INHIBITORY POTENTIAL OF HONEY ON THE ENZYMATIC ACTIVITY OF *HELICOBACTER PYLORI* UREASE

by

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DECLARATION

I, hereby, declare that this dissertation submitted to the University of Fort Hare for the degree of Masters of Science in Biochemistry in the Faculty of Science and Agriculture, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirety for the award of any degree.

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DEDICATION

This dissertation is dedicated to the loving memory of my grandmother, the late Eniya Matongo and to my sister, the late Givemore Matongo who both passed away while I was away from home for this study. Thanks for nurturing me and giving me the love I needed to be successful. You both knew I would always get an MSc, but when? It is only in God's Time, as it was God's time for you my grandma and you my sister to pass on to your heavenly home.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AHA	Acetohydroxamic acid
BSA	Bovine serum albumin
Cag A	Cytotoxin associated gene A
$CHCl_3$	Chloroform
EDTA	Ethylene diamine tetraacetic acid
EU	Extracellular urease
FPLC	Fast protein liquid chromatography
G + C	Guanine and cytosine composition
GLDH	Glutamate dehydrogenase
GOx	Glucose oxidase
H. pylori	*Helicobacter pylori*
Hsp B	Heat shock protein B
IC_{50}	50 % Inhibitory concentration
IU	Intracellular urease
Kb	Kilobase pairs
K_m	Michaelis constant
LPS	Lipopolysaccharide
MALT Lymphoma	Mucosa–associated lymphoid tissue lymphoma
Mbp	Megabase pairs
NADH	Nicotinamide adenine dinucleotide hydrogenase
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
U	Units
UMF	Unique Manuka Factor
VacA	Vacuolating cytotoxin gene A
V_{max}	Maximum velocity

ABSTRACT

Urease of *Helicobacter pylori* is an important virulence factor implicated in the pathogenesis of many clinical conditions, such as chronic gastritis, peptic ulceration, and gastric cancer. Many urease inhibitors have been discovered, like phosphorodiamidates, hydroxamic acid derivatives, and imidazoles. Despite good activities at the enzyme level and excellent kinetic properties most of them have not been used as therapeutic agents *in vivo* because of their side effects, toxicity and instability.

This has led to much attention to focus on exploring the novel urease inhibitory activities of natural products because of their low toxicity and good bioavailability. Honey, a natural product has been used in folk medicine due to its antitumor, antioxidant, antimicrobial and anti-inflammatory properties. The aims of this study were to isolate, characterise, purify urease produced by *H. pylori* and investigate the inhibitory effects of solvent honey extracts on its enzymatic activity.

Urease was found to be both surface-associated and cytoplasmic. Maximum cytoplasmic urease activity was found to occur after 72 hr whereas maximum extracellular urease activities were found to occur after 96 hr. Characterization of the crude cytoplasmic urease revealed optimal activity at a pH of 7.5 and temperature of 40°C. The kinetic parameters V_{max} and K_m were 45.32 U ml^{-1} and 61.11 mM respectively.The honey extracts inhibited the activity of the crude urease in a concentration dependent manner. The Lineweaver-Burk plots indicated a non-competitive type of inhibition against *H. pylori* urease. The two honey extracts gave promising inhibitory activities against urease of *H. pylori*. Thus the results of this study delineates that inhibition of urease can ease development in therapeutic and preventative approaches based on the enzymatic activity of this *Helicobacter* protein.

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Chapter 1 : INTRODUCTION AND LITERATURE REVIEW

This chapter first gives an overview of *Helicobacter pylori* and its urease, followed by giving four parts of literature review – biochemistry and physiology of the urease, the biological implications of *H. pylori* urease activity and the medical use of honey.

1.1 Overview

The bacterium *Helicobacter pylori*, the well-studied gastric pathogen is unique in its ability to colonize the acidic stomach which other gastrointestinal organisms can hardly pass through. *Helicobacter pylori* synthesizes a predominantly active urease which allows it to function in the harsh acidic conditions of the stomach and accordingly, inhibition of this active urease is an important area of pharmaceutical and medical research. *H. pylori*, one of the most common bacterial pathogens of humans causes chronic gastritis, duodenal ulceration, gastric ulcers and gastric cancer in later life. The bacterial urease of *H. pylori* plays an important role in the pathogenesis of these human diseases. The gastric pathogen colonizes the human stomach by producing large amounts of urease, which hydrolyzes urea present in the gastric lumen which is at a concentration that is approximately equal to that in plasma. This reaction generates ammonia which protects the organism by buffering the gastric acid. Accordingly, inhibition of urease is a suitable and applicable target for development of novel therapies to eradicate *H. pylori* infection.

1.1.1 *H. pylori*

H. pylori is a gram-negative, spiral-shaped bacterium with bluntly rounded ends in gastric biopsy specimens, measuring 2 to 4 µm in length and 0.5 to 1 µm in width. Although usually spiral-shaped when cultured on solid media, the bacterium can assume a rod-like shape, though coccoid forms typically predominate after prolonged in vitro culture or antibiotic treatment (Dunn *et al*., 1997; Kusters *et al*., 1997).

The bacterium has 2 to 6 polar-sheathed flagella and exhibits a distinctive bulb at the end which is an extension of flagellar sheath (O'Toole *et al*., 2000). The flagella help it move freely in viscous solutions such as the mucus layer that superimposes the gastric epithelial cells (O'Toole *et al*., 2000). The cell wall of this bacterial pathogen has a variety of putative outer membrane proteins. Heat shock protein B (Hsp B) and urease are profuse in the outer membrane protein of *H. pylori*. Studies have revealed that HspB and urease are located in the cytoplasm in the early log phase of bacterial growth. But, in the late log phase, these two proteins become associated with the bacterium surface upon spontaneous lysis of some of the bacteria followed by adsorption of the proteins onto the surface of the remaining intact bacteria. This process is referred to as altruistic autolysis. The function of this process is not yet understood but is thought to aid in protection against environmental stresses thus benefiting the remaining viable bacteria *in vivo*. It is also considered to be crucial in pathogenesis (Phadnis *et al*., 1996). The Lipopolysaccharide (LPS) of this organism has been found to have low biological activity but is thought to abet in the persistence of infection.

Studies by Dunn *et al*., 1997 revealed that the O specific chain of this pathogen's lipopolysaccharide mimicked Lewis blood group antigen in configuration and this molecular mimicry should perhaps contribute to its pathogenesis.

1.1.2 Genome, plasmids, and strain diversity

The size of the two sequenced *H. pylori* genomes ranges from 1.6-1.73 Mbp and the G+C composition ranges from 35 to 40%. The determination of the complete genome sequence of *H. pylori* strain 26695 showed the existence of 1,587 genes, whereas the genome of strain J99 showed only the presence of 1,491 genes (Alm *et al*., 1999; Boneca *et al*., 2003). However, both genomes have two copies of the 16S, 23S, and 5S rRNA genes.

Quite the opposite of other bacterial pathogens, *H. pylori* is genetically heterogeneous. This genetic heterogeneity is probably an adaptation of the pathogen to the harsh acidic conditions of its host (Kuipers *et al*., 2000). Investigations have shown that about 40% of this bacterium's isolates contain plasmids which range in size from 1.5 to 23.3 Kb, though these plasmids do not contain recognized virulence factors (Dunn *et al*., 1997). The bacterium displays significant phenotypic and sequence diversity. Studies by Dunn *et al*., 1997 indicated considerable sequence diversity in multiple genes encoding urease, structural and accessory proteins, flagellin, vacuolating cytotoxin and Cag A.

1.1.3 Urease

The urease produced by the *H. pylori* is a well-defined virulence protein. Theories suggest that this enzyme contributes to the pathogenesis of this gastrointestinal bacterium throughout the time it is present in the stomach. This metal-containing enzyme is a key element in the bacterium's resistance to acidity.

It enables this bacterium to survive in hostile environments of extreme acidity in the stomach during colonisation which leads to severe gastric diseases like gastric and peptic ulcer, gastric cancer and MALT Lymphoma. The important role that urease plays suggests that inhibition of this enzyme might represent a novel opportunity for therapeutic intervention in *Helicobacte*r infections, either through the use of a urease inhibitor alone or, more likely, in combination with a conventional antibiotic.

Moreover, the structure and catalytic mechanism of urease are of much interest, because of its large enhancement (10^{14} times faster than the uncatalysed reaction) of the rate of urea hydrolysis and the presence of a dinuclear nickel active site with a carbamylated lysine residue, which is distinctive amongst hydrolytic enzymes.

Therefore, understanding the urease inhibition mechanisms may confer the solution to a simple and efficacious form of treatment and prevention of colonization. Hypotheses assume that the enzyme plays an essential role in the bacterium's colonization of the gastric mucosa; however, the exact mechanism by which this occurs remains controversial. The unavailability of a simple, consistent, and high yield method of purification of this enzyme has been the main limit in revealing this essential role (Dunn *et al*., 1990).

1.1.4 Acid resistance mechanism of *H. pylori* urease

The ammonia produced is thought to play a role in the gastritis that inevitably follows infection. Research has indicated that urease is synthesized constitutively by the bacterium accounting for about 15% of protein synthesis; making the enzyme dependable for the acid resistance mechanism of *H. pylori* (Clyne *et al*., 1995).

In view of this, it appears that if acid resistance is eliminated by a selective inhibitor of urease then a novel therapeutic agent for eradication would be available (Weeks *et al*., 2000) which would target the acid resistance mechanism of *H. pylori*.

1.1.5 Theories of acid resistance

The effect of *H. pylori's* urease on acid resistance became and is still the most studied attribute of bacterial ureases. Urease and acid resistance: Where does it take place? At first, it was speculated that that *H. pylori's* urease was a surface-associated protein that was involved in acid resistance of the bacterium leading to microenvironmental neutrality (Krishnamurthy *et al*., 1998). The pH was thought to increase through the association of H^+ ions with non-charged ammonia released by urease activity (Marshall *et al*., 1990). Marcus and Scott, 2001 later demonstrated that the urease was a cytoplasmic enzyme and became surface-associated during the stationary-phase, when a considerable proportion of the cells lyse and the released urease binds to the surface of non-lysed cells.

The theory of how urease contributes to acid resistance of *H. pylori* then moved on to a second hypothesis, which postulated that cytoplasmic urease activity was critical for acid survival (Voland *et al*., 2003). Conforming to this hypothesis, the non-charged, urease-produced ammonia was speculated to leave the cytoplasm and bind protons in the periplasm, leading to an increase of the periplasmic pH (Voland *et al*., 2003).

The third hypothesis speculated that uncharged cytoplasmic urease-produced ammonia molecules bind protons in the periplasm, elevating the periplasmic pH and preventing protons to reach the cytoplasm (Stingl *et al*., 2002).

This model implied that a putative ammonium transporter would export charged ammonium ions from the cytoplasm thus creating a neutral cytoplasm. Though up to present, no ammonium transporter has been identified in *Helicobacter* species (Stingl *et al*., 2002). Though it is now widely accepted that cytoplasmic urease protects *H. pylori* against acidity, there is still a debate about the compartment which is buffered by the produced ammonia.

1.2 BIOCHEMISTRY OF UREASE

There are many fascinating aspects of urease biochemistry. The following sections will discuss what is known about urease assays, kinetic behavior of urease, urease structural properties, purification studies, active-site structure and catalytic mechanism, properties and functions of urease-related accessory proteins that facilitate metallocenter assembly.

1.2.1 Urease enzymology

1.2.1.1 Enzymatic cleavage of urea

Urease (urea amidohydrolase; EC 3.5.1.5) is a nickel dependent enzyme that catalyzes the hydrolysis of urea to produce ammonia and carbamate. The product, carbamic acid, being unstable undergoes spontaneous hydrolysis to yield a second molecule of ammonia and carbonic acid but now in an uncatalyzed reaction.

$$NH_2CONH_2 + H_2O \xrightarrow{\text{Urease}} NH_3 + NH_2COOH$$

$$NH_2COOH + H_2O \longrightarrow NH_3 + H_2CO_3$$

At physiological pH, the produced carbonic acid dissociates into the aqueous environment to produce hydroxide ions and the two molecules of ammonia produced get in equilibrium with their deprotonated and protonated forms as well.

The end result of the hydrolysis of urea by this enzyme is increase in pH (Burne and Chen, 2000).

$$H_2CO_3 \longrightarrow H^+ + HCO_3^-$$

$$2\ NH_3 + 2\ H_2O \longrightarrow 2NH_4^+ + 2OH^-$$

This role of acid neutralization has been associated with pathogenesis of *H. pylori* and has made the urease of this bacterium one of the most carefully analyzed ureases in the medical research.

1.2.1.2 Urease assays

Several excellent techniques are available for quantitating urease activity and kinetic analysis. There are advantages and disadvantages for each method, and these different assays are different in their sensitivities, ease of use, and susceptibility to interference. Thus, the selection of the best assay method will depend on the particular experiment of interest.

Indophenol assay

The ammonia released during the reaction can be reacted with phenol-hypochlorite at high pH to form indophenol, providing a simple and quantitative spectrophotometric assay (Weatherburn, 1967). This method is very sensitive, can easily detect <0.02 µmol of ammonia and is very useful in analyzing kinetic behavior. Nonetheless, this method has the disadvantage of being a fixed-time-point assay which requires multiple samples of each assay. Furthermore, several urease inhibitors have been found to interfere with this method.

Nesslerization reaction

It is a fixed-time-point spectrophotometric assay, in which ammonia reacts with Nessler reagent, containing KI and HgI, (Sigma ammonia color reagent). Instead of the intense blue color observed in the indophenol assay, a two- to four-fold less intense orange color develops. This assay is convenient because it is rapid and easily performed; however, it has the disadvantage of having low sensitivity and interference by cellular components.

Coupled enzyme assays

Coupled urease-glutamate dehydrogenase (GDH) assay

A continuous spectrophotometric assay method which uses glutamate dehydrogenase to couple ammonia release to reduced nicotinamide adenine dinucleotide oxidation has been widely used (Kaltwasser and Schlegel, 1966). The production of ammonia by urease stimulates the oxidation of NADH in this coupled enzyme assay and the reaction can be monitored by measuring extinction at 340 nm. This method can detect 0.003 U of urease per ml (1 U hydrolyzes 1 µmol of urea per min), which means that 0.02 µmol of ammonia would be released in 3.3 min from a 1-ml reaction.

In view of that, the coupled enzyme assay and the indophenol assay are generally comparable in sensitivity. Nevertheless, some drawbacks to this coupled system have been noted (Kaltwasser and Schlegel, 1966). For instance, glutamate dehydrogenase has a higher pH optimum (pH 8.3) than most ureases; the coupling enzyme may be inhibited by compounds being tested as urease inhibitors; a delay is observed in reduced nicotinamide adenine dinucleotide oxidation, requiring that the

enzyme rate be approximated by a tangent to the time course; and cellular reduced nicotinamide adenine dinucleotide oxidase activity may be present in crude fractions.

Coupled urease-horseradish peroxidase assay

The ammonia released by urease remarkably stimulates horseradish peroxidase-catalyzed peroxidation of *o*-dianisidine, causing an increase in absorbance at 460 nm (Stutts and Fridovich, 1964). Drawbacks to the method have also been of concern, amongst them are; the nonlinearity of the absorbance changes and the requirement for basic buffer conditions (pH 9.3).

pH indicator assay

The catalysis of urea hydrolysis by urease results in an increase in pH. The rate of pH change can be monitored spectrophotometrically in the presence of a pH indicator such as phenol red (Hamilton-Miller and Gargan, 1979). This assay is very convenient and suitable for routine comparison of urease rates, but is not recommended for detailed kinetic analysis. In assays containing 7 µg of phenol red per ml, a nearly linear change with time at 560 nm was observed between 0.15 and 0.5 absorbance unit when initiated at pH 6.8 (Mobley *et al.,* 1986). However, a serious drawback to this method is that the pH of the assay solution will increase during the analysis and affect enzyme activity.

Potentiometric assays

Ammonium ion-selective electrodes are also used to quantitate urease activity by directly measuring ammonium ion production (Hamilton-Miller and Gargan 1979; Katz, 1964; Montalvo, 1970). One of the drawbacks to the method is low sensitivity (0.1 mM ammonium ion is the lower limit), and it does not provide a linear response.

A further setback with this electrode is interference by potassium and other monovalent ions. A pH-Stat can also be utilized to monitor direct alkalinization in a very sensitive way (Blakeley *et al*, 1969). A pH-Stat is an apparatus that is used to maintain a constant pH in an aqueous liquid mixture in which hydrogen ions are being liberated or taken up. This method is superb and unaffected by inhibitors; nonetheless, no buffer is used in the assay and the ionic strength of the solution changes during the analysis, which may possibly affect the activity of the enzyme.

Urease activity in gels

Several methods have also been adapted or devised to detect urease activity in native agarose or acrylamide gels. The simplest technique is to equilibrate the gel in a buffer of pH 6 containing a pH indicator, followed by transfer of the gel to a solution containing urea (Blattler *et al*, 1967). As the urea is hydrolyzed, the pH indicator changes color within the gel at the location of the urease activity.

1.2.1.3 Kinetic properties of Urease

H. pylori like other ureases exhibit simple Michaelis-Menten-type kinetic behaviour. Up to present, there has been no evidence for substrate inhibition or allosteric behaviour associated with this enzyme. For the cytoplasmic urease, enzyme activity is measured using lysed cells to reduce problems caused by urea or ammonia transport across the cytoplasmic membrane.

Low urease activity is probably due to inhibition of the enzyme in cruder preparations by unidentified cellular components. As a general rule, the specific activity of urease depends on the state of purification, on the specific activity of the homogeneous protein, and on the conditions of assay.

However, in most cases, the *Km* values of highly purified ureases agree quite well with the values observed for crude urease extracts; hence, the determination of *Km* values does not require the purified enzyme. But it must be kept in mind that the specific activity of urease is strongly pH dependent, as some studies have shown urease inhibition by thiols, borate, phosphate, and other buffer components leading to inaccurate K_m values.

The urease of *H. pylori* has been purified and its kinetic parameters have been determined by measuring the rates of hydrolysis over a range of urea concentrations from 0 to 5 mM at 23°C. The reported values of K_m ranged from 0.17 to 0.48 mM of urea (Dunn *et al*., 1990; Evans *et al*., 1991; Hu and Mobley 1990). Under saturating conditions of substrate the purified enzyme has been found to possess specific activity ranging from 1,100 to 1,700 µmol of urea/min/mg protein (Dunn *et al*., 1990; Hu and Mobley 1990).

