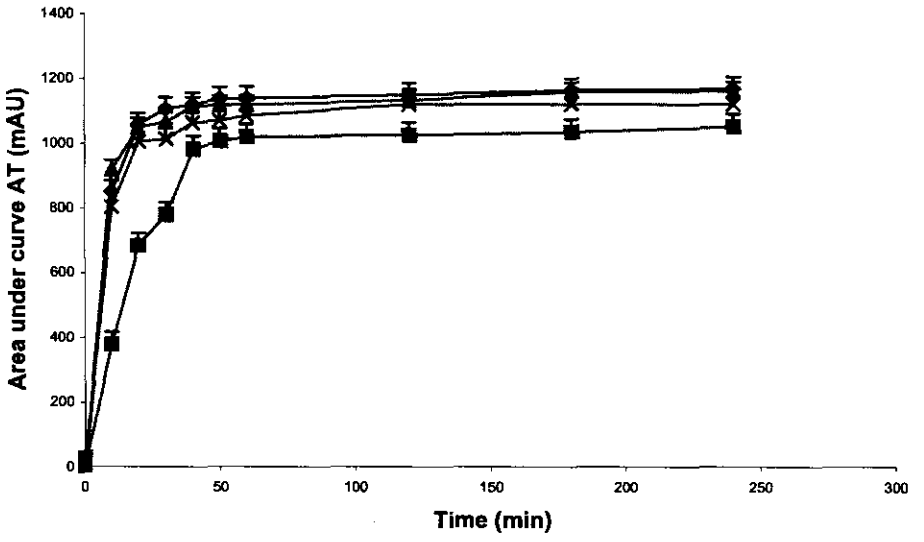

Figure 5.4 The formation of *N*-Acetyl-L-Tyrosine (AT) due to the degradation of *N*-Acetyl-L-Tyrosine ethyl ester (ATEE) by α-chymotrypsin in the presence of *N,N*-dicarboxymethyl chitosan (DCMC). ♦, Control: ▲, 0.25 % (w/v) DCMC: ×, 0.5 % (w/v) DCMC and ■, 1.0 % (w/v) DCMC.

Even though the molecule display characteristics of an enzyme inhibitor, it may be because of various reasons that it lacks proper enzyme inhibitory properties. It may be that the long polymer chain is too compact so that it can't properly react with bi-valent organic ions or simply react with too few bi-valent organic ions. For what ever the reason may be, DCMC still display some ability to inhibit enzymes and therefore needs further study.

5.3.3 High molecular weight *O,N*-carboxymethyl chitosan

As described in chapter 3 *O,N*-carboxymethyl chitosan is a water-soluble carboxymethylated chitosan polymer with carboxylic groups in the *O*- and *N*-positions. The *O,N*-carboxymethyl chitosan tested for enzyme inhibition was received from V-LABS, Inc. (Covington, Louisiana, USA). It was synthesised from chitosan which was 76.1 % deacetylated with a viscosity of 10 cps (See Annexure A). An enzyme inhibition study of *O,N*-carboxymethyl chitosan on α-chymotrypsin gave the results represented in table 5.3 and Figure 5.5. The polymer was tested at concentrations of 0.25 % (w/v), 0.5 % (w/v) and 1.0 % (w/v).

Table 5.3 The formation of N-Acetyl-L-Tyrosine (AT) in the presence of *O,N*-carboxymethyl chitosan.