The K_m value of *H. pylori* urease deserves special mention. The urease of this gastrointestinal pathogen has one of the lowest K_m values (0.17 mM) of all microbial ureases and this reflects a very high affinity for substrate. Since the bacterium occupies the gastric mucosal lining, a region where low concentrations of urea are supplied from the bloodstream (1.7 to 3.4 mM in urea), this low kinetic constant seems to be suitable to the ecological niche of the pathogen as it allows this enzyme to function under close to saturation conditions, thus working at its V_{max} despite the low substrate concentration.

1.2.1.4 Urease Inhibitors

A realistic approach to the control of urease-related pathogenesis of *H. pylori* infections is to exploit potent and highly specific inhibitors of this enzyme. A number of classes of urease inhibitors have been discovered and examined for their pharmacological value (Mobley and Hausinger, 1989; Rosenstein and Hamilton-Miller, 1984). Inhibitors of this enzyme thus have potential value in medicine. In view of this, the study of urease inhibitors can present insight into selected aspects of the enzyme mechanism and active-site structure.

Substrate analogs

A number of urea analogs have been studied as urease inhibitors, amongst them are alkylated ureas, various thioureas, hydroxyurea, and hydroxamic acids. Crude enzyme preparations rather than purified enzymes have mostly been used for substrate analog studies.

Substituted ureas and thioureas

Alkyl-substituted derivatives of urea have been evaluated for their effect on urease activity. The urea analogs tested: methylurea, ethylurea, and phenylurea all gave no significant urease inhibition. Studies have shown that despite the close structural similarity between urea and thiourea, the latter compound is not a substrate for urease.

Hydroxyurea

Hydroxyurea is a substrate of the urease. When tested on a urease sample rapid inhibition is observed followed by slow recovery of activity as hydroxyurea is hydrolyzed (Nakano *et al*., 1984).

Hydroxamic acids

Hydroxamic acids characterized by a terminal functionality are distinguished inhibitors of urease discovered by Kobashi *et al*. in 1962. They have been examined against the ureases of microbial origin. The best-studied hydroxamate is acetohydroxamic acid (AHA), and has been shown to inhibit several microbial ureases including *H. pylori*. Hydroxamic acids are excellent metal chelators. Spectroscopic studies have shown that the mechanism of inhibition involves binding to the active site nickel ion. Hydroxamic acid binds in a time-dependent nature and has very slow inhibitor dissociation rate. Kinetic analysis of acetohydroxamic acid inhibition have been done and AHA was found to be a reversible, competitive inhibitor of urease, with a slow dissociation rate (k_{-1}, 8.4 x 10^{-5}/s) and a K_i of approximately 4 µM at pH 7.0 to 7.1 and 25°C (Mobley and Hausinger, 1989).

Phosphoroamides

Phosphoroamides are another class of synthetic inhibitors. There are apparently slow, tight-binding inhibitors that are extremely potent inhibitors of urease, actually more than AHA (Millner *et al.*, 1982). Several studies have demonstrated the inhibition of urease activity by phenylphosphorodiamidate (Kobashi *et al*., 1985). N-acyl phosphoric triamides have shown significant urease inhibition in cell extracts of many microbes (Kobashi *et al*., 1985). Phosphoroamide compounds are thought to act as transition state analogs because of their tetrahedral geometry that is thought to mimic an intermediate state in enzymatic catalysis. Spectroscopic studies have shown that phosphoramidate binds to the active site nickel (Mobley and Hausinger, 1989).

Thiols

Thiol-compounds compete with urea for the active site and appear to inhibit urease activity by chelating the nickel atoms involved in the catalysis process. Though they are weak inhibitors of urease, they have been very useful for better characterization of the enzyme metallocenters (Mobley and Hausinger, 1989).

1.2.1.5 Mechanism of urease inhibition

Urease inhibitors can be classified into two major classes based on their modes of binding:

I. Inhibitors that bind in a substrate like or active-site directed mode

II. Inhibitors that bind in a non-substrate like or mechanism-based directed mode (Amtul *et al*., 2002).

Substrate –like /Active-site directed inhibitors

The active site directed inhibitors and their analogs are currently the most studied compounds in urease inhibition. These compounds share a common feature, each inhibitor bridges the two paramagnetic nickel ions in the active site of the enzyme. So, the nickel ions and the amino acid residues in the active site are in an orientation similar to those of the substrate of urease. The main examples of the substrate-like urease inhibitors are thiourea and hydroxyurea. The majority of the first known inhibitors of urease contain strongly basic groups such as mimics of the amide bond in the urea molecule. Hydroxamic acid derivatives and phosphazenes are also known as substrate-like urease inhibitors (Amtul *et al*., 2002).

Thiourea Hydroxyurea

Figure 1.1 Structures of some substrate-like urease inhibitors

Non-substrate like /Mechanism based inhibitors

With this class of inhibitors, there is no close structural similarity between non-substrate like inhibitors and the enzyme's substrate (urea). Rather they are designed to interfere with the enzyme's mechanism of action leading to enzyme inactivation. The mechanism-based inhibitors are unreactive compounds with structures dissimilar to the substrate or the product of the target enzyme. Typical examples of this class are phosphorodiamidates and imidazoles (Amtul *et al*., 2002).

The enzyme converts them into reactive forms, which then inactivates the target enzyme, prior to its release from the active site.ref. Mechanism-based inhibitors have shown a lot of potential in drug design. They have a lot of advantages over competitive reversible inhibitors. This is because their steady-state concentrations do not need to be maintained in order to sustain the inhibitory effects since they are generally irreversible inhibitors.

1.2.1.6 Inhibitory concentration

The inhibitory concentration (IC) is the concentration of a substance that causes a defined inhibition of a given system. The inhibitory potential of an inhibitor is described either as the concentration of an inhibitor at which the enzyme activity falls to 50 % of its original value or as the molar concentration of the compound required to inhibit 50% of the urease activity i.e. IC_{50}. The IC_{50} values are similar to the K_i (enzyme inhibitor equilibrium dissociation constant) values; but, the correlation between these depends on the mode of inhibition, the enzyme concentration and the substrate concentration (Tsou, 1988). Thus IC_{50} values are not a good measure of efficacy.

Nonetheless, IC_{50} values are not very helpful in giving insights into the mechanism of inhibition, provided standard equilibrium conditions are reached. If the type of inhibition is also known, then the overall dissociation constant for inhibitor binding could be related to their IC_{50} values by taking into account the substrate and enzyme (Mobley and Hausinger, 1989). In contrast, the ratio of inhibitor binding(K_s) and dissociation(K_6) i.e. (K_s/K_6) is a useful measure of efficacy when comparing different inhibitors.

1.2.2 Structure of urease

In 1926, James Sumner demonstrated that urease could be isolated and crystallized in pure form, a prestigious piece of work never accomplished by any person up till at that time. His research also showed that the pure urease was a protein. This was the first investigational proof that an enzyme is a protein, which was a very controversial question at the time. The protein structure of urease from *Klebsiella aerogenes* was then first solved in 1995 by Andrew Karplus using X-ray crystal technology (Jabri *et al*., 1995).

Since then several other structures of the enzyme have been determined, including urease structures from *Helicobacter pylori* (Ha *et al*., 2001). Additional insights into the structure of urease are available from work in other organisms.

1.2.2.1 Primary structure

The urease of *H. pylori* is a two-subunit enzyme. *H. pylori* urease consists of two different structural subunits of 61.7 kDa (α) and 26.5 kDa (β), unlike most other microbial ureases that have three different structural subunits (α, β and γ) and the plant urease, jack bean urease that is a single polypeptide (Hu and Mobley, 1990). The primary structure of the enzyme is conserved among all *H. pylori* species. Evans *et al*., 1991 supposed that the *H. pylori* urease forms a huge complex whose molecular mass is estimated to be ~ 600 kDa. The enzyme has appeared under transmission electron microscopy (TEM) as a complex structure with a diameter of 13 nm that displays a three-fold rotational symmetry (Austin *et al*., 1992).

1.2.2.2 Quaternary structure

The urease of *H. pylori* is a heteropolymeric enzyme that possesses a native *M*r of 380,000 to 600,000 (Hu and Mobley, 1990). There are no differences in subunit stoichiometries in this two-subunit enzyme. The urease of *H. pylori* possesses equal numbers of each of the distinct subunit polypeptides. Though there are differences in quaternary structure between eukaryotes and prokaryotes, all urease sequences are highly conserved and the metallocenters are thought to be identical. The metal ions seem to be deeply buried in the enzyme in such a way that they are inaccessible to chelators. This raises the question of how the metals and the carbamate become incorporated into the protein.

1.2.2.3 Crystal structure

The crystal structure of *H. pylori* urease has been determined and found to be a giant 1.1 MDa , $[(UreAB)_3]_4$ supramolecular assembly of α- and β-subunits.

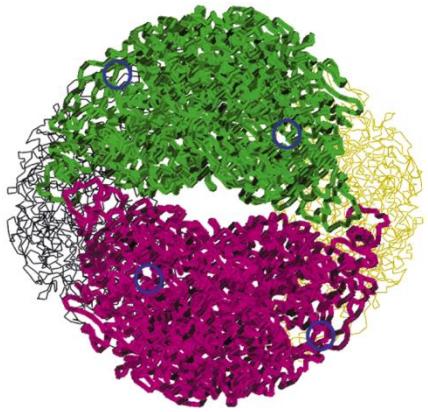

Figure 1.2 Crystal structure of *H. pylori* urease (Adapted from Ha *et al*., 2001)

Each UreAB catalytic unit contains a buried dinuclear nickel active site (Ha *et al*., 2001). Structure-based investigators have postulated that this molecular design is adapted for acid resistance allowing the survival of the enzyme at low pH. This complex enzyme structure together with the low K_m value of this enzyme is likely to allow this gastrointestinal bacterium to effectively inhabit the host niche.

1.2.3 Urease metallocenter assembly

The moment the bacterium enters the gastric mucosal lining, the assembly of the urease metalloenzyme commences. The synthesis of a catalytically active urease is a laborious process, requiring the presence of two structural proteins, nickel (Ni) ions, accessory proteins (UreEFGH), carbon dioxide (for carbamylation) and GTP.

The accessory proteins (UreFGH) are crucial for urease activity (Ferrero *et al*., 1992), while UreE delivers nickel to the urease apoprotein complex. UreG is a GTP-binding accessory protein that is part of the urease enzyme complex, functioning in the energy-dependent assembly of the holoenzyme (Moncrief and Hausinger, 1997). UreH polypeptide is thought to stabilize the apourease (Park *et al*., 1994) whereas the UreF polypeptide aids in the carbamylation of the Ni^{2+}-bridging lysine residue and prevents premature binding of Ni^{2+} to the active site (Moncrief and Hausinger, 1996). The hydrogenase accessory proteins HypA and HypB, encoded by hypA and hypB in a separate gene cluster, are needed for Ni^{2+} insertion into hydrogenase (Olson *et al*., 2001). Ni^{2+} ions are imported into *H. pylori* cells via NixA, a high-affinity metal permease. The transport protein is a 331 amino acid, 37 kDa integral membrane protein composed of eight transmembrane domains (Fulkerson *et al.*, 1998).

1.2.3.1 In vitro activation of urease apoprotein

Studies of the *H. pylori* GroES homologue carried out by Kansau and colleagues have shown that ribosomal synthesis and chaperonin-facilitated folding of the urease apoprotein UreAB initiate urease activation (Kansau *et al.,* 1996). UreAB forms complexes with the UreF, UreG, and UreH accessory proteins, encoded adjacent to the structural genes in the *ureABIEFGH* cluster (Moncrief and Hausinger, 1997, 1996; Park *et al*., 1994; Park and Hausinger, 1995; Soriano and Hausinger, 1999). Seemingly, following ribosomal synthesis and chaperonin-assisted folding of UreAB, the structure of the newly formed apoprotein is identical to that of the native enzyme, apart from lacking nickel and carbamylation.

The apoprotein subsequently forms a series of complexes with the three essential urease accessory proteins, UreFGH, already in complex with each other. Figure 1.3 shows the current understanding of the key events in cellular urease activation.

The urease apoprotein-UreFGH exhibits GTP-dependent urease activation associated with a nucleotide-binding site located on the UreG component in the presence of nickel ions. Ni^{2+} delivery to the urease apoprotein-UreFGH complex is thought to occur via the metallochaperone, UreE through interaction between UreE and UreG. It is at this point that the enzyme complex becomes fully activated. The CO_2, Ni-UreE, and GTP-dependent activation process results in production of the urease holoprotein and dissociation of the accessory proteins.

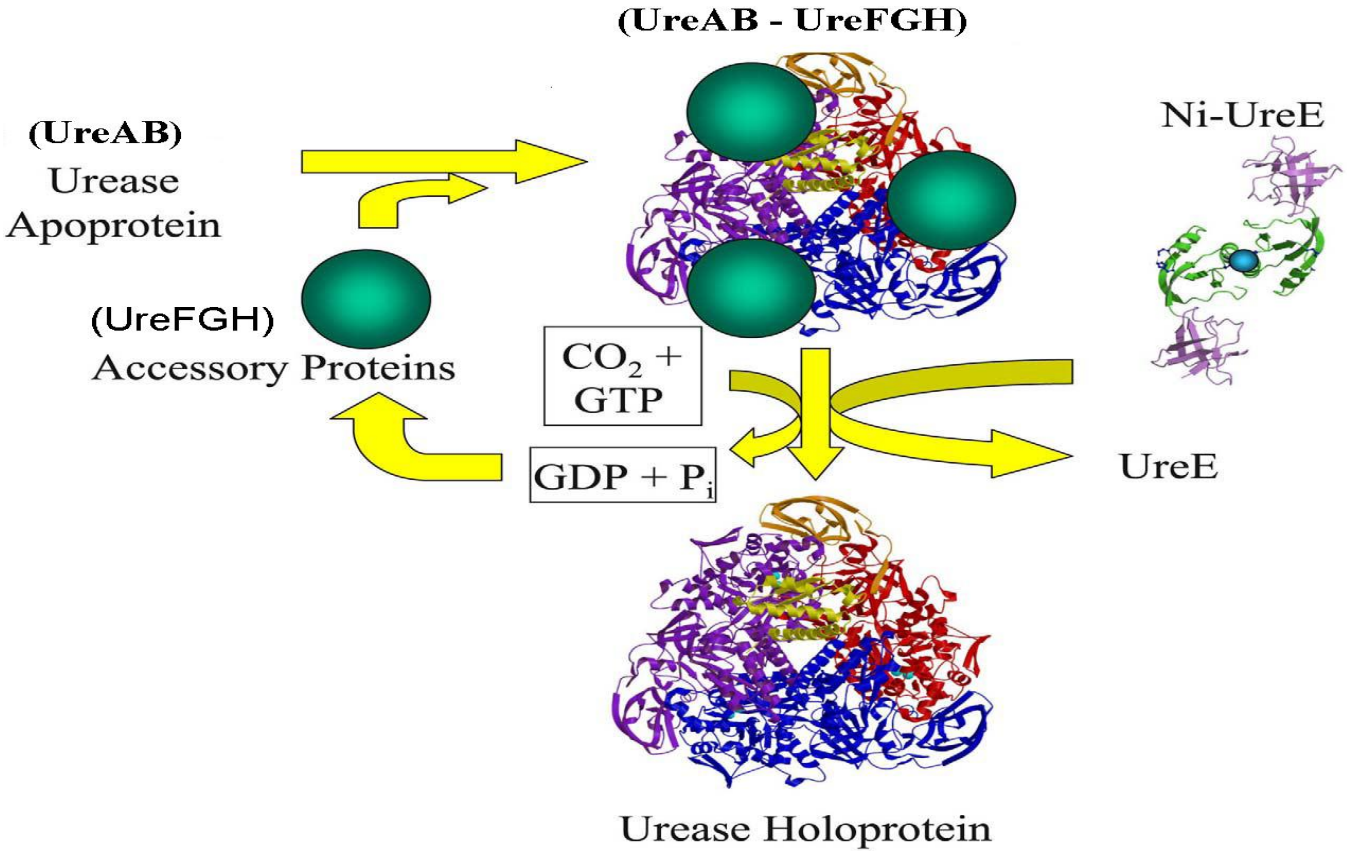

Figure 1.3 Model for synthesis of active *H. pylori* urease (Mulrooney and Hausinger, 2003).

1.2.4 Active-site studies of urease

Site-directed mutagenesis experiments (Martin and Hausinger, 1992; Park *and* Hausinger 1993; Sriwanthana *et al*., 1993), apoprotein activation studies (Park *and* Hausinger 1995), and structural determinations by X-ray crystallography (Jabri *et al*., 1995) have given a comprehensive view of the urease catalytic center.

H. pylori urease had previously been considered to be a hexamer on the basis of size-exclusion chromatography. The *H. pylori* urease is apparently a dodecamer protein, consisting of α and β-subunits (Ha *et al*., 2001). The active site of this enzyme has been found to be located in the UreB subunit.

It consists of amino acid residues found throughout the primary structure that have been brought into proximity in the tertiary structure (Jabri *et al*., 1995). The enzyme is a metalloenzyme whose active site possesses two nickel ions in the β-subunit correctly positioned 3.5 Å apart. The amino acid residues of *H. pylori* UreB subunit: His-136, His-138, Lys-219, His-248, His-274, and Asp-362 strongly coordinate with the nickel ions and come in direct contact with the urea and a water molecule within the active site. His-322 is near the active site and in close proximity to the ions acting as a general base in the catalysis. The active site of the *H. pylori* urease contains seven histidine residues and one cysteine residue which also aid in the binding of the nickel ions (Labigne *et al*, 1991). Figure 1.4 shows a close view around the active site of the urease. Red is the histidine residue, Yellow: Cys 321 (aa 123 of UreB), Blue ball: nickel ion. There are seven histidine residues and one cysteine residue around two nickel ions. His 136, 138, 248, and 274 strongly coordinate with the nickel ions. His 221 and 323 locates very close to the ions. Cys 321 is also present in a close position to the ions.