Time (min)	Area under curve AT (mAU)			
	Control	0.25 %	0.50 %	1.00 %
0	0	0	0	0
10	238.4 ± 11.5	235.0 ± 32.2	202.5 ± 59.2	198.7 ± 24.3
20	374.9 ± 12.9	354.9 ± 68.5	308.1 ± 65.2	286.5 ± 29.5
30	509.1 ± 7.0	497.2 ± 89.4	364.2 ± 21.4	297.4 ± 105.3
40	631.1 ± 61.7	508.3 ± 52.5	496.3 ± 110.1	466.2 ± 64.4
50	729.4 ± 30.1	629.3 ± 110.0	633.6 ± 14.7	492.9 ± 5.0
60	801.2 ± 13.2	747.8 ± 5.7	649.3 ± 100.1	574.5± 130.9
120	915.8 ± 43.0	820 ± 7.5	801.5 ± 86.1	743.3 ± 20.8
180	1004.9 ± 29.1	815.1 ± 2.6	859.4 ± 113.8	778 ± 29.1
240	1058.7 ± 15.8	972.8 ± 95.8	930.5 ±17.3	786.6± 5.2

Each value represents the mean ± standard deviation of 3 experiments.

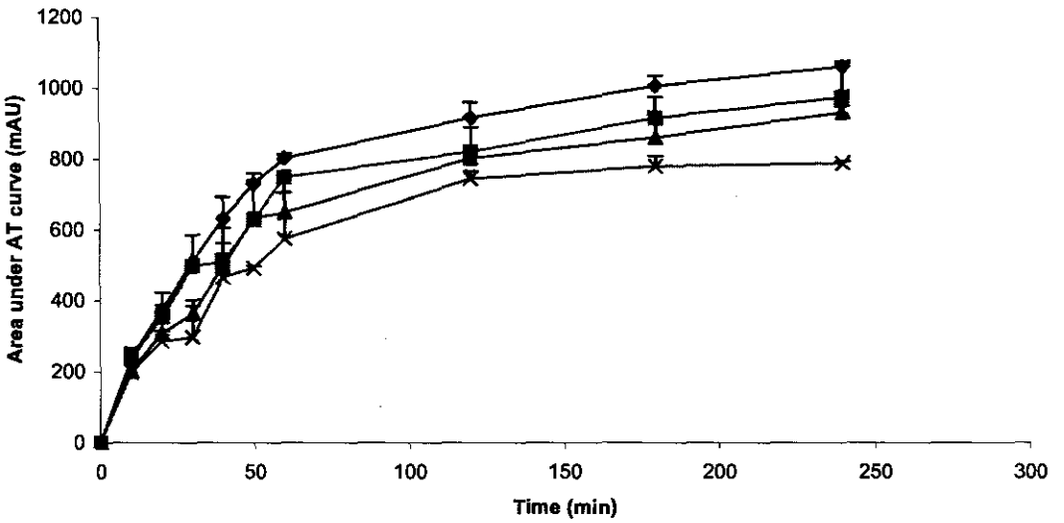

Figure 5.5 The formation of *N*-Acetyl-L-Tyrosine (AT) due to the degradation of *N*-Acetyl-L-Tyrosine ethyl ester (ATEE) by α-chymotrypsin in the presence of *O,N*-carboxymethyl chitosan. ♦, Control: ■, 0.25 % (w/v) *O,N*-carboxymethyl chitosan: ▲, 0.5 % (w/v) *O,N*- carboxymethyl chitosan and, ✕ 1.0 % (w/v) *O,N*- carboxymethyl chitosan.

As the concentration of the *O,N*-carboxymethylated chitosan increases the amount of product (AT) formed over time decreases. It is clear that *O,N*-carboxymethyl chitosan exhibits inhibitory properties on α-chymotrypsin proportionally to an increase in concentration of the polymer. Inhibition of α-chymotrypsin appears to be a fraction higher than with *N,N*-dicarboxymethyl chitosan. This may possibly be because of the carboxylic substitution on the *O* as well as the *N* atoms, which allows a better chance for reactivity towards bivalent organic ions needed for enzyme activity. The polymer therefore acts competitively against the enzyme by binding organic ions.

5.3.4 Low molecular weight *N,N*-dicarboxymethyl chitosan oligomer (DCMO)

As described in chapter 3 a low molecular weight, short chain *N,N*-dicarboxymethylated chitosan oligomer was synthesised. The polymer is also anionic with two carboxylic groups substituted on each nitrogen atom. The enzyme inhibition study carried out with DCMO gave the results presented in table 5.4 and Figure 5.6. DCMO was tested at concentrations of 0.25 %, 0.5 % and 1.0 % (w/v). It is evident from Figure 5.6 that the formation of AT is inhibited. The inhibition is directly proportional to the concentration of DCMO. It seems that the length of the polymer chain has none or little effect on the enzyme inhibitory effects of the polymer when comparing it with that of the long chain, high molecular weight DCMC and *O,N*-carboxymethyl chitosan. Inhibition of the formation of AT by DCMO appears to be a fraction higher than that of DCMC especially at lower concentrations of the polymer. It may be because the shorter polymer chain has better manoeuvrability or elasticity and higher access to the enzyme molecule resulting in a more complete reaction and thus better inhibition of the enzyme. For whatever the reason may be for the difference in the inhibitory effect of DCMC and DCMO is very small and would have to be studied further.

Table 5.4 The formation of N-Acetyl-L-Tyrosine (AT) in the presence of *N,N*-dicarboxymethyl chitosan oligomer (DCMO).

Time (min)	Area under curve AT (mAU)			
	Control	0.25 %	0.50 %	1.00 %
0	0	0	0	0
10	538.8 ± 60.0	611.7 ± 39.4	611.8 ± 23.8	700.4 ± 13.0
20	848.4 ± 17.1	838.0 ± 6.7	835.1 ± 22.1	862.8 ± 74.7
30	973.9 ± 33.0	979.6 ± 25.2	596.2 ± 12.7	874.7 ± 75.9
40	1055.5 ± 38.8	1003.1 ± 39.6	936.3 ± 23.6	951.7 ±25.6
50	1097.3 ± 30.7	1018.0 ± 31.1	955.6 ± 36.7	937.4 ± 13.4
60	1105.7 ± 40.9	1036.5 ± 25.9	949.5 ± 47.4	943.8 ± 13.2
120	1175.5 ± 26.2	1087.9 ± 51.5	1015.1 ± 38.3	950.8 ± 7.4
180	1205.2 ± 46.5	1138.4 ± 45.9	1051.6 ± 27.4	959.6 ±23.3
240	1234.8 ± 46.1	1157.4 ± 36.0	1054.9 ± 38.5	1000.9 ± 11.0

Each value represents the mean ± standard deviation of 3 experiments.