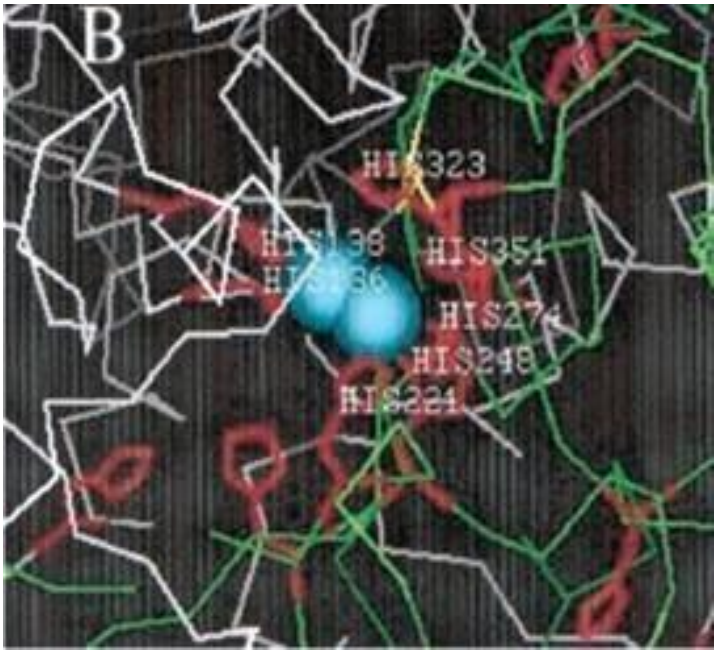

Figure 1.4 Active site of *H. pylori* urease (Adapted from Fujii *et al*., 2004).

The structure of *H. pylori* urease was determined by Ha *et al*. (2001). However, the knowledge on this urease active site was provided by the crystal structures resolved for bacterial urease from *Klebsiella aerogenes* (Chang *et al*., 2004).

1.2.4.1 Urease mechanism

The mechanism for urease catalysis follows the scheme proposed by Zerner and colleagues for the plant urease (Dixon *et al.,* 1980). The ligands to the bi-nuclear metallocenter in the urease include a bridging lysine carbamate, a partially bridging water molecule, four histidine imidazoles with two bound to each nickel ion, and an aspartic acid residue bound to one nickel ion (Jabri *et al*., 1995, Park and Hausinger 1993, Park and Hausinger 1995, Wang *et al.,* 1994). Urea is suggested to bind in O-coordination to one nickel ion facilitated by His-221, resulting in polarization of the urea carbonyl group. A carboxylate group has been suggested to stabilize a particular tautomer of the bound substrate.

The amino acid residue His-219 not shown in the figure is thought to further stabilize substrate binding. Nucleophilic attack on the urea carbonyl by the metal-coordinated hydroxyl group gives rise to a tetrahedral intermediate that decomposes to carbamate with elimination of ammonia. The water then displaces the carbamate completing the cycle. His-320, which is in close proximity to the ions acts as a general base by activating the water molecule (or hydroxyl group) which is coordinated to the second nickel ion (Mobley *et al*., 1995).

1.3 PHYSIOLOGY OF UREASE

The complete genomic sequence of *H. pylori* (Tomb *et al*., 1997) has given further insight into the studies of physiology and metabolism of this interesting pathogen. Several investigations have shown that *H. pylori* lacks most of the biosynthetic pathways generally found in less specialized bacteria, such as many enteric bacteria (Doig *et al*.,1999; Tomb *et al*., 1997). Genomic comparisons and metabolic studies have further deduced that *H. pylori* has a metabolic route which lacks biosynthetic pathways for some amino acids.

So, *H. pylori* only grows in chemically defined medium supplemented with amino acids arginine, histidine, isoleucine, leucine, methionine, phenylalanine and valine, and some strains also require alanine and/or serine (Nedenskov, 1994; Reynolds and Penn, 1994). *H. pylori* has the capacity *to* catabolize glucose. Genomic and metabolic reports show that *H. pylori* cannot utilize any other sugars either fermentatively or oxidatively. However, the pathogen exhibits glucose kinase activity, which is associated with cell membrane-bound enzymes for the pentose phosphate pathway (Doig *et al*., 1999; Dunn *et al*., 1997). The lack of genes encoding the corresponding enzymes for these metabolic routes has been implicated with such biochemical deficiencies (Berg *et al*., 1997; Kelly, 2001).

1.3.1 Urease regulation

Genes encoding urease of *H. pylori* have been amplified, cloned and sequenced. Urease has till recently been regarded as a constitutive and permanently active enzyme. However, recent advances have shown that urease activity of this gastrointestinal pathogen is subtly modulated at different levels.

Regulatory mechanisms

i. The expression of the UreA and UreB structural subunits is regulated by acidity and the nickel-responsive transcriptional regulator, NikR.

ii. Ni^{2+} incorporation mediated by the urease accessory proteins, UreEFGH (expression modulated by pH-dependent mRNA stability), and two of the hydrogenase accessory proteins, HypA and HypB

iii. The availability of Ni^{2+} in the cytoplasm is influenced by the NixA importer (the expression of which is down-regulated under acidic conditions) and the amount of histidine-rich proteins such as Hpn (the expression of which is up-regulated by NikR) and possibly by HspA.

iv. The availability of urease substrate, urea, which is controlled via the acid-gated UreI urea channel (Stingl and De Reuse, 2005).

1.3.2 Enzyme location

The outer membrane of this pathogen has an extraordinary property; it has the ability to incorporate urease which is strictly cytoplasmic in other bacteria (Hawtin *et al*., 1990). Interestingly, considering that urease is a large oligomeric protein (Austin *et al*., 1992) one would normally not expect it to traverse the bacterial outer membrane.

Cryo-immunolocalization techniques have shown that the enzyme is strictly cytoplasmic in early log-phase growth of *H. pylori*. At the end of the log-phase, a significant fraction of this protein becomes surface associated or extracellular (Phadnis *et al*., 1996).

Other proteins of this pathogen are known to have specific secretion pathways, but the mechanism whereby urease, become associated with the cell surface is controversial. Amazingly, unlike other bacterial ureases which are cytoplasmic, *H. pylori* urease is exported. Also noteworthy is that the export of these ureases requires the presence of a nucleotide consensus leader sequence which is absent from both *H. pylori* urease subunits (Clayton *et al*., 1990). In species of Enterobacteriaceae, this lack of a consensus leader sequence results in confinement of urease in the cytoplasm. This implies that this bacterium may possibly have a distinct pathway for the export of urease subunits. It will be of startling interest to establish whether this pathway is limited to the urease subunits or is generally applicable to *H. pylori* proteins. Studies by Cussac *et al*. 1992 and Ferrero *et al*. 1992 have suggested that since the urease of this pathogen lacks leader peptides, alternative transport mechanisms must exist. Investigations by Phadnis *et al*. 1996 have shown that urease of *H. pylori*, is an intrinsic cytoplasmic protein in log-phase bacteria. This protein becomes surface-associated when it is secreted from the cytoplasm by autolysis.

1.3.3 Urea availability

The liver is an organ that synthesizes urea which is found in serum, saliva, and gastric juice at low concentrations (about 10 mM). However, it is excreted at high concentrations (400 to 500 mM) in urine (Griffith *et al*., 1976).

The arginase (RocF)-mediated hydrolysis of L-arginine to L-ornithine also supplies urea to *H. pylori* though in little amounts (McGee *et al*., 1999). Weeks *et al*. 2000 demonstrated that the protein, UreI seems to form a urea-specific pore in the cytoplasmic membrane. This channel regulates urea availability to cytoplasmic urease by opening at low pH and closing at high pH.

1.3.4 Use of ammonia generated by urea hydrolysis

Ammonia generated by urea hydrolysis is assimilated into protein and other nitrogenous compounds by a single pathway (Reitzer and Magasanik, 1987). Glutamine synthetase (EC 6.3.1.2) catalyzes the reaction:

$$NH_3 + \text{glutamate} + ATP \xrightarrow{\text{Glutamine synthetase}} \text{glutamine} + ADP + P_i$$

Glutamine produced serves as nitrogen donor for other nitrogenous compounds. i.e. histidine, serine, tryptophan, AMP, and glucosamine 6-phosphate. It is postulated that glutamine synthetase is crucial for viability and nitrogen assimilation in *H. pylori* (Garner *et al*., 1998). To confirm this, glutamine synthetase-deficient mutants generated by allelic exchange could not be isolated.

1.3.5 Nickel transport

Generally each active site of urease contains two nickel ions. Since *H. pylori* urease has six active sites then it has 12 nickel ions. *H. pylori* has a high-affinity system to acquire nickel ions which is adapted to overcome the nickel limitation that probably occurs in the host. Ni^{2+} ions are thought to be transported into *H. pylori* by at least two mechanisms.

i. A cytoplasmic membrane-bound protein, NixA which transports nickel ions with a K_T of 11.3 nM (Mobley *et al*., 1995). Topology studies by Fulkerson and Mobley (2000) revealed that the protein has eight transmembrane domains and a periplasmic loop with both the N terminus and C terminus residing in the cytosol. Negatively charged residues of Glu, Asp and His which are located in the transmembrane domains were found to be crucial for active transport of nickel ions (Fulkerson *et al*., 1998).

ii. A mutant in which *nixA* was insertionally inactivated showed a significant decrease in both the rate of nickel transport and urease activity following culture *in vitro* (Bauerfeind *et al*., 1996). Since nickel transport and urease activity were not fully abolished in *nixA* mutants, these findings puts forward the possibility of a second mechanism of nickel transport that has yet to be elucidated.

1.3.6 Metal-binding proteins that may affect urease activity

Bauerfeind *et al*. 1996 showed that catalytic activity of urease requires Ni^{2+} ions, nevertheless; a *H. pylori* nixA mutant still retained some urease activity though at a reduced rate. These findings showed that auxiliary proteins are likely to be involved in nickel binding or transport. There are proteins of *H. pylori* that have been identified with predicted nickel-binding or general metal-binding properties.

i. Hpn, a protein of 60 amino acid residues long with a molecular size of 7.1 kDa (Gilbert *et al*., 1995). Histidyls alone constitute 47% of the protein's residues making Hpn a strong binder of nickel and zinc ions. So Hpn might be used to activate urease.

ii. A P-type ATPase of 686-amino acid residues long known as CadA has been identified in *H. pylori* (Melchers *et al*., 1996). It contains segments rich in histidine, methionine, glutamate, and aspartate residues at the N terminus which may act as a nickel-binding domain. This ATPase has been revealed to be a heavy metal ion (Cd^{2+}, Zn^{2+}, Co^{2+}) exporter which exploits ATP as an energy source for transport. Mutation of the gene encoding the CadA appears to diminish but not abolish urease activity. These findings thus suggest that this protein contributes to high levels of urease activity by exporting divalent metal cations that may interfere with Ni^{2+}-metalloenzyme formation (Herrmann *et al*., 1999).

iii. HspA (heat shock protein A), a protein of 118 amino acid residues long has been identified in *H. pylori*. Mutation of the gene encoding the HspA appears to be lethal for *H. pylori*. For this reason, the role of this heat shock protein in urease activity cannot be tested directly in the native organism. However, expression studies of hspA in E. coli have resulted in slight increase of urease activity, signifying that HspA may possibly affect urease activity in wild-type *H. pylori* (Suerbaum *et al*., 1994).

1.4 MOLECULAR PATHOGENESIS

1.4.1 Urease as virulence factor in gastrointestinal infections

A urease-positive bacterium, *H. pylori*, discovered 28 years ago, has remained the most prevalent infectious agent in the world. Urease is central to the virulence elicited by this bacterium. The involvement of this metalloenzyme in gastritis and peptic ulceration has been well documented (Cover and Blaser, 1995).

Helicobacter pylori urease has been identified as a novel virulence factor, leading to the production of pro-inflammatory cytokines. This protein could thus be a future target of therapy since there is no ideal first-line eradication of *H. pylori* infection.

1.4.2 Urease as an antigen

Urease, the most prominent protein component of *H. pylori* (Dunn *et al*., 1997), serves as a powerful immunogen for this organism (Czinn *et al*., 1989). Patients with active gastritis due to *H. pylori* show significantly elevated immunoglobulins G and A along with urease in serum when compared with preinfected levels. Enzyme-linked immunosorbent assay systems have been developed to measure these immune responses (Perez-Perez and Dunn, 1989). Such tests are useful for diagnosing acute infection, monitoring level of *H. pylori* during the antibiotic therapy and for epidemiological studies.

1.4.3 Avoidance of host defense

Urease is one of the bacterial factors critical for *H. pylori* colonization of the gastric mucosa (Eaton *et al*., 1991). *H. pylori* is relatively sensitive to low pH *in-vitro* (Hazell and Lee, 1985), unless urea is present (Marshall *et al*., 1988). The colonization of the gastric mucosa with a low pH (i.e. pH ≤ 3) would be intricate for the organism unless it has the capacity to protect itself from the hostile acid conditions.

It appears that the bacterium hydrolyzes urea to ammonia, which neutralizes acid and allowing survival and initial colonization. To confirm this, urease-negative mutants of *H. pylori* have been constructed by chemical mutagenesis (Perez-Perez and Dunn, 1989; Hazell and Lee, 1985); by selection of naturally occurring mutants (Marshall *et al*., 1988); by allelic exchange of *in-vitro* constructed deletion mutations (Blaser *et al*., 1990).

1.4.4 Direct toxicity to the host

Ammonium hydroxide generated by urea hydrolysis has been found to contribute significantly to histologic damage. The damage actually results from the hydroxide ions produced by the equilibration of ammonia with water. Studies to demonstrate the role of urease in the cytotoxic effects on human gastric adenocarcinoma cell line have been done (Hazell and Lee, 1985). Cell viability was established to be inversely proportional to ammonia concentrations generated by urea hydrolysis. However, cell viability was enhanced when the urease inhibitor acetohydroxamic acid (AHA) was incubated with the culture before the exposure to *H. pylori*. Acetohydroxamic acid was shown to slow down the release of ammonia and reduce the cytotoxic effect. These findings suggested that the histological damage observed might be a direct result of localized ammonia generated by urea hydrolysis (Hazell and Lee, 1985).

1.4.5 Host damage induced by the immune response

This enzyme may damage the gastric epithelium via its interaction with the immune system. Suzuki *et al*. 1992 demonstrated that *H. pylori* cells have the ability to stimulate an oxidative burst in human neutrophils.

When rabbit fetal gastric mucosal cells were incubated with neutrophils, *H. pylori* and urea, shrunken gastric cells were observed which evidenced cytotoxicity. In another study, urea-free medium incubated with the inhibitor AHA no cytotoxicity effects were observed.

These findings suggested that H_2O_2 from the oxidative burst in neutrophils causes the oxidation of the chlorine ions which then react with ammonia generated by urea hydrolysis to give the highly toxic monochloroamine.

The *in vitro* cytotoxicity activity of urease towards gastric epithelial cells could be mimicked by the addition of monochloroamine to the cells.

The enzyme itself was shown to activate monocytes and polymorphonuclear leukocytes and stimulate them to produce inflammatory cytokines. The secreted inflammatory cytokines play a role in mediating the inflammatory response in the gastric epithelium. This recruitment of inflammatory response cells thus leads to an in indirect damage to the gastric epithelial cells (Harris *et al*., 1996).

1.5 DIAGNOSTIC TESTS FOR *H. PYLORI* INFECTION

Since *H. pylori* is implicated in several gastrointestinal conditions, the demand for treatment of infection, and the interest for reliable diagnostic tests have increased tremendously. However, the choice of test depends on issues such as cost, availability, clinical situation and factors such as the use of use of antibiotics which may influence the test results. The diagnostic tests are classified as either invasive or non-invasive.

Invasive tests are those that require an endoscopic biopsy of gastric mucosa i.e. culture, histological examination, rapid urease test, polymerase chain reaction (PCR) of biopsy samples and Fluorescence *in situ* hybridization (FISH). Under non-invasive tests are serology, ^{13}C- and ^{14}C- urea breath tests or stool antigen assays, dental plaque and saliva PCR assays.

1.5.1 Invasive techniques

1.5.1.1 Culture

H. pylori is cultured in a variety of selective (Skirrow's supplement) and non-selective (chocolate agar) commercially available media (Mobley *et al*., 2003). The criterion should be followed as close as possible because sensitivity can be affected by sampling methods, sub-optimal laboratory conditions, delayed transport of biopsy samples or even exposure to air (Moayyedi and Dixon 1998). There are, however, clear drawbacks that should be considered i.e. to culture *H. pylori* is complicated, time-consuming and endoscopy is costly, and requires trained and motivated personnel. However, it is the only method which permits determination of antimicrobial susceptibility (Buckley and O'Morain, 1998).

1.5.1.2 Histology

Histologic analysis of biopsy samples is common and accepted all over the world as the "gold standard" for accurate diagnosis of *H. pylori* infection. Histopathology in *H. pylori* identification also allows for the determination of the degree of inflammation, and the presence/absence of MALT-lymphoma and gastric cancers in patients (Hardin and Wright, 2002). This method is generally more sensitive than culture and it allows direct visualization of organism and extent of the nature of tissue involvement. However, limitations can arise if inadequate numbers of biopsy specimens are obtained, or if there is a failure to obtain specimens from different areas of the stomach (Hardin and Wright, 2002).

1.5.1.3 Rapid urease test

The basis of this test is that *H. pylori* produces urease in abundance. In an *H. pylori* infected patient, urease is produced beneath the gastric mucus layer where it is protected from luminal acid and remains fully active. If urease is present in the specimen it will cleave the urea into ammonia and carbon dioxide. The ammonia released results in pH elevation which is detected and shown by a colour change in a pH indicator. The test is inexpensive, fast, widely available, accurate and easy to perform. However, the sensitivity of the test depends on the bacterial load in the stomach (Burette, 1998).

1.5.1.4 Polymerase chain reaction

Polymerase chain reaction (PCR) is useful in research, particularly in molecular epidemiology and finger printing *H. pylori* isolates. With PCR, *H. pylori* can be identified in small samples with only a few bacteria present (Hardin and Wright, 2002). If the target sequence is present in the sample tissue then *H. pylori* DNA will be amplified. PCR assays have shown high sensitivities and specificities on DNA fragments from the ureA, ureC, and 16SrRNA. Like any method there are drawbacks i.e. only few laboratories can carry out such assays, and test accuracy is easily affected by the target DNA and choice of primers, specimen preparation, bacterial density, and technical issues regarding the PCR protocol (Dunn *et al*., 1997).

1.5.1.5 **Fluorescence *in situ* hybridization (FISH)**

This method is an established non-culture dependent molecular technique that simultaneously detects the presence of *H. pylori*. The technique is used to detect and localize the presence or absence of specific DNA sequences on chromosomes through hybridization or fluorescence-labeled DNA probes to denatured chromosomal DNA (Demiray *et al*., 2006).

FISH uses fluorescent molecules to brightly mark genes on chromosomes. Short sequences of single stranded DNA, called probes, which are complementary to the DNA sequences that researchers wish to mark and examine are prepared. These probes hybridize to the complementary DNA. Since the probes are labeled with fluorescent tags this enables researchers to spot the location of those sequences of DNA (Demiray *et al*., 2006).