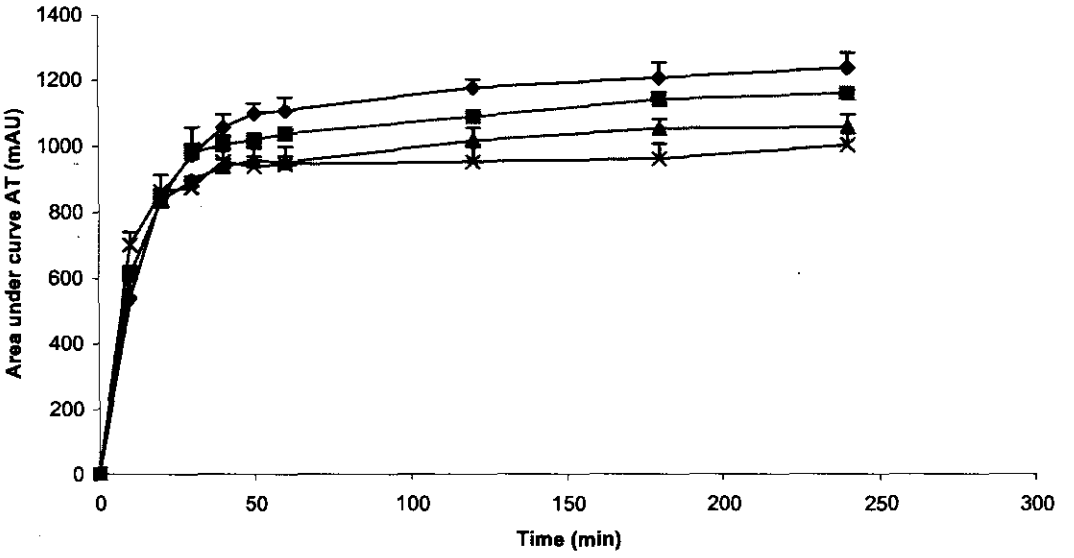

Figure 5.6 The formation of *N*-Acetyl-L-Tyrosine (AT) due to the degradation of *N*-Acetyl-L-Tyrosine ethyl ester (ATEE) by α-chymotrypsin in the presence of *N,N*-dicarboxymethyl chitosan oligomer (DCMO). ♦, Control: ■, 0.25 % (w/v) DCMO: ▲, 0.5 % (w/v) DCMO and, ✕ 1.0 % (w/v) DCMO.

5.3.5 Cationic *N*-trimethyl chitosan chloride (TMC)

To illustrate the difference between the interaction of the enzyme with anionic and cationic polymers, *N*-trimethyl chitosan chloride or TMC (degree of quaternisation = 48.75 %) was also tested for any enzyme inhibitory properties. Table 5.5 and Figure 5.7 shows the results found with the experiment done with TMC at concentrations of 0.25 %, 0.5 % and 1.0 % (w/v). It is evident that cationic and anionic polymers react totally different with enzyme molecules. Enzymes react with anionic polymers to form complexes. TMC did not react with the enzyme and did not in any way hindered the formation of AT. Instead, TMC promoted the formation of AT. It may be that TMC reacted with other molecules (inhibiting factors) that may inhibit the enzyme in other

ways. The formation of AT appears to be directly proportional to the concentration of TMC. AT is formed at a faster rate as well as in higher quantities over time in the presence of TMC.

Table 5.5 The formation of N-Acetyl-L-Tyrosine (AT) in the presence of *N*-trimethyl chitosan chloride (TMC).

Time (min)	Area under curve AT (mAU)			
	Control	0.25 %	0.50 %	1.00 %
0	0	0	0	0
10	345.2 ±12.8	778.5 ± 5.1	927.9 ± 19.4	786.8 ± 13.0
20	601.3 ± 18.0	983.2 ± 17.0	1009.0 ± 38.5	961.2 ± 3.2
30	718.6 ± 36.2	965.0 ± 44.3	989.5 ± 42.0	1046.8 ± 12.3
40	769.4 ± 39.3	981.9 ± 36.0	1028.3 ± 23.8	1032.5 ± 25.6
50	824.7 ± 36.4	970.3± 25.7	991.8 ± 32.6	1026.0 ± 42.4
60	860.8 ± 27.9	970.1 ± 32.7	1000.9 ± 22.1	1025.5 ± 49.1
120	973.5 ± 78.0	970.0 ± 73.4	1022.7 ± 42.2	1054.9 ± 44.2
180	1004.4± 67.6	1043.0 ± 26.4	1085.0 ± 14.4	1109.3 ± 6.1
240	1064.8 ± 24.9	1067.9 ± 28.9	1129.6 ± 16.0	1104.8 ± 26.5

Each value represents the mean ± standard deviation of 3 experiments.