There are many advantages of FISH over other diagnostic tests. Firstly, the use of the rRNA-targeted fluorescence-labeled oligonucleotide probes for determination of antibiotic susceptibility gives the clinician essential information needed to make a proper treatment recommendation (Demiray *et al*., 2006). Secondly, FISH is a highly sensitive, reliable, specific and fast technique for the diagnosis of *H. pylori* infection such that test results are available within 3 h after an endoscopy. Thirdly, the probes used are commercially available, and the technique can be applied in any laboratory without the need for special equipment and facilities, apart from a fluorescence microscope (Demiray *et al*., 2006).

1.5.2 Non-invasive testing

Several non-invasive methods for detection of *H. pylori* infection are available like serology, urea breath test, and stool antigen test.

1.5.2.1 Serology tests

These tests are based on the fact that infection of the gastric mucosa with *H. pylori* results in immune responses. The responses include elevated levels of specific IgA and IgG in serum and elevated levels of secretory IgG and IgM in the stomach (Dunn *et al*., 1997). There are a variety of serological tests known to date, however most tests available commercially are enzyme-linked immunosorbent assays (ELISA)

(Dominguez–Bello *et al*., 2001). These tests are however less reliable and are not recommended for clinical use. Although these tests are fast, simple and relatively inexpensive none of the tests for IgM and IgA to diagnose *H. pylori* infection have been officially approved.

The sensitivity and specificity of these tests have been too low for them to be recommended. Non-specific cross-reactions also give false positive results. As of such it is recommended that antibody tests be used only as screening tests, and that a positive or negative result depending on the state of the patient being examined should be confirmed (Mobley *et al*., 2003).

1.5.2.2 Stool antigen testing

H. pylori infection can be identified by detection of *H. pylori* protein antigens (HpSA) in stool. Enzyme immunoassay kits are used for the detection of these protein antigens. The stool antigen test is of high importance as a diagnostic test in children because it is easy to perform, reliable and non-invasive (Van Doorn *et al*., 2001). Because of the fastidious nature of *H. pylori*, culturing the bacterium from stool has proven tedious and with a very low yield. The high sensitivity of PCR allows detection of the organism even when it is present in low numbers. However, a major drawback of this diagnostic technique is the high incidence of false negative results which are caused by inhibition factors that are present in stool (Van Doorn *et al*., 2001).

1.5.2.3 Urea breath test (UBT)

Although *H. pylori* itself cannot be detected noninvasively, its urease activity can be detected by way of a breath test just like with the rapid urease test. In order to detect gastric urease, urea that is radioactively labeled with ^{13}C and ^{14}C- is ingested.

If *H. pylori* urease is present, urea is split into NH^{4+} and $^{13}CO_2$ or $^{14}CO_2$, which can be detected in the breath. NH^{4+} ions are sequestered in the acidic stomach and isotope labelled $C0_2$ enters the bloodstream where it is carried to the lungs and rapidly expired.

1.5.2.4 ^{14}C – urea breath test

For this diagnostic test a certain amount of expired CO_2 (1 or 2 mmol) is captured in a solution that a carbon dioxide trapping agent called hyamine hydroxide. This hydroxide is dissolved in either ethanol or methanol, and has thymolphthalein added to it as a pH indicator (Henze *et al*., 1990). Sample readings are taken on a liquid scintillation counter. This test is less costly and yet more sensitive than the ^{13}C-UBT, however, though radiation doses are relatively low, it remains inappropriate for diagnosis in children and pregnant women.

1.5.2.5 ^{13}C – urea breath test

With this test, a patient fasts overnight and a breath sample of 75-200 mg of 13C-urea is given to patient simultaneously with a test meal. Breath sampling is done at various time intervals. The amount of labelled CO_2 liberated as a result of *H. pylori* urease activity is quantified as the increase in $^{13}CO_2/^{12}CO_2$ ratio.

Determining the $^{13}CO_2/^{12}CO_2$ ratio by isotope dilution mass spectrometry can be very difficult due to the high precision required. However, this test has a very high sensitivity and specificity when compared to other non-invasive tests and it is the only non-invasive test that can detect active *H. pylori* infection. It is the considered the ideal test to determine if treatment was successful in killing the pathogen. Not like serology, UBT is extremely accurate in children (Koletzko and Feydt-Schmidt, 2001).

1.6 TREATMENT AND CONTROL

The colonization of the stomach by *H. pylori* causes peptic ulcer disease, gastric adenocarcinomas and gastric mucosa–associated lymphoid tissue (MALT) lymphomas (Chong *et al*., 2008). The mechanism by which *H. pylori* stimulates peptic ulcer disease is partly understood but is thought to mainly involve a combination of virulence factors of the organism (e.g., VacA and CagA proteins), genetic predisposition of the host, mechanical injury to the mucosa, and alterations of gastric secretions (Cohen, 2000).

H. pylori infection presents an extraordinary therapeutic challenge. Efforts to establish optimal treatment regimens have been thwarted by the fact that the pathogen lives in an environment (acidic stomach) not easily accessible to many therapeutics. Presently, it is accepted that *H. pylori* infection is the major culprit in peptic ulcer disease and for eradication; most clinicians use a triple drug (at least two antibiotics and either bismuth or a proton pump inhibitor) or even quadruple drug approach. A proton pump inhibitor is administered in combination with clarithromycin or amoxicillin and metronidazole twice daily. These drugs synergize to eradicate the bacterium (Malfertheiner *et al*., 2000).

In South Africa, the treatment regimen contains clarithromycin, amoxicillin, metronidazole and doxycycline and supposedly should exhibit 85 - 95% efficacy (Aboderin *et al*, 2007). Several antibiotics are thus used worldwide to treat *H. pylori* infections, but emerging bacterial antibiotic resistance presents an added challenge and continues to plague attempts to eliminate these infections. This problem is a very serious public health concern in Africa where there is high prevalence of multidrug-resistant strains (Asrat*et al*., 2004; Lwai-lume *et al.* 2005; Ndip *et al*., 2008).

Bacterial resistance against the different antibiotics used is common in many countries but varies between countries and may even change with time and geographical location (Ndip *et al*., 2008).

Moreover, these treatment regimens have put up substantial problems such as high cost, adverse side effects and poor compliance amongst patients often leading to relapse. (Ndip *et al*., 2008). These, therefore, result in significant levels of treatment failure and contraindications amongst patients. In view of these challenges and other obstacles there is need for novel therapeutic approaches to prevent or eliminate *H. pylori* infections using natural products, which have desired anti-bacterial properties but at the same time are relatively non-toxic, cheap and have less or no side effects.

This has led to the re-evaluation of the therapeutic use of honey which is a widely used medicine worldwide for the treatment of different disorders, including gastrointestinal diseases. Several authors have previously reported that honey inhibit the activity of bacteria *in vitro* and *in vivo*, and thus possess therapeutic potential (Somal *et al*., 1994; Ali, 1995). Studies with different models of experimentally induced acute and chronic gastric lesions in rats have shown the molecular mechanisms of honey (Mobarok Ali, 2003).

The antibacterial properties of honey have been attributed to hydrogen peroxide, osmotic effect, low pH, low protein content and non-peroxide factors like flavonoids and phenolic acids (Malika *et al*., 2004). The concentration of these non-peroxide components is usually low in the honey but on extraction with organic solvents they become more concentrated and thus exhibit increased levels of activity (Aljadi and Yusoff, 2003). Consequently, we investigated the effect of honey on *Helicobacter pylori* urease activity.

1.7 HONEY

All the perceived health concerns and genuine limitations with antibiotic use that we just described have led to many studies to find alternative treatments. Recently, there has been substantial interest in the therapeutic use of honey as an alternative treatment. Natural products such as honey have been previously reported to have antibacterial activity. Honey is a substance that has been used as traditional medicine in a number of customs for decades (Ransome, 1937). The first research which demonstrated the antibacterial action of honey was undertaken in 1892 by the Dutch scientist Van Ketel. Nowadays, researchers have proven that there are several compounds present in honey that possess antibacterial activity (Waikato Honey Research Unit 2005).

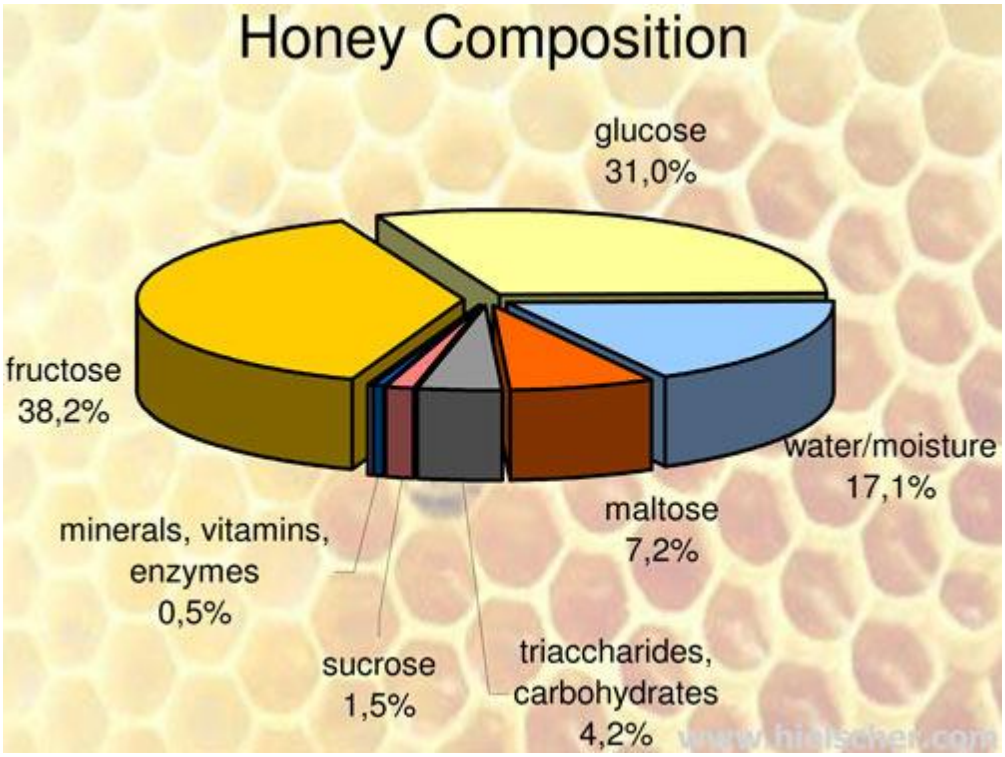

Figure 1.5 The chemical composition of honey (Source: www.hielschr.com)

Honey is a highly concentrated, complicated mixture of carbohydrates, of which the monosaccharides form by far the greater part. The composition of honey is very complex and differs widely according to its source. It contains more than 70% sugars and less than 20% water. Because of the relatively low water content, the caloric value of honey is very high. The overabundance of sugar makes honey unstable.

1.7.1 Mechanism of action

The mechanism of antimicrobial action of honey is apparently a combination of many different modes of action (Molan, 1992a). This antibacterial activity is attributed to its high osmolarity, low pH, low water activity, hydrogen peroxide content and other uncharacterised compounds.

1.7.1.1. Glucose oxidase and hydrogen peroxide

Glucose oxidase (GOx) secreted by bees is an oxido-reductase that catalyses oxidation of glucose to form gluconic acid and hydrogen peroxide (H_2O_2) (Fig. 9) (Meyer and Wohlfahrt, 2006). H_2O_2 is the main antimicrobial agent responsible for the antibacterial activity shown in most types of honey (Airborne Honey, 2003). The glucose oxidase activity is generally measured by the production of H_2O_2 and is highly variable between different types of honey (Airborne Honey, 2003). The concentration of H_2O_2 in honey varies and this results in major variations seen in overall antibacterial activity of honey (National Honey Board, 2006). Light, room temperature, a small amount of visible light and heat (> 50°C) have been found to reduce GOx activity (Airborne Honey, 2003).

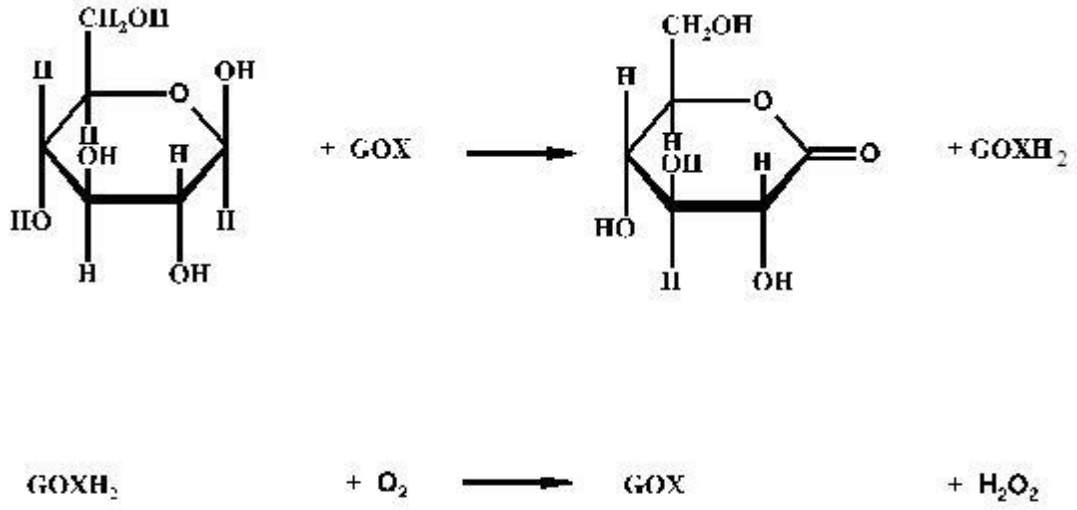

Figure 1.6 The enzymatic reaction catalysed by glucose oxidase (Adapted from Meyer and Wohlfahrt, 2006)

H_2O_2 was initially hailed for its antibacterial and cleansing properties when it was first introduced into clinical practice. In more recent times it has lost favor because it damages tissue and causes inflammation. However, the H_2O_2 concentration produced in honey when diluted is typically around 1 mmol/l, which is around 1,000 times less strong than the 3 % solution commonly used as an antiseptic solution but is equally effective. It reduces inflammation and does not damage tissue cells. The harmful effects of H_2O_2 are further reduced because honey sequesters and inactivates the free iron which catalyses the formation of oxygen free radicals produced by $H_2O_2$and its antioxidant components help to clear up oxygen free radicals.

1.7.1.2. Gluconic acid

Other than carbohydrates, honey contains a variety of amino acids (0.05-0.1%) and organic acids (0.57%). Formic, acetic, butyric, citric, formic, gluconic, lactic, maleic, oxalic, pyroglutamic and succinic acids have all been identified in honeys (Nelson and Mottern, 1931). Gucollic, α-ketoglutaric and pyruvic acids have been found in honeys as well (Malika *et al*., 2004). Honey is mildly acidic with its pH being between 3.2 and 4.5 (National Honey Board, 2006). The main organic acid present in honey is gluconic acid ($C_6H_{12}O_7$) that is formed when the first carbon of glucose is oxidised (National Honey Board, 2006). The antibacterial activity of honey has been shown to be also due to its acidity level. Investigations have shown that it can inhibit most bacteria, among them *Helicobacter pylori*, *Escherichia coli*, *Salmonella spp*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* (National Honey Board, 2006).

Many studies have been done on the antibacterial activity of honey on *Helicobacter pylori*, but there is no information available on the antiurease activity of honey. Therefore, one aim of this project was to investigate if honey is an effective inhibitor against urease activity.

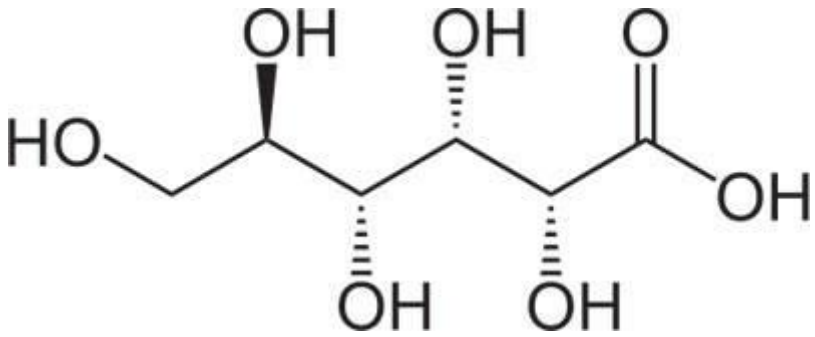

Figure 1.7 The chemical structure of D-Gluconic acid (Adapted from Wikipedia 2006e).

1.7.1.3. Osmotic effect

Honey is a supersaturated solution of two monosaccharides (glucose and fructose) with a low water activity and high osmolarity. This mixture has a low osmotic pressure since most of the water molecules bind with the sugars leaving little available to support the growth of microorganisms. Therefore, the high osmolarity is a valuable property in the treatment of established infections, because it prevents the growth of bacteria (Malika *et al*., 2004).

Manuka honey is a high quality honey made in New Zealand by bees which gather nectar only from the flowers of the manuka bush. The manuka tree (*Leptospermum scoparium)* is a New Zealand native plant well-known for its medicinal properties (Honey New Zealand, 2006). The H_2O_2 antibacterial property of the active manuka honey has higher effectiveness compared to other honeys (Molan, 1992).

Studies by Allen *et al.* (1991) on 345 samples of New Zealand honeys from 26 different floral sources showed that when catalase was added to any of these honeys to destroy the H_2O_2, only manuka honey retained a significant amount of antibacterial activity. This meant that manuka honey has an additional bioactivity that is not present in other honeys.

This unique activity was first discovered by Dr. Peter Molan from the Waikato Honey Research Unit (Healing Honey, 2005). This bioactivity was named Unique Manuka Factor (UMF). It is very stable towards light, heat and body enzymes (Honey New Zealand, 2006) and it is also resistant to heat (Waikato Honey Research Unit, 2005). The UMF activity subsists in addition to the antibacterial properties of H_2O_2. Collectively, these two factors thus behave synergistically (Waikato Honey Research Unit, 2005).

Studies have established that UMF effectively inhibits the growth of a wide range of bacteria, such as *Helicobacter pylori, Staphylococcus aureus, E. coli* and *S. pyogenes* (API Health Ltd, 2006).

1.8 THE PROPOSED INVESTIGATION

1. The discovery of a potent and safe *H. pylori* urease inhibitor has been and remains a very important area of pharmaceutical research because of the cascade of surprisingly simple biological processes initiated by this urease-producing pathogen in the gastric mucosa. In view of that, the importance of urease inhibitors as drugs is enormous given that these molecules can be used for treating *H. pylori* infections. Nonetheless, up to present, no inhibitor has been approved as a drug for use in clinical trials. We will therefore, focus our efforts on to investigate new *H. pylori* urease inhibitors with maximum therapeutic impact and minimal side effects. This avenue of research is likely to provide answers to many other questions of these inhibitors particularly focusing on their type and mechanism of inhibition at the molecular level. Moreover, this might as well help deduce the urease mechanism of action further.

2. Several excellent techniques are available to assess urease activity. In this work, our approach was to screen new urease inhibitors via a continuous spectrophotometric assay which uses glutamate dehydrogenase to couple ammonia release to reduced nicotinamide adenine dinucleotide oxidation.

1.9 PREVIOUS WORK DONE IN THE RESEARCH GROUP

In previous years, three locally produced honeys (Pure honey, Citrus blossom and Gold crest) from different regions in South Africa were screened for anti-*H. pylori* activity at four different concentrations using the agar well diffusion technique (Manyi *et al*., 2010). All honeys and their solvent extracts demonstrated anti-*H. pylori* activity and were most active at 75% v/v. Chloroform extract recorded the lowest MIC_{95} values that ranged from 0.156-5% v/v confirming this extract to be the most active. These antibacterial properties were attributed to presence of potential compounds with therapeutic activity that could be further exploited as lead molecules in the treatment of *H. pylori* infections (Manyi *et al.*, 2010).

The observation that these honeys can inhibit the growth of *H. pylori* is an important finding. However, no studies in the world have been reported the antiurease activity of honey and its organic solvent extracts on *H. pylori* isolates, bearing in mind that the organism is ubiquitous worldwide, responsible for significant morbidity and mortality (Samie *et al*., 2007) with an increasing trend in antibiotic resistant strains (Tanih *et al*., 2010). Therefore, a survey of different honey varieties (Pure honey and Manuka honey) of different geographic origin may reveal a honey with considerable antiurease activity since honeys of different geographic origin have different antimicrobial activities (Ndip *et al*., 2007; Basson and Grobler, 2008). For this reason, in this study, the main aim was to assess and compare the inhibitory potential of Pure honey and Manuka honey extracts on the urease activity of *H. pylori* isolates from South Africa. This could give light to identification of organic compounds in honey that are derived from numerous biosynthetic pathways which contribute in the aromatic properties of the honey in addition to its floral and geographical origin determination.

1.10 HYPOTHESIS

Urease plays an important role in the biological activity of *H. pylori*. Its inhibition will suppress *H. pylori* activity, consequently reducing the infection.

1.11 OBJECTIVES OF THIS RESEARCH

The following principal objectives guided the investigation:

a. To isolate and study the enzymatic activity of *H. pylori* urease.

b. To determine the inhibitory effects of 'Pure honey'(South Africa) and Manuka honey (New Zealand) extracts on the activity of *H. pylori* urease

c. To investigate and compare the inhibitory effect of two different varieties of honeys of different geographic origin on *H. pylori* urease

d. To purify and characterize the *H. pylori* urease enzyme

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Chapter 2 : PRODUCTION AND ISOLATION OF *H. PYLORI* UREASE

2.1 INTRODUCTION

2.1.1 Bacterial growth

When bacteria are grown in a batch culture, four phases of growth are typically observed:

a) Lag phase - Instantly after inoculation of bacterial cells into fresh medium, the cell population remains conditionally unchanged as cells initially adjust to the new medium. During this phase cells increase in mass or volume, synthesize enzymes, RNA as metabolic activity increases but there is no apparent cell division occurring. The length of the lag phase is apparently dependent on several factors such as inoculums size, bacterial strain, culturing conditions, time necessary to recuperate from shock encountered in the transfer; time required for synthesis of division factors (Todar, 2004).

b) Exponential (log, logarithmic) phase – This physiological state is marked by rapid division of cells by binary fission as growth increases by geometric progression. The rate of cell division is dependent on growth medium composition and the incubation conditions.

c) Stationary phase – Classically defined as a physiological state where the rate of cell division equals the rate of cell death, thus viable cell number remains constant. Population growth becomes limited by: exhaustion of available nutrients, accumulation of inhibitory metabolites and/or end products and lack of "biological space".

d) Autolysis (death, exponential/logarithmic decline) phase – If bacterial cells are incubated beyond the stationary phase, a death phase follows, in which the viable cell population starts to decline as lysozymes are released into the cytoplasm. During this physiological state, the number of viable cells decreases exponentially.

2.1.2 Production of urease by *H. pylori*

When bacteria grow under specific conditions they produce the highest percentage of the enzyme of interest. Enzymes are usually classified as inducible or constitutive. Inducible enzymes are produced in response to the availability of a homologous substrate in the culture medium (Todar, 2004). These enzymes are only produced when they are needed. Conversely, constitutive enzymes are produced in constant amounts without regard to the physiological demand or the concentration of the substrate. Therefore, they are not controlled by induction or repression and are present in relatively the same concentration in cells at all times. The ability of plants, bacteria, fungi, algae and invertebrates to synthesize urease (urea amidohydrolases, EC 3.5.1.5) has been studied extensively. The ureases though of different protein structures, they are all nickel-containing metalloenzymes that have a single catalytic function, urea-hydrolysis to its final products, ammonia and carbonic acid (Karplus *et al*., 1997; Sumner, 1926).

Amongst the several ureolytic bacteria, of special interest to the scientific community are those that are pathogenic to humans and animals. The pathogenesis is due to the end products of urea hydrolysis, which bring about pH elevation (up to about 9.2) and the toxicity of the released ammonia and its derivatives (Burne and Chen, 2000). The substrate urea for the reaction is readily available as a nitrogenous metabolic waste product of most terrestrial animals with a serum concentration in humans of about 1–10 mM (Burne and Chen, 2000).

This makes urea readily available to ureolytic bacteria making the intestinal tract one of the most common sites of ureolytic bacteria infections in humans (Burne and Chen, 2000). *H. pylori* is the principal ureolytic bacterium infecting the gastrointestinal tract (Burne and Chen, 2000). The bacterium colonizes the mucosal lining of the stomach, a highly acidic environment which is considered the first line of defense against most gastrointestinal pathogens. It survives in this hostile environment by synthesising vast quantities of urease (accounting for about 10% of the total protein content of the cell) which hydrolyses urea into ammonia and $C0_2$, which generate a pH neutral micro-environment (Bauerfeind *et al*., 1997).

H. pylori appears unique in that it has the ability to incorporate urease in its outer membrane which is strictly cytoplasmic in other bacteria (Hawtin *et al*., 1990). Interestingly, considering that urease is a large oligomeric protein (Austin *et al*., 1992) one would normally not expect it to traverse the bacterial outer membrane. Though so evident that urease is located on the surface of late log phase *H. pylori*, the mechanisms whereby surface-association occurs have not been clearly elucidated.

2.1.2.1 H. pylori urease as a potential therapeutic target for the treatment of gastric infections

The urease of this gastrointestinal pathogen is a conspicuous biochemical marker which can be targeted for intervention therapy. In light of this, urease inhibitors have attracted much attention as potential new antiulcer drugs since the virulence of the pathogen can be controlled using a substance that inhibits this enzyme's activity (Eaton *et al.,* 1991).

2.2 Materials and methods

2.2.1 Media

All media were suspended in distilled water, mixed well and dissolved by heating with frequent agitation. Boiling was then carried out for about one minute to allow complete dissolution. The media was then sterilized in an autoclave at 100 kPa (121°C) for 15 minutes.

2.2.2 Buffers

The preparation of all buffers is explicitly mentioned in Appendix A.

2.2.3 Source of micro-organism

Two strains of *Helicobacter pylori*, a standard ATCC 43526 (American Type Culture Collection, Manassas, VA, USA) and one clinical isolate (*H. pylori* 369C) cultured from gastric corpus biopsy specimen of a patient with gastroduodenal disease attending the endoscopy unit of Livingston hospital, Port Elizabeth, Eastern Cape Province were used for this study. Primary isolation of the clinical strains was performed on Columbia Blood Agar (Oxoid Ltd, Basingstoke, Hants, UK) supplemented with Skirrow's antibiotics (Appendix B) and de-fibrinated horse blood 7% (v\v). The plates were incubated microaerophilically (85% N_2, 10% CO_2 and 5% O_2) (Helico-Campy Pack gas-generating envelopes, Oxoid Ltd, Basingstoke, Hants, UK) at 37^{o}C for 5 days. *H. pylori* bacteria cells were identified according to colony morphology, Gram staining, microaerophilic growth at 37°C, and confirmed by the presence of oxidase, catalase, and urease. *H. pylori* reference strain, ATCC 43526 was used as positive control.

2.2.4 Time course survey

For the intracellular and extracellular enzyme determinations to be meaningful, it was necessary to standardize on an incubation time that would give as maximum levels of the enzyme as possible. The strains were grown in brain heart infusion broth supplemented with 10% heat-inactivated horse serum for culture times ranging from 24-96 hours at 37^{o}C under microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2) (Helico-Campy Pack gas-generating envelopes) on a rotary shaker at 120 rpm as previously described by Shibata *et al* (2003). The levels of intracellular and extracellular ureases were evaluated in aliquots removed from 24 hr, 48 hr, 72 hr, and 96 hr old cultures by monitoring the enzyme activity as established by Kaltwasser *et al.* (1966). The cells that gave the highest urease activities were then used for all subsequent experiments in order to prepare the crude cell-free extracts.

2.2.5 Crude Urease preparations

The *H. pylori* cells were harvested by centrifugation (5000 g, 4^{o}C and 20 mins) and used for isolation and purification of the intracellular enzyme; whereas the supernatant was used for the isolation and purification of the extracellular enzyme.

2.2.5.1 Extracellular crude urease

Broth cultures (3 Litres) of 2.0 x 10^8 CFU/ml were centrifuged (5 000g, 4^{o}C and 10 mins). The pellet and supernatant were separated and designated PLT1 and SNT1 respectively. SNT1 was assayed for protein and extracellular urease activity. The pellet (PLT1) was washed twice with phosphate-buffered saline (pH 7.4) and used for intracellular urease isolation.

2.2.5.2 Intracellular crude urease

H. pylori cells (17.530 g wet weight) (PLT) were resuspended in 100 ml buffer, supplemented with Complete, Mini, EDTA-free Protease Inhibitors (Roche Diagnostics, South Africa). The suspended cells were divided into 20 Falcon tubes. Bacterial cell disruption was achieved using an Omni Sonic Ruptor Ultrasonic Homogenizer (Omni International, Kennesaw, USA) on an ice bath. Following centrifugation at 25,000 g for 20 mins at 4^{o}C to remove the cell debris, the supernatant obtained was designated SNT 2. This cell free extract was assayed for protein concentration and intracellular urease activity.

2.2.6 Urease activity and protein determination

2.2.6.1 Protein Determination

Protein concentrations were determined according to Bradford (Bradford, 1976) with bovine serum albumin as a standard (Appendix C). The method relies on the shift in maximum absorbance from 465 to 595 nm when Coomassie Brilliant Blue G- 250 binds to the protein in acid solution.

2.2.6.2 Urease Assay

Urease activity was assayed spectrophotometrically by measuring the reduction in NADH in the coupled urease-glutamate dehydrogenase (GDH) system using the protocol established by Kaltwasser *et al.* (1966).

$$\text{Urea} + H_2O + 2H^+ \xrightarrow{\text{Urease}} 2\,NH^{4+} + CO_2$$

$$2NH^{4+} + 2\alpha\text{-ketoglutarate} + 2NADH \xrightarrow{\text{GLDH}} 2\text{Glutamate} + 2NAD^+ + 2H_2O$$

The assay cocktail was made up by pipetting the following: 0.1M potassium phosphate buffer, pH 7.6 (1.2 ml), 0.023M ADP (0.05 ml), 0.0072M NADH (0.05 ml), 0.026M α-ketoglutarate (0.05 ml), 1.8M Urea (0.05 ml) and glutamate dehydrogenase, 500 Units/ml (0.05 ml) into a cuvette. The assay cocktail was equilibrated at 25°C for 10 min prior to the addition of the enzyme preparation. The decrease in NADH absorbance was recorded at 340nm every 30 s for 8 minutes.

Enzyme Activity Calculation

The change in absorbance per minute was plotted over the linear range of the curve and was used in the calculation of urease activity. One enzyme unit was defined as that amount of urease that catalyzes the oxidation of 1 µmol of NADH per minute at 25°C and pH 7.6. Enzyme activity and specific activity were calculated according to equations below.

$$\text{Units/ml} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank})\ (\text{Volume of assay, ml})}{(6.22)\ (\text{Volume of enzyme used, ml})}$$

$$\text{Units/mg} = \frac{\text{Units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

6.22 : extinction coefficient of NADH at 340 nm

The decrease in absorbance at 340 nm, due to the oxidation of NADH, is proportional to the ammonia concentration. The ammonia produced reacts with α-ketoglutaric acid and NADH in the presence of glutamate dehydrogenase to form glutamate and NAD^+. Glutamate dehydrogenase reacts specifically with ammonia. Specific activity of urease was calculated as µmol of urea hydrolysed per min/mg of protein.

2.2.7 Enzyme location

H. pylori cells (17.5303 grams wet weight) were harvested as described earlier in section 2.2.3. The pellet and supernatant were separated and designated PLT1 and SNT1 respectively. SNT 1 and PLT 1 were assayed for protein concentration and urease activity. The pellet was then re-suspended in distilled water and then disrupted by sonication as described earlier. Cell debris designated (PLT 2) and cell free extract designated (SNT 2) were obtained by centrifugation (25,000 xg, 20 mins, 4^{o}C). PLT 2 and SNT 2 were also assayed for protein concentration and urease activity.

2.2.8 Optimization of cytoplasmic urease extraction

H. pylori cells were harvested as described earlier in 2.2.5.1 to yield a pellet (17.5303 grams wet weight). The pellet was washed twice with phosphate-buffered saline (pH 7.4) and then re-suspended in buffer. The cells were divided into 10 tubes of equal volumes. Five tubes were subjected to sonication on ice at an amplitude of 40W for times ranging from 1 – 5 minutes. The sonicated samples were centrifugated (15,000 xg, 20 mins, 4^{o}C) and the cell free extracts obtained were tested for protein concentration and urease activity. The other five tubes were subjected to a sonication time of 5 minutes for power amplitudes ranging from 10 – 50W. The sonicated samples were centrifuged and the cell free extracts obtained were assayed for protein concentration and urease activity as described earlier.

2.2.9 Statistical analysis

Throughout the entire study, the test results and data were recorded and analyzed using the SAS software Version 9 statistical package. The differences were considered significant when the *p* value was less than 0.05.

2.3 Results and Discussion

2.3.1 Time course survey

A time course survey to determine the highest cytoplasmic and extracellular urease activities during the growth of *H. pylori* was carried out. The highest cytoplasmic urease activity was observed at 72 hr (late exponential phase) (Figure 2.1).

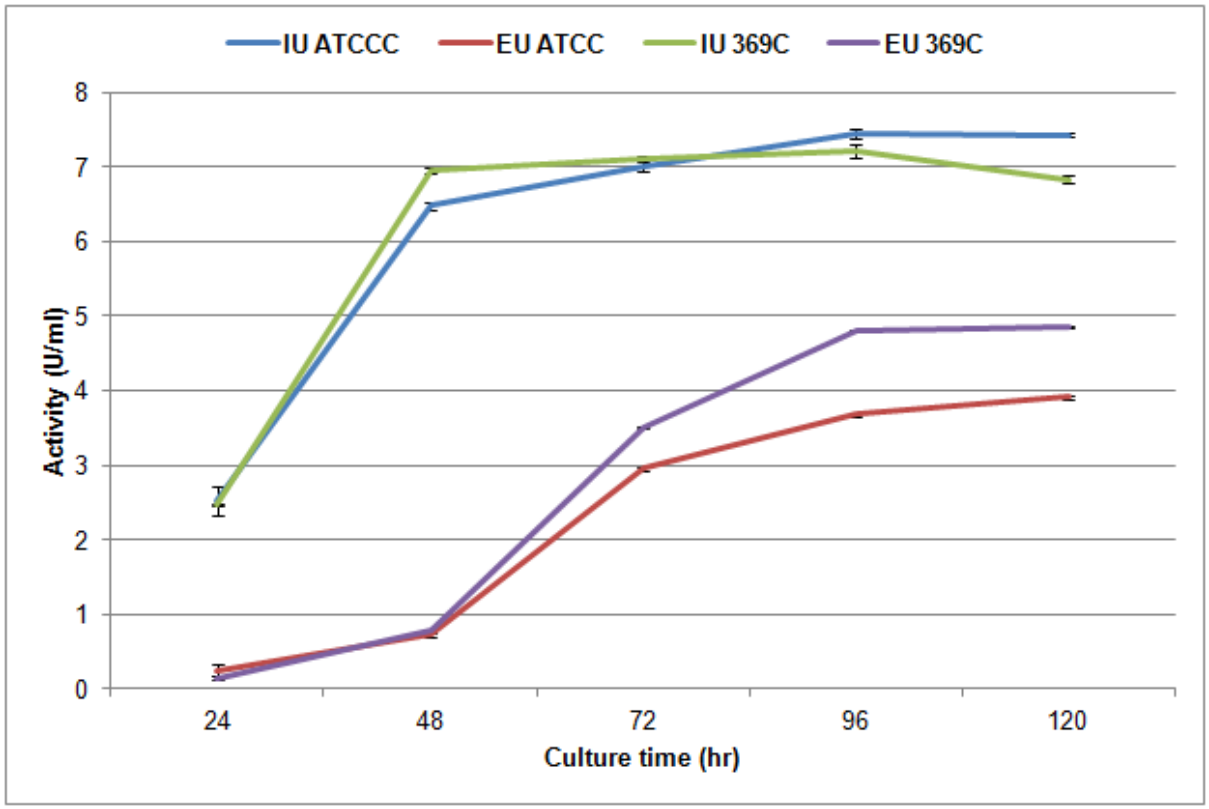

Figure 2.1 Production of cytoplasmic and extracellular urease by *H. pylori* over time. IU means intracellular urease whereas EU means extracellular urease.369C – clinical isolate. ATCC – reference strain. The data are the average of three experiments and bars indicate standard deviations.

These findings are similar to previous observations which showed that urease is only localized in the cytoplasm during the exponential growth phase in *H. pylori in vitro* cultures (Phadnis *et al*., 1996).

The highest extracellular urease activity was observed at 96 hr (Figure 2.1) and this corresponds with the stationary phase of *H. pylori* growth (Phadnis *et al*., 1996). *H. pylori* cultivated for 96 hrs demonstrated significant amounts of extracellular urease activity, probably due to significant amounts of surface-bound enzyme. These observations indicate that there is extracellular release of cytoplasmic urease with aging cultures. This dependence of urease release on the phase of growth in vitro appears to imply that the mechanism of autolysis process is regulated genetically (Mobley, 2001). The importance of autolysis in this bacterium's pathogenesis remains an intriguing hypothesis to explain its adaptation to an acidic environment.