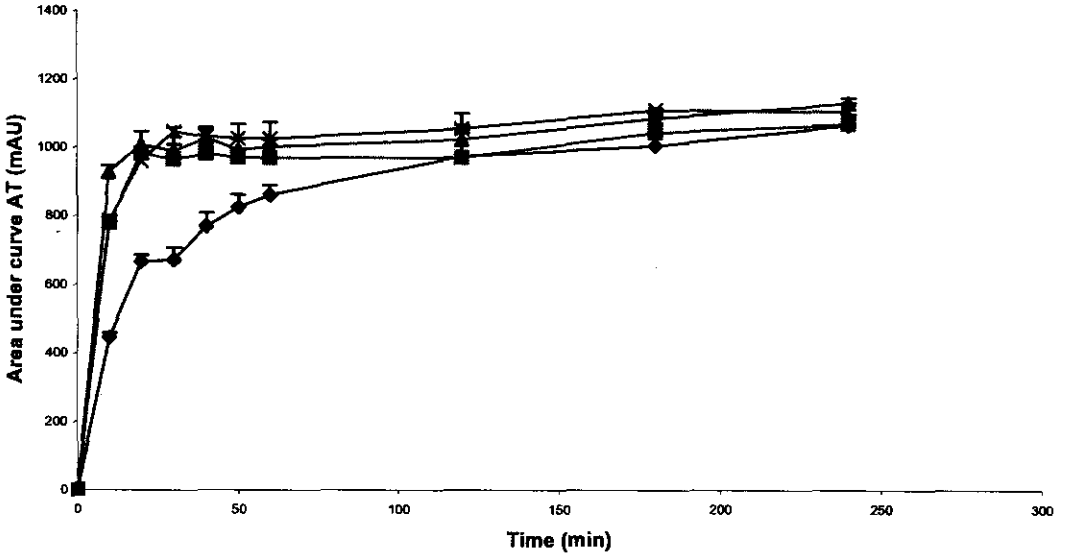

Figure 5.7 The formation of *N*-Acetyl-L-Tyrosine (AT) due to the degradation of *N*-Acetyl-L-Tyrosine ethyl ester (ATEE) by α-chymotrypsin in the presence of *N*-trimethyl chitosan chloride (TMC). ♦, Control: ■, 0.25 % (w/v) TMC: ▲, 0.5 % (w/v) TMC and, ✕ 1.0 % (w/v) TMC.

5.4 Conclusion

In this chapter the enzyme inhibitory properties of several chitosan derivatives was described and compared with those of a well-known enzyme inhibitor, Carbopol 934P®. It was seen that Carbopol 934P® inhibited α-chymotrypsin by reducing the formation of *N*-Acetyl-L-Tyrosine from N-Acetyl-L-Tyrosine ethyl ester (ATEE) in the presence of the polymer. Major inhibition was found at concentrations as low as 0.1 % (w/v) of the polymer. All the anionic chitosan derivatives displayed some inhibition abilities on α-chymotrypsin. No or little inhibition by DCMC at low concentrations (0.25 – 0.5 % w/v) of the polymer was found but DCMC showed definite inhibition at a concentration of 1.0 % (w/v). High molecular weight *O,N*-carboxymethyl chitosan and low molecular weight *N,N*-dicarboxymethyl chitosan oligomer (DCMO) showed inhibition even at low concentrations (0.25 % w/v) of the polymers and inhibition proved to be directly proportional to polymer concentration. All the anionic chitosan derivatives posses enzyme inhibitory properties with definite inhibition at a concentration of 1.0 %.

To investigate the possibility of enzyme inhibition by cationic chitosan derivatives *N*-trimethyl chitosan chloride or TMC was also tested. TMC do not inhibit α-chymotrypsin but rather promote the formation of *N*-Acetyl-L-Tyrosine throughout the experiment. The increase in formation of AT over time seemed to be directly proportional to the concentration of TMC.

It may be concluded from these experiments that carboxymethylated chitosans (anionic chitosan derivatives) have an inhibitory effect on enzymes such as α-chymotrypsin but that they cannot be classified as enzyme inhibitors because inhibition is achieved at fairly

high concentrations of the polymers. TMC does not inhibit enzymes such as α-chymotrypsin and can also not be classified as such.

Chapter 6

Summary and future prospects

The concept of enzymes have come a long way and the theories of enzyme action and function are well established in modern science. It was as early as 1880 that Wurtz proposed that an enzyme reacts with a substrate to form an enzyme-substrate complex. Enzymes are proteins that catalyse numerous physiological processes in the human body which makes life possible. Enzymes require co-factors for activity of which these co-factors include bi-valent cations such as Fe^{2+}, Mg^{2+}, Zn^{2+} or Mn^{2+}. In the absence of these cations enzymes are incapable of proper function (Nelson & Cox, 2000:244).