Probably, older cultures are more virulent than the young ones. If that is the case then this indicates that autolysis is sensitive to the phase of growth in *H. pylori*. These observations potentially represent an important mechanism for *H. pylori* to modulate its environment within the host.

2.3.2 Production of protein during *H. pylori* growth

The protein concentrations in the extracellular fraction (SNT 1) and intracellular fraction (SNT 2) were evaluated in aliquots removed from 24h, 48h, 72h, and 96h cultures by the Bradford assay. A typical profile for biosynthesis of proteins during bacterial growth was observed (Figure 2.2). Maximal production of protein was obtained during incubation time of 24 – 72 hours.

This culture time corresponds to the exponential growth phase of *H. pylori* (Phadnis *et al*., 1996), in which bacterial cells are increasing hastily as cell proteins (amino acids, enzymes, transporter molecules, etc) are synthesized. From the observed fluctuations we are to think that there is a regulatory pathway or mechanism that controls protein levels within bacterial cells at any given time during their lifetime.

This mechanism will likely ensure that the cells produce proteins that are necessary for the immediate use at a particular time and those proteins not required at that particular time are not produced. This corresponds to work by Todar (2004) who illustrates that bacterial cells have developed complex mechanisms for the regulation and expression of enzymes.

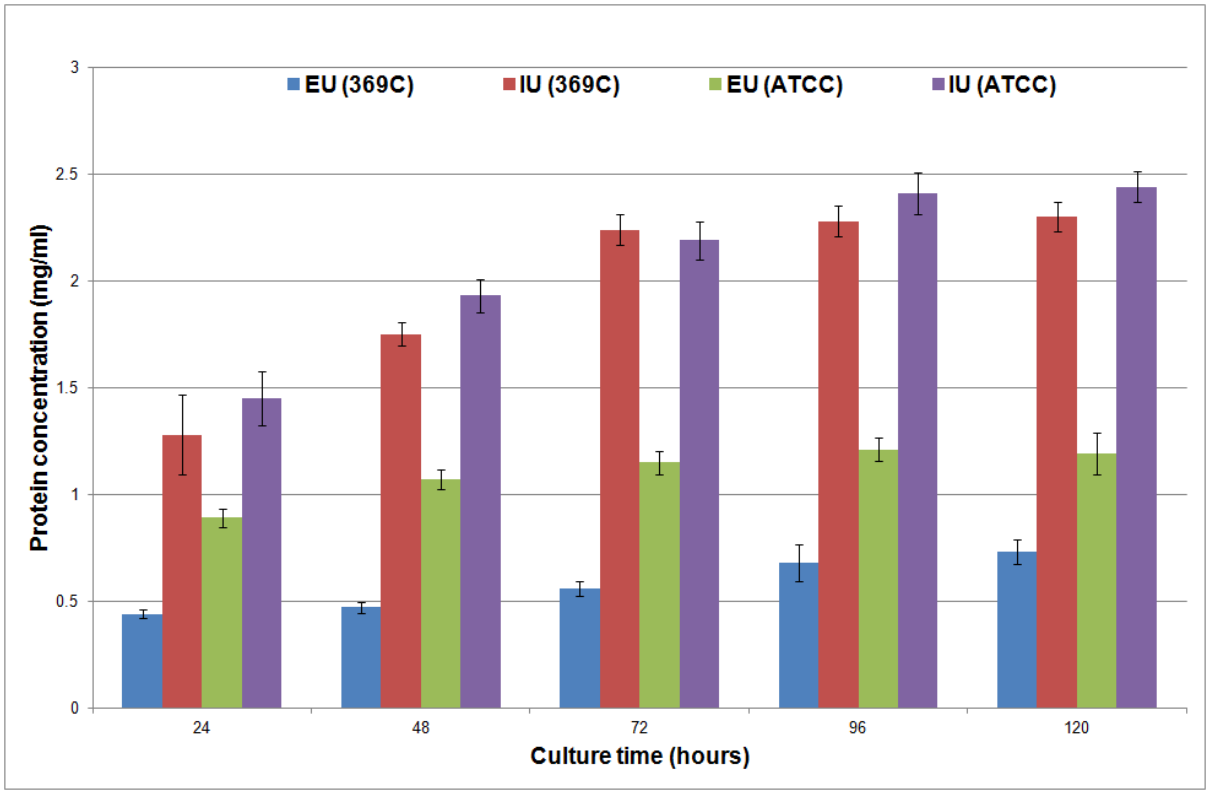

Figure 2.2 Production of protein by *H. pylori* over time. IU means intracellular urease whereas EU means extracellular urease.369C – clinical isolate. ATCC – reference strain. The data are the average of three experiments and bars indicate standard deviations.

2.3.3 Localization of urease enzyme

To allow comparison of the urease activity of the different cell fractions, enzyme activity was expressed as a comparative percentage. The whole pellet fraction was considered as 100% since it represents the activity of the cell in its natural state. Analysis revealed urease activity in both *H. pylori* strains; urease activity was associated with both the cell surface and the cytoplasm. Urease activity showed no correlation with localization of activity.

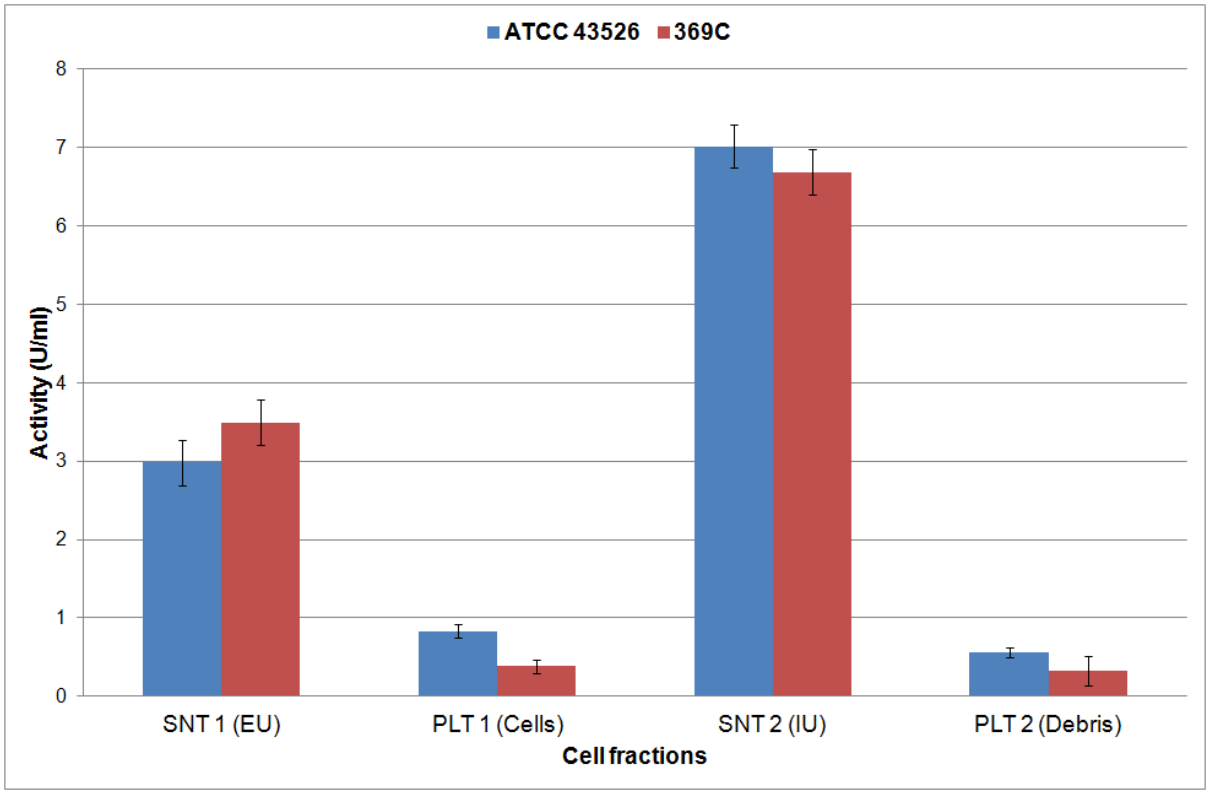

Figure 2.3 Activity of urease enzyme (72 hr culture) in different cell fractions. SNT1=Supernatant before sonication; PLT1= Control (pellet before sonication); SNT2= supernatant after sonication; PLT2= pellet after sonication. 369C – clinical isolate. ATCC 43526 – reference strain. The data are the average of three experiments and bars indicate standard deviations.

Our results demonstrate that *H. pylori* urease is not uniform in all *H. pylori* strains. Because of the differences in activity we are to think that localization of urease activity may account for different virulence activities among the strains.

2.3.4 Optimization of cytoplasmic urease extraction by sonication

Optimising enzyme release by sonication is very important. According to the Omni Sonic Ruptor Ultrasonic Homogenizer (Omni International, Kennesaw, USA) Manual, a power of 50W is the extreme and 40W is recommended for sonication of bacterial cells. In this study, sonication was done at 4ºC and the cells were suspended in buffer.

As presented in the graph, a power setting of 40W was used and the exposure time to the ultrasound was varied between 1-5 minutes. A time setting of 5 minutes was also chosen and kept constant whilst the power applied was varied between the two extremes (10- 50W).

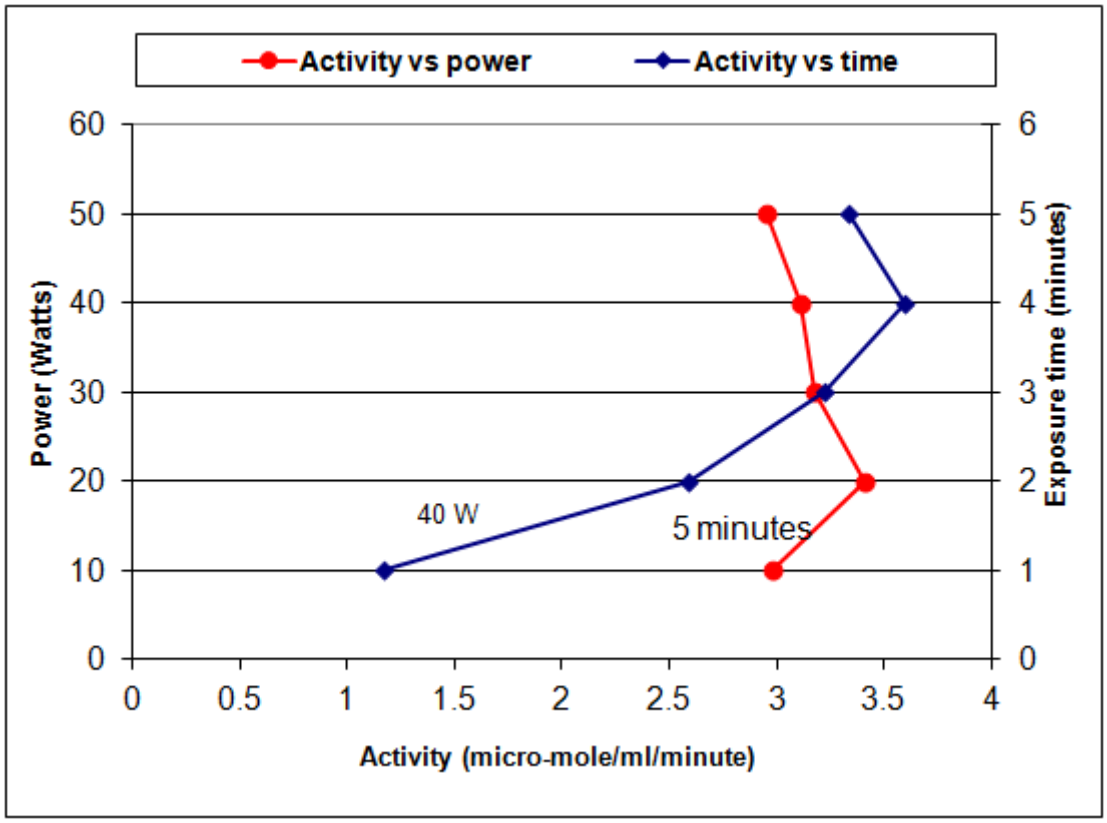

Figure 2.4 Sonication effects on urease activities. The data are the average of three experiments.

The sonication conditions that resulted in the most significant urease activity were found to be amplitude of 40W and a time of 4 minutes. A power setting of 20W and exposure time of the cells for 5 minutes also gave significant enzyme activity though slightly lower (Figure 2.7). From enzyme activity values presented above it shows that doubling the power from 20W to 40W and increasing exposure time from 4 minutes to 5 minutes only increases urease activity by about 5.6%.

% difference = (3.6 - 3.41)/3.41*100%

= 5.6 %

For this reason, the best sonication conditions were thought to be amplitude of 20W and a time of 5 minutes. In any case it would be better to choose a lower amplitude as to minimize the risks of enzyme denaturation as heat is generated during the process which would reduce the sonication efficiency.

We also found some observation of interest. The recommended machine input is 40W for bacterial samples and the urease activity is also highest at 40W implying that there could be a resonance effect. Resonance is when the frequency of oscillations of the input signal equals the natural frequency of the substance being investigated.

2.4 Conclusions

In summary, the following conclusions were deduced from this chapter:

a. Maximum extracellular urease activity is attained on culturing for 96 hr.

b. Maximum intracellular urease activity is attained on culturing for 72 hr.

c. Maximal production of protein is obtained during the exponential phase of *H. pylori* in broth cultures (24 – 72 hr).

d. The significant urease activity observed after sonication of the cells indicates that the enzyme is mainly located in the cytoplasm.

e. Sonication conditions that release the greatest amount of enzyme into the supernatant are a power of 20W and a time of 5 minutes but however denaturation of enzyme cannot be ruled out.

The findings of the optimal time at which maximum urease is produced by *H. pylori* led to the experiments conducted in Chapter 3 and Chapter 4.

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Chapter 3 : PURIFICATION AND CHARACTERIZATION OF *H. PYLORI* UREASE

3.1 Materials and Methods

3.1.1 Materials

The lyophilized urease of specific activity ≥ 5 Units/mg protein was obtained from Merck (South Africa (Pty) Ltd). The Glutamate dehydrogenase (GLDH) from bovine liver of specific activity 40 Units mg−1 protein, NADH, ADP, α-ketoglutaric acid, urea and acetohydroxamic acid were all obtained from Sigma-Aldrich Co. (South Africa (Pty) Ltd). All the chemicals were of analytical grade and were prepared in 0.10 M Phosphate buffer, pH 7.6. Protease inhibitors (Complete mini EDTA-free) were purchased from Roche Diagnostics (Pty) Ltd, South Africa. Horse serum was from Oxoid Ltd (Hampshire, UK). Dialysis tubing (cellulose membrane, 10 kDa MWCO), SDS-PAGE standard markers (Prosieve QuadColor Protein ™ Markers; 4.6 – 300kDa, Maine, USA), Brilliant Blue R-Staining Solution (B-6529), acrylamide, N, N - methylene-bis-acrylamide were also purchased from Sigma-Aldrich Co. (South Africa (Pty) Ltd).

3.2 Purification methods

3.2.1 Bacterial Culture

Two strains of *Helicobacter pylori*, a standard ATCC 43526 and one clinical isolate (*H. pylori* 369C) cultured from gastric corpus biopsy specimen of a patient with gastroduodenal diseases attending the endoscopy unit of Livingston hospital, Port Elizabeth, Eastern Cape Province were cultured and harvested as previously described (Sections 2.2.3).

3.2.2 Harvesting of cells and preparation of cell free extracts

Cell free extracts (CFE) were obtained as described earlier in section 2.2.5 of Chapter 2 and purified.

3.2.3 Urease activity and protein determination

3.2.3.1 Protein Determination

Protein concentrations in the cell free extracts and purified enzyme fractions were determined in replicates according to Bradford (Bradford, 1976) with bovine serum albumin as a standard as previously described (Appendix C).

3.2.3.2 Urease Assay

Urease activity was assayed spectrophotometrically by measuring the reduction in NADH in the coupled urease-glutamate dehydrogenase (GDH) system as described earlier in Section 2.2.6.2 of Chapter 2.

3.2.4 Cell-free extract concentration

Concentration of the crude extract is necessary prior to protein purification using chromatographic techniques, in order to increase protein content and decrease the volume. Two extract concentration methods were employed in this study. The cell-free extract (100 ml) was divided into two equivalent parts designated CFE1 and CFE2; CFE1 was concentrated by ammonium sulfate precipitation and CFE2 by freeze drying. Several attempts were made in this study before best results could be obtained.

3.2.4.1 Ammonium Sulfate Precipitation

CFE1 (50 ml) was precipitated by slow addition of solid ammonium sulfate to 70% saturation (Appendix E) with constant stirring on a magnetic stirrer at room temperature over a period of 30 min. The precipitates were separated by centrifugation (10 000 g, 4°C and 30 min). Then the precipitated proteins were re-suspended in 10ml of 0.1M potassium phosphate buffer (pH 7.6) and dialyzed against excess of the same buffer. This dialysate was designated as ammonium sulphate fraction (ASF) and was analyzed for protein concentration and urease activity.

3.2.4.2 Concentration by freeze-drying

CFE2 (50 ml) was transferred into a freeze-drying flask and frozen by gently swirling the flask in acetone. The freeze-drying flask was then transferred onto a freeze dryer (New Brunswick Scientific Co, New Jersey, USA) and the extract freeze dried at - 70°C overnight. The powdered extract was weighed and re-suspended in 6 ml of potassium phosphate buffer (0.1 M, pH 7.6) (1:2 w/v) and assayed for protein and urease activity. This fraction was designated as freeze-dried fraction (FDF).

3.2.4.3 Dialysis

The two concentrated samples were sealed in dialysis tubing (Pierce Biotechnology, Rockford, Illinois, USA) and dialyzed overnight in 10 times sample volume potassium phosphate buffer (0.1M, pH 7.6) to ensure complete removal of ammonium sulfate and contaminating salts. The samples were assayed for protein and urease activity.

3.2.5 Chromatographic instrument

A fast protein liquid chromatography, AKTA-FPLC system (pump P-900, monitor UPC-900 and fraction collector Frac-900) with ultra-violet (UV), conductivity and pH sensors was used. Protein detection was done by reading absorbance at 280nm. The system was controlled by Unicorn software version 5.1 installed on the computer. Sample injection was done using a sample loop (Pharmacia). Fractions were collected automatically according to obtained UV reading peak values.