Because of the importance of these proteins in human physiology and biochemistry the manipulation of enzyme function will have a huge impact on the human body. Many diseases are being treated today by substances which inhibit various specific enzymes (Silverman, 1992:147). It was also found that there exists various types of enzyme inhibition of which some are reversible reactions (competitive and non-competitive enzyme inhibition) and some are non-reversible reactions.

N,N-dicarboxymethyl chitosan is a much more water-soluble product than chitosan and a very good chelating agent of bi-valent cations. Because of this interaction anionic chitosan derivatives may have the ability to interact with enzymes and alter their function directly. Walker *et al* (1996:6) concluded that the inhibition of trypsin by Carbomer 934P® is the result of an enzyme-polymer interaction reducing the free concentration of trypsin and in part denaturing the enzyme. It was also found that Carbomer 934P® inhibits chymotrypsin and it was determined that inhibition is due to the enzyme-polymer interaction. Anionic chitosan derivatives are likely to interact in exactly the same way

with protease enzymes such as α-chymotrypsin. *N,N*-dicarboxymethylated chitosan polymers of both high- and low molecular weight were synthesised and their ability to inhibit α-chymotrypsin was investigated. These polymers were analysed and characterised with ^{1}H-NMR, infrared spectrometry and differential calorimetry and defined as being di-substituted by carboxylic groups on the nitrogen atom of each polymer.

The analysis used in the enzyme inhibitory studies was done by High Performance Liquid Chromatography (HPLC). A validation of the analysis method was carried out by which the method was found to be well within the boundaries of the acceptance criteria for requirements such as specificity, linearity, stability, accuracy and precision and therefore the HPLC analysis was found to be valid for use of it in the experimental procedure used in this study.

Enzyme inhibitory experiments were carried out by which the basic enzymatic reaction where the enzyme α-chymotrypsin react with the substrate *N*-Acetyl-L-Tyrosine ethyl ester (ATEE) to form *N*-Acetyl-L-Tyrosine (AT), the product, was monitored under the addition of different concentrations of the polymers. The polymers used were Carbomer 934P®, high molecular weight *N,N*-dicarboxymethyl chitosan (DCMC), low molecular weight *N,N*-dicarboxymethylated chitosan oligomer (DCMO), high molecular weight *O,N*-dicarboxymethylated chitosan and cationic *N*-trimethyl chitosan chloride (TMC). Carbomer 934P® is known to inhibit α-chymotrypsin (Lueßen *et al.*, 1996:124). It was seen in the experiments that Carbomer 934P® inhibit α-chymotrypsin even at low concentrations of the polymer (0.1 %) and that it is a good enzyme inhibitor.

All the anionic chitosan derivatives displayed some inhibition abilities on α-chymotrypsin. High molecular weight *N,N*-dicarboxymethyl chitosan (DCMC) proved not to be such a good enzyme inhibitor at low concentrations of the polymer but DCMC showed definite inhibition at a concentration of 1.0 % (w/v). High molecular weight *O,N*-carboxymethyl chitosan and low molecular weight *N,N*-dicarboxymethyl chitosan oligomer (DCMO) showed inhibition even at low concentrations (0.25 % w/v) of the

polymers and inhibition proved to be directly proportional to polymer concentration. All the anionic chitosan derivatives posses enzyme inhibitory properties with definite inhibition at a concentration of 1.0 %.

TMC did not inhibit α-chymotrypsin but rather promote the formation of *N*-Acetyl-L-Tyrosine (AT), the product, throughout the experiment. The increase in formation of AT over time seemed to be directly proportional to the concentration of the polymer TMC.