3.2.6 Column chromatography

All purification steps were carried out at room temperature (ca. 25°C), and the urease activity of every fraction collected was evaluated as described in Section 3.2.6.2. The purified enzyme fraction was obtained after one-step procedure described next.

3.2.6.1 Size-exclusion chromatography on Superdex 200 pg

Concentrated samples (4 ml each) from ammonium sulphate precipitation and freeze-drying were applied separately onto a HiLoad 16/60 Superdex 200 pg (GE Healthcare). The column was pre-washed with gel-permeation buffer [1X: (50mM NaH_2PO_4 , 150mM NaCl, 1 mM EDTA); pH 7] and equilibrated until A_{280} of elute had reached baseline. The flow-rate was set at 1 ml /min and samples were eluted with the same buffer. Fractions (1 ml) eluted at 1 ml min^{-1} were screened for urease.

3.2.6.2 Urease Activity Selection

After the chromatography run, each fraction (20 µl) was mixed with testing buffer (300 µl) composed of 3 mM NaH_2PO_4, 110 mM urea, and 7 µg ml^{-1} phenol red adjusted to pH 7.6 in a 96-well microtiter plate using a multichannel pipette. To blank wells, chromatography buffer (20µl) was added. The plate was incubated at 37ºC.

Quantitative determination of the absorbance at 560 nm was done in a microplate reader (SynergyMx, BioTek Instruments, USA) by serial measurement at different time points for up to 20 min.

3.2.6.3 Urease specific activity determination

Determination of protein content in the fractions was done according to Bradford (Bradford, 1976) with bovine serum albumin as a standard as previously described (Appendix C). Urease specific activity was determined spectrophotometrically using the coupled urease-glutamate dehydrogenase (GDH) system as described earlier in Section 2.2.6.2 of Chapter 2.

3.2.7 Determination of purity

To monitor the progress of purification of the enzyme, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on samples exhibiting urease activity. Samples (25 µl) from each purification step and a standard molecular weight maker (4.6 – 300 kDa, Merck, South Africa) were applied on 12% SDS-PAGE (Appendix D) and electrophoresed at 200 V for 50 minutes. The gels were stained with Coomassie Brilliant Blue R-250 Staining solution (Appendix D), then destained in acetic acid, methanol and water (1:3:6 v/v/v) destaining solution overnight (Appendix D). The distance moved by the partially purified protein was measured and its corresponding molecular weight calculated from the calibration curve of log molecular weight versus distance migrated (Appendix D).

3.3 Results and Discussion

3.3.1 Purification of Urease

Each concentrated extract was filtered through a 0.45 µm-pore-size filter. Each filtrate was then subjected to size-exclusion chromatography on a HiLoad 16/60 Superdex 200 pg column, which has a separation range of M_r 10 000 - 600 000, and a V_o of 40 ml. The fractions were screened for urease. The fractions that were urease-positive were pooled and assayed for activity.

The purification of urease from our isolate, *H. pylori* 369C was compared with the purification of urease from the reference strain, *H. pylori* ATCC 43526. In attempting to concentrate and purify urease we employed freeze-drying and fractional precipitation with ammonium sulphate. The effect of these two concentration methods on urease activity was thus investigated. Several attempts to concentrate the 369C urease with ammonium sulphate precipitation resulted in significant loss of enzyme activity; as a result freeze drying was the preferred method for the purification of the 369C. On the other hand the ATCC 43526 urease that was concentrated by fractional precipitation with ammonium sulphate had higher enzyme activity than the ATCC 43526 urease concentrated with freeze-drying thus it was chosen for purification of the ATCC 43526.

3.3.1.1 Size-exclusion chromatography of 369C enzyme extract after freeze-drying

The chromatogram in Fig. 3.1 starts with the injection of the sample and shows the absorbance at 280 nm. The maximum urease activity was found in the first peak (45.57 ml retention). The other contaminating peaks apparently indicative of several other different proteins were not further characterized.

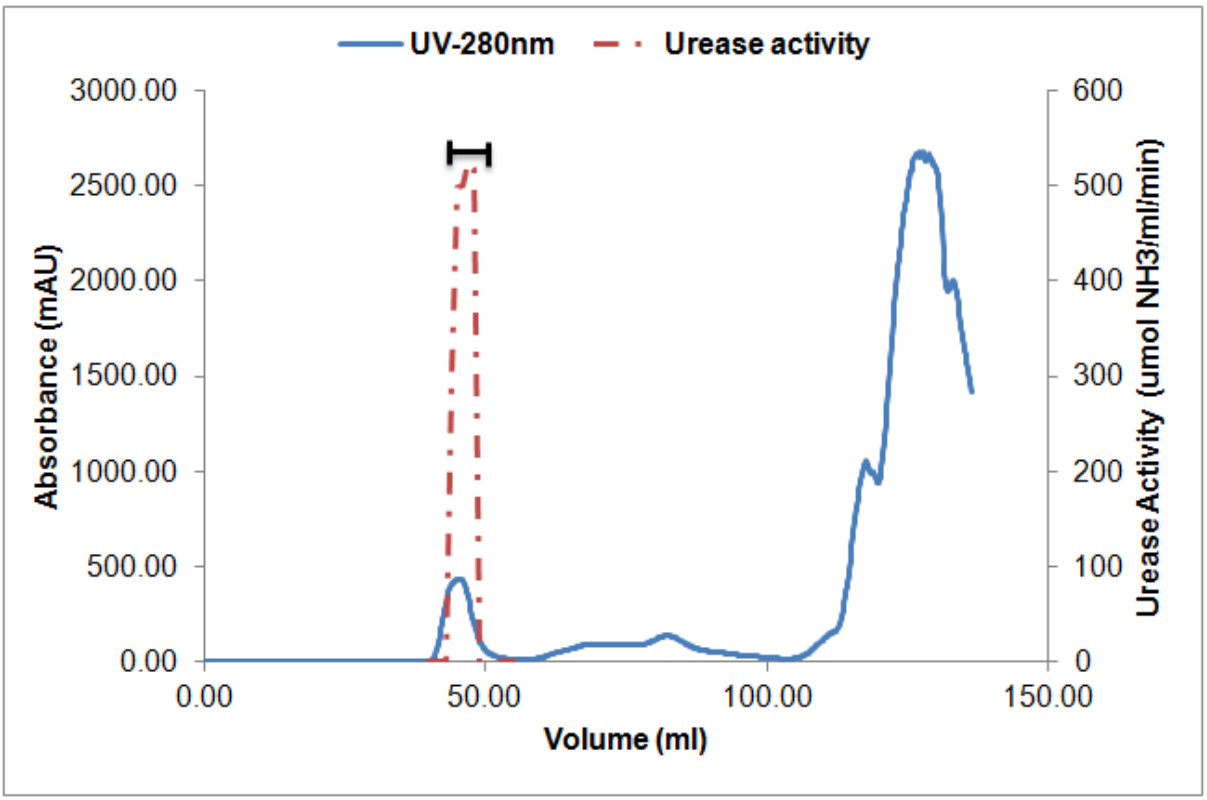


Figure 3.1 Size-exclusion chromatography of 369C urease after freeze-drying on a HiLoad 16/60 Superdex 200 prep grade column equilibrated with running buffer (50mM NaH_2PO_4, 150mM NaCl, 1 mM EDTA); pH 7] at a flow-rate of 1 ml/min. H indicates pooled fractions.

After the size-exclusion chromatography, urease-positive fractions were pooled, evaluated for protein content and assayed for activity. Using this data, specific activity, fold purification and enzyme recovery were calculated. The results of this purification are summarized in Table 3.1.

Table 3.1 Urease purification from *H. pylori* strain 369C

Purification step	Specific Activity (µmol of urea hydrolysed/min/mg)	Purification (fold)	Total Activity (µmol/min)	Total protein (mg)	Enzyme recovery (%)
Crude extract	53.4	1	7145	133.8	100
Freeze-drying	89.0	1.46	3904	43.9	55
Dialysis	95.4	1.54	3572	37.4	50
Superdex 200 pg	203.7	14.3	2608	12.8	36

The crude enzyme extract obtained by sonication containing 133.8 mg total protein and 7144.92 µmol/min total activity was concentrated by freeze drying. Purification by freeze drying increased the specific activity from 53.4 to 89.0 µmol of urea hydrolysed/min/mg as water was removed creating more concentrated areas of solute (urease). The marked decrease in protein concentration from 133.8 to 43.87 mg is also an indication that contaminating proteins were removed. The freeze-dried extract had higher levels of activity than the extract concentrated by ammonium sulphate fractionation perhaps because very low levels of heat are involved in freeze-drying. The dialysis step removed salts from the buffer and other small molecules that had been freeze dried. This technique increased specific activity from 89.0 to 95.4 and the fold purification from 1.46 to 1.54. This slight increase in urease activity is probably as a result of salts removal from enzyme extract. The size-exclusion step removed most of the contaminating proteins as shown by a marked increase in specific activity (from 95.4 to 203.7 µmol of urea hydrolysed/min/mg.

3.3.1.2 Size-exclusion chromatography of ATCC 43526 enzyme extract after ammonium sulphate fractionation

The chromatogram in Fig. 3.2 starts with the injection of the sample and shows the absorbance at 280 nm.

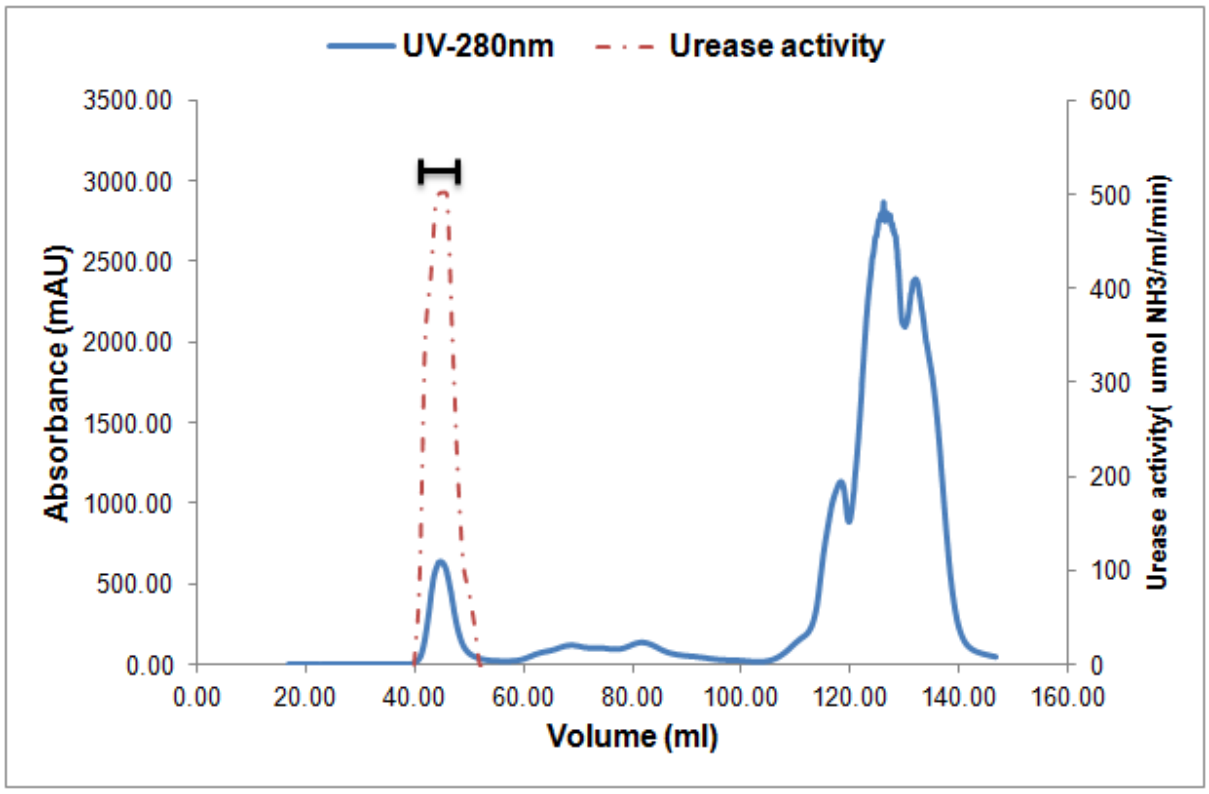


Figure 3.2. Size-exclusion chromatography of ATCC 43526 urease after ammonium sulphate precipitation on a HiLoad 16/60 Superdex 200 prep grade column equilibrated with running buffer (50mM NaH_2PO_4 , 150mM NaCl, 1 mM EDTA); pH 7] at a flow-rate of 1 ml /min. H indicates pooled fractions.

The maximum urease activity was found in the first peak (44.53 ml retention). The other several peaks which are suggestive of several other different proteins were not further characterized. For this urease from the reference strain, ATCC 43526, the urease-positive fractions were pooled, evaluated for protein content and assayed for activity as well. The results of this purification are summarized in Table 3.2.

Table 3.2 Urease purification from *H. pylori* strain ATCC 43526

Purification step	Specific Activity (µmol of urea hydrolysed/min/mg)	Purification (fold)	Total Activity (µmol/min)	Total protein (mg)	Enzyme recovery (%)
Crude extract	46.8	1	7144.92	147.2	100
Ammonium Sulphate Fractionation	98.6	2.1	3794	38.5	55
Dialysis	106.4	2.3	3422	32.2	50
Superdex 200 pg	264.7	5.7	2580	9.6	37

Fractional precipitation of ATCC 43526 crude extract with ammonium sulphate increased the specific activity from 46.8 to 98.6 µmol of urea hydrolysed/min/mg (Table 3.2). Ammonium sulphate draws water molecules away from the non-polar units of the proteins. This decrease in available water molecules increases the surface tension and enhances hydrophobic interactions, thus allowing the protein to precipitate from a solution. The fold purification increased from 1 to 2.107.

Ammonium sulphate maintains the protein in a folded state and also its low heat of solubilisation avoids the risk of protein denaturation that can occur when the sample temperature increases. The dialysis step removed salts and ions (that may limit the activity of the enzyme) through osmosis from a highly concentrated solution (enzyme extract) to the dilute solution (buffer).

This is supported by the purification fold which increased from 2.107 to 2.280. The Superdex 200 pg step 'cleaned up' the target protein as shown by the significant increase in specific activity (from 106.4 to 264.7 µmol of urea hydrolysed/min/mg). From both purification tables of the urease from the two *H. pylori* strains there was a noticeable drop in the enzyme recovered as a result of the size-exclusion step. However, chromatography on Superdex 200 pg does significantly increase the fold purification based on protein thus indicating that this is a high yield method of purification of this enzyme.

The two concentration methods gave concentrates of almost the same activity and had almost the same retention times when chromatographed on Superdex 200 pg. The first peak that was found with the freeze dried extract (369C) should be the same enzyme that is present in the first peak of the ammonium sulphate fractionated extract (ATCC 43526).

Confirmatory studies were performed with SDS-PAGE to determine the molecular weight of the urease enzyme.

3.3.2 SDS-PAGE molecular weight analysis

The ureases purified from this study were derived from two different strains: 369C (a clinical isolate from a hospital in South Africa) and ATCC 43526.

3.3.2.1 Purified urease from H. pylori ATCC 43526

The molecular weight markers, crude extract, and ASF extract were loaded in lanes 1, 2 and 3 respectively, while lane 4 was loaded with pooled fractions obtained from the Superdex 200 pg chromatography.

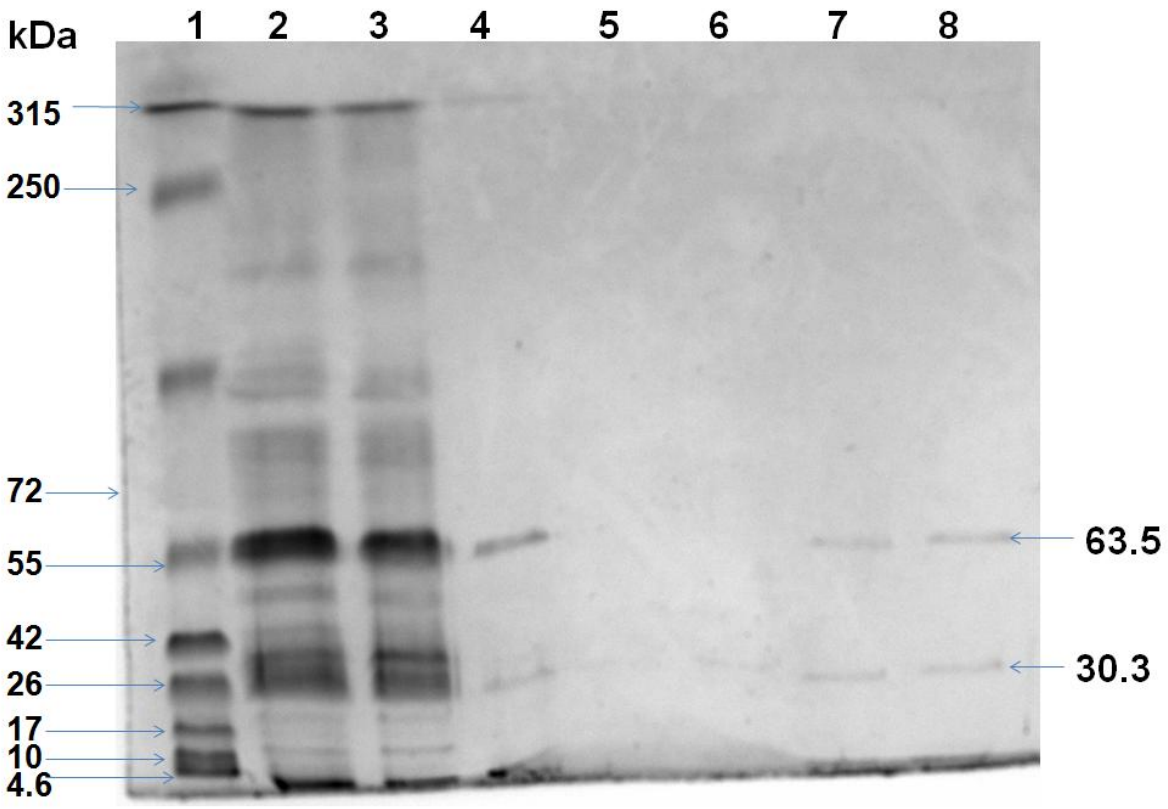


Figure 3.3 SDS-PAGE (12 %) analysis of purified urease from *H. pylori* ATCC 43526. Lane 1: Molecular weight markers. Lane 2: Crude extract. Lane 3: Ammonium sulphate fractionation (ASF) extract. Lane 4: Pooled fractions (Tubes 45 – 48) from Superdex 200 pg size exclusion. Lane 5: Fraction 45. Lane 6: Fraction 46. Lane 7: Fraction 47. Lane 8: Fraction 48.