Inhibiting protease enzymes which break down peptide drugs is a definite contribution to enhancing the absorption of such drugs. Extensive studies should be done on specific formulations, where attention should be given to effective concentrations of enzyme inhibiting polymers and compatibility with such formulations as well as toxicity and safety studies in the use of such a polymer in a specific formulation. It should also be established how other enzymes related to the breakdown of peptide drugs or are involved in any of the digestive processes that are affected by these enzyme inhibitors. Inhibition of other protease enzymes such as trypsin, carboxypeptidase or leucin may further contribute to the absorption of peroral administered peptide drugs.

Even though not classified as enzyme inhibitors, anionic chitosan derivatives may still be useful in formulations for the fact that they do inhibit protease enzymes. Future research will establish the concentrations of such polymers to be used in specific formulations. If such formulations would include large amounts of anionic chitosan polymers it might be possible that inhibition of protease enzymes by these polymers will be sufficient to consider enzyme inhibition as an commodity of such a formulation. It is therefore conclusive that to include such a polymer in a formulation in sufficient amounts where the absorption of such a drug needs to be raised or its bioavailability to be improved, will be of an advantage to such a formulation.

Annexure A

Certificate of analysis
ChitoClear™

PRODUCT: Fine grade chitosan made from fresh shrimp shells. Batch no: TD 012.

CHEMICAL PARAMETERS:

DRY MATTER:	92,1 %
ASH:	0,1 %
DEGREE OF DEACETYLATION:	97,6 % *(Titration- method)*
SOLUBILITY:	> 99,9 % *(in 1% acetic acid)*
TURBIDITY:	6 NTUs
VISCOSITY:	552 (mPa·s(cP)) *1% solutions in 1% acetic acid measured on a Brookfield RV DV-I/+ viscometer, 25C, spindle no:RV2 at 60 rpm.*

FEATURE:

FLAKES:	0,1–6 mm

MICROBIOLOGICAL PARAMETERS:

TOTAL PLATE COUNT:	< 1 000/g
ESCHERICHIA COLI:	absent
COLIFORME BACTERIA:	absent
SALMONELLA PR. 25 GRAM:	absent
MOULD AND YEAST:	< 100/g

Primex Ingredients ASA

Christian Horst
Laboratory Manager

Certificate of Analysis for Chitosan oligosaccharide

Product: Fine grade chitosan oligosaccharide made from fresh shrimp shells. Batch no.: 1118372

Chemical parameters:

Dry matter:	88.8 %
Ash:	0.4 %
Degree of deacetylation:	79.1 % (UV method)
Viscosity:	1.4 (mPa.s(cP)) *1.0 % solutions in 1.0 % acetic acid measured on a Brookfield LV-II+ viscometer, 25C, spindle no: ula at 60 rpm*
Turbidity:	8 NTUs
Density (Tapped):	0.56 g/cc (USP method)

Microbiological parameters:

Total plate count:	< 1000/g
Escherichia coli:	Absent
Coliforme bacteria:	Absent
Salmonella PR. 25g:	Absent
Mould and yeast:	<100/g

Primex Ingredients ASA

Christian Horst
Laboratory Manager

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Product name: Carboxymethylchitosan Product number: 108I

Chemical name: Carboxymethylchitosan

Solubility in water: Soluble (1-15 %)

Appearance and odour: White to pale yellow flake or powder, no significant odour.

Bulk density: Approx. 0.4 – 0.8 g/cc

Percent Volatile by weight (%): <10 %

Principle hazardous components: 0 %

Stability: Stable

Acknowledgements

GOD, for all His love, patience, mercy and the cross.

Prof. Awie Kotzé, for all his guidance and ideas and his faith in me.

Miss. Chriselle Jonker, for her guidance in the synthesis of the *N,N*-dicarboxymethylated chitosan polymers.

Mr. Dewald Snyman, for all his help, guidance, understanding and patience throughout this study.

Dr. J.L. du Preez, for all his time, effort and guidance with the High Performance Liquid Chromatography analysis and method development.

My Dad, for all his love, support and understanding.

Teresa Grobler, for her love and friendship through all the good and bad times.

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