A faint band at molecular weight 63.5 and 30.3 kDa was seen (Figure 3.3) with the pooled fractions from the size-exclusion chromatography, while very distinct bands were observed in the crude extract (obtained after sonication) and the ammonium sulphate fraction (ASF). The molecular weight values of these subunits are 63.5 and 30.3 kDa. The values are similar to those reported by Dunn *et al*. (1990) and Hu and Mobley, 1990).

3.3.2.2 Purified urease from H. pylori 369C

The molecular weight markers, crude extract, and freeze-dried extract were loaded in lanes 1, 2 and 3 respectively, while lane 4 was loaded with pooled fractions obtained from the Superdex 200 pg chromatography.

No bands were seen (Figure 3.4) with the pooled fractions from the size-exclusion chromatography (Lane 4). However, like with the other electrophoretogram, two distinct bands were observed in the crude extract (obtained after sonication) and freeze drying. The bands observed correspond to molecular weight values of about 60 and 26.5 kDa. Lanes 5 – 8 which are representatives of Fraction 43 – 46 showed distinct bands at molecular weight values of 60 and 26.5 kDa. Surprisingly with the pooled fractions (Lane 4), no band could be visualized. Another SDS gel was run but to no avail, no bands were visualized in Lane 4 (pooled fractions). This suggests probable contamination during loading of samples. There are also bands of molecular weight values of about 315 kDa appearing in Lanes 2 and 3 on both electrophoretograms.

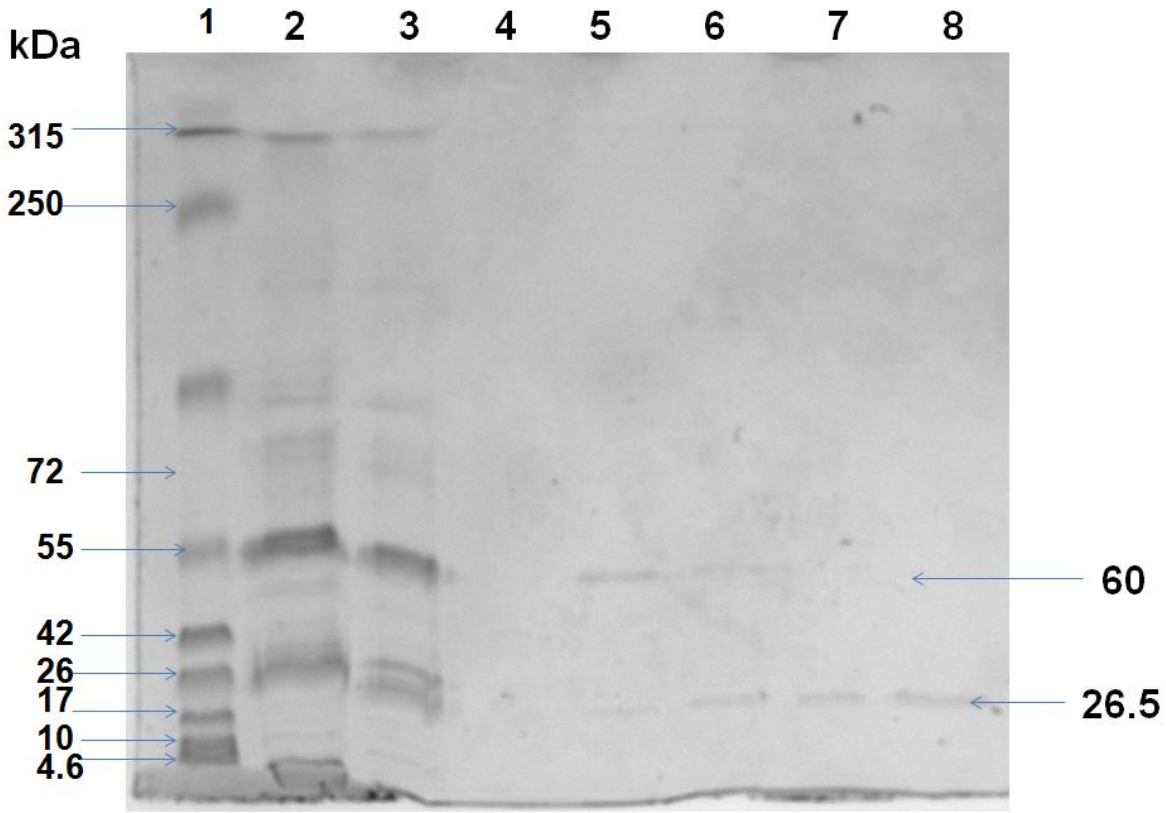


Figure 3.4 SDS-PAGE (12 %) analysis of purified urease from *H. pylori* 369C. Lane 1: Molecular weight markers. Lane 2: Crude extract. Lane 3: Freeze-dried extract. Lane 4: Pooled fractions (Tubes 43 – 46) from Superdex 200 pg size exclusion. Lane 5: Fraction 43. Lane 6: Fraction 44. Lane 7: Fraction 45. Lane 8: Fraction 46.

This observation is probably caused by aggregation of urease forming macromolecular assemblies which is in agreement with previous studies (Austin *et al*., 1991). This postulation is also supported by the fact that Superdex 200 pg has a separation range of M_r 10,000 – 600,000.

3.4 Summary

Urease purification from *H. pylori* proved successful. Total protein and enzyme activity decrease with each step in enzyme purification, starting with low levels of urease activity probably left minute amounts of the urease after Superdex 200 pg size exclusion chromatography.

Other likely reason for the low yield or recovery in this purification might be the nature of the source for the microorganism and proteolysis during the course of the study. Though protease inhibitors were introduced in the crude extract, proteolysis cannot be ruled out.

Although all the purification procedures were performed at low temperatures, degradation of the target protein cannot be ruled out during the course of the study. In spite of all these challenges, appreciable levels of purification are shown in the purification tables.

3.5 Conclusions

1. The urease of *H. pylori* purified in this study is a multimeric enzyme composed of polypeptide subunits. These subunits were in agreement with those previously reported for *H. pylori* urease.

2. We can safely conclude that the molecular weights of ureases of *H. pylori* are similar and quite independent on the particular culture of the bacteria and culturing conditions.

3. Ureases in different strains tend to be uniformly distributed in the *H. pylori*.

References

Austin JW, Doig P, Stewart M and Trust TJ (1991). Macromolecular structure and aggregation of *Helicobacter pylori* urease. *J.Bacteriol.* **173** (18): 5663-5667

Bradford MM (1976). A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**: 248-251.

Dunn BE, Campbell GP, Perez-Perez GI and Blaser MJ (1990). Purification and characterization of urease from *Helicobacter pylori*. J. Biol. Chem. **265:**9464–9469.

Hu LT and Mobley HLT (1990). Purification and N-terminal analysis of urease from *Helicobacter pylori*. *Infect. Immun*. **58**: 992–998.

Chapter 4 : INHIBITORY POTENTIAL OF HONEY ON *H. PYLORI* UREASE ACTIVITY

4.1 Enzyme inhibition kinetics

Several substances can bring about a reduction in the rate of an enzyme catalysed reaction. Some substances are non-specific protein denaturants (e.g. urea) whereas others act in a quite specific manner and are known as inhibitors (e.g. cyanide and heavy metals). The loss of enzyme activity may be either reversible, when the activity may be restored by the removal of the inhibitor, or irreversible, when the loss of enzyme activity is time-dependent and cannot be recovered during the timescale of interest (Copeland, 2000). Therefore enzyme inhibitors fall into two broad classes: those causing reversible inactivation of enzymes (reversible inhibitors) and those whose inhibitory effects can be un-reversed (irreversible inhibitors).

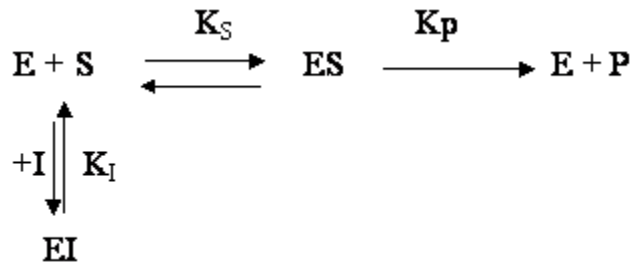
4.1.1 Reversible inhibitors

A typical property of all reversible inhibitors is that when the inhibitor concentration falls, the enzyme activity is regenerated. These inhibitors typically bind to enzymes by non-covalent forces and the inhibitor keeps up a reversible equilibrium with the enzyme. Reversible inhibitors fall into four classes: competitive inhibitors, non-competitive inhibitors, uncompetitive and mixed inhibitors (Meleti, 1986).

4.1.1.1 Competitive inhibitors

The substrate and competitive inhibitors both compete and bind at the same active site. If the inhibitor binds in the enzyme active site, then the enzyme does not react with its substrate.

This kinetic behaviour can be shown as:



Scheme 1: Adapted from Bugg (2004)

Where:

E is the active enzyme;

I is the inhibitor

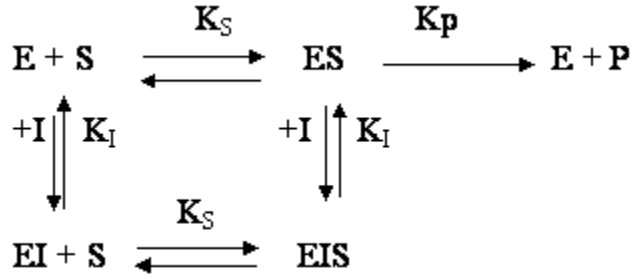
EI is the reversible enzyme-inhibitor complex

K_I is the inhibition constant

K_S is the thermodynamic constant

4.1.1.2 Non-competitive inhibitors

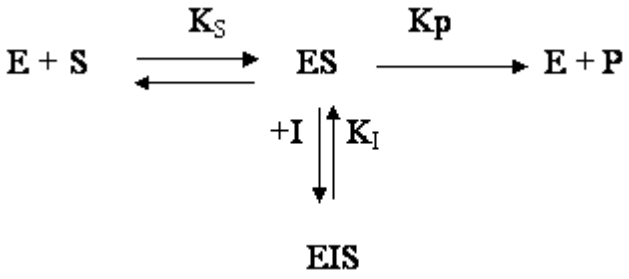
The non competitive inhibitor and substrate react at different active sites.



Scheme2: Adapted from Bugg (2004)

4.1.1.3 Uncompetitive inhibitors

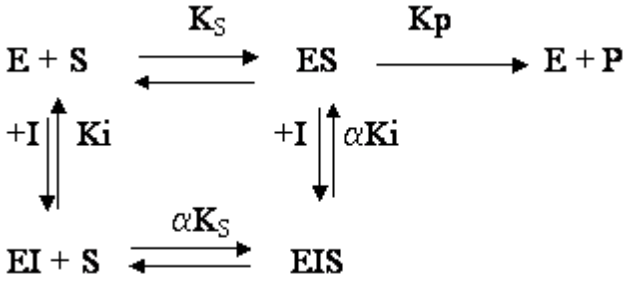
Uncompetitive inhibition occurs when the inhibitor binds only to the enzyme–substrate complex, not to the free enzyme; the EIS complex is catalytically inactive.



Scheme 3: Adapted from Bugg (2004)

4.1.1.4 Mixed inhibitor

Mixed-type inhibitors bind to both E and ES, but their affinities for these two forms of the enzyme are different ($K_i \neq K_i'$). Thus, mixed-type inhibitors interfere with substrate binding (increase K_M) and hamper catalysis in the ES complex (decrease V_{max}).

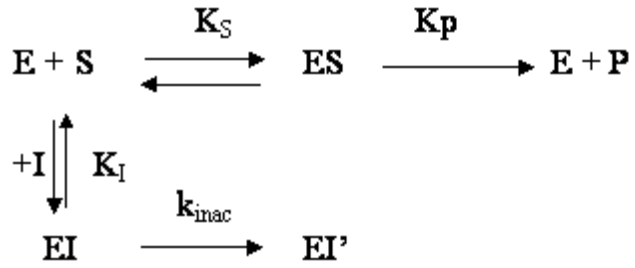


Scheme 4: Adapted from Bugg (2004)

4.1.2 Irreversible inhibitors

Unlike reversible inhibitors that dissociate from the enzyme, an irreversible inhibitor forms a covalent bond with the enzyme molecule and does not dissociate. As shown below, irreversible inhibitors form a reversible non-covalent complex with the enzyme (EI) and this then reacts to produce the covalently modified complex, EI'.

The rate at which EI' is formed is thus the inactivation rate or k_{inact}. The enzyme is therefore permanently inactivated due to the irreversible interaction between the inhibitor and enzyme.



Scheme 5: Adapted from Bugg (2004)

where:

E is the active enzyme and **I** is the inhibitor

EI is the reversible enzyme-inhibitor complex

EI' is the irreversible enzyme-inhibitor complex

$\mathbf{K_I}$ is a thermodynamic constant that describes the affinity of the inhibitor for the active zone of the enzyme

$\mathbf{k_{inac}}$ is a kinetic constant that describes the rate of binding of inhibitor to the enzyme active site.

4.2 Materials and methods

3.2.1 Materials

The commercial enzyme (Jack Bean urease) and substrate (urea) were purchased from Merck (South Africa (Pty) Ltd). A locally produced natural honey called “Pure Honey” (Multiflora, non-irradiated, 100% SA dawn, Bee farms) was purchased from Port Alfred, South Africa. Manuka honey was purchased from Littleover Apiaries (Derbyshire, New Zealand). Chloroform and Diethyl-ether were purchased from Merck (South Africa (Pty) Ltd) and were of analytical grade.

4.2.2 Solvent extracts preparation

Chloroform was used for extraction of ‘Pure Honey’ and Diethyl-ether was used for extraction of Manuka honey. The extraction of each honey sample was performed using the method adapted from Zaghloul *et al*. (2001). The crude honey (100g) was diluted with sterile distilled water (150 ml) in a 500 ml flask. The diluted crude honey was successively extracted with 150 ml of solvent in three steps using 50 ml of solvent each time. The mixture was shaken for 15 min for each extraction process. The mixture was then allowed to stand to allow separation of the solvent layer from the aqueous layer. The aqueous layer was returned to the flask and another batch of the same extraction solvent (50 ml) was added and extracted for 15 min. This was done as three successive extractions. The solvent layers from each extraction process were pooled, and filtered over anhydrous sodium sulfate to remove water contaminating extracts. The solvent extract was concentrated by evaporation with a rotary evaporator (Steroglass, Strike 202, Padua, Italy), under reduced pressure at 40ºC. The extracts were kept at room temperature prior to the urease inhibition assay.

4.2.3 Urease Inhibition

The solvent extracts were tested for their inhibitory effects on partially purified intracellular ureases. The intracellular ureases used for inhibition studies were those isolated previously in Chapter 2. One extracted from the clinical isolate (*H. pylori* 369C) and the other from ATCC 43526. The Jack bean urease was used as control.

4.2.3.1 Why focus on the inhibition of intracellular urease?

It was speculated that extracellular urease is involved in acid resistance of the bacterium (Krishnamurthy *et al*., 1998). However, studies by Marcus and Scott, 2001 have shown that urease is a cytoplasmic protein, but it is then found on the surface of stationary-phase bacteria probably due to lysis of a subpopulation. These investigators have put through strong arguments that extracellular urease does not contribute to acid resistance of *H. pylori* and that the enzyme is irreversibly inactivated at gastric pH levels (less than 4). Since it is now widely accepted that intracellular urease protects *H. pylori* against acidity and not extracellular urease we thus decided to focus only on the inhibition of intracellular urease.

4.2.3.2 Urease Assay

Urease activity was assayed spectrophotometrically by measuring the reduction in NADH in the coupled urease-glutamate dehydrogenase (GDH) system as described earlier in Section 2.2.6.2 of Chapter 2.

4.2.3.3 Urease Linearity Assays

A certain volume of assay buffer [0.1 M Phosphate buffer, pH 7.6] was added to a solution of ADP (100 µL, 0.023 M), NADH (100 µL, 0.0072 M), ketoglutarate (100 µL, 0.026 M), urea (100 µL, 1.8 M) and GLDH (100 µL, 500 Units/ml) in a UV cuvette, followed by the addition of aliquots (0 to 100 µL) of urease which started the

reaction. The assay buffer volume was adjusted to give a constant total assay volume (3000 µL). The spectrophotometer was set to measure the decrease in absorbance at 340nm, at 30s intervals for 5 mins at 25°C as described earlier in Chapter 2 (Section 2.2.6.2). All of the urease linearity assays were carried out in triplicate.

4.2.3.4 Urea Dependence Assays

A solution of urea (0 to 100 µL, 1.8 M) was added to a certain volume of assay buffer in a UV cuvette containing the other assay cocktail components: ADP (100 µL, 0.023 M), NADH (100 µL, 0.0072 M), ketoglutarate (100 µL, 0.026 M), urea (100 µL, 1.8 M) and GLDH (100 µL), followed by the addition of a certain volume of urease which started the reaction. The assay buffer volume was adjusted to give a constant total assay volume (3000 µL). The spectrophotometer was set to measure the decrease in absorbance at 340nm, at 30s intervals for 5 mins at 25°C as described earlier. All of the urea dependence assays were also carried out in triplicate.

4.2.3.5 Urease Inhibition Assay using Honey Solvent Extracts

Acetohydroxamic acid was used as the standard inhibitor of urease. The commercial urease was also used as the standard enzyme. The partially purified urease isolated from the ATCC 43526 strain and the *H. pylori* 369C were incubated in the assay cocktail with honey extracts in potassium phosphate buffer prior to the reading. A solution of urease was mixed with varying volumes of the honey chloroform extract. Aliquots were then added to a certain volume of assay buffer (adjusted to give a constant total assay volume of 3000 µL) in a UV cuvette containing the other assay cocktail components as described earlier. The reaction was started by addition of the substrate, urea (100 µL, 1.8M). The buffer solution (3000 µL) was used as the blank and acetohydroxamic acid (50 µL) was used as the control.

The spectrophotometer was set to measure the decrease in absorbance at 340nm, at 30s intervals for 5 mins at 25°C. The inhibition percentage (I %) for each solvent extract tested was calculated by comparing the NADH oxidation rate before and after incubation with the solvent extract according to:

$$I\ (\%) = A_0 - A_i / A_0 \times 100$$

where A_0 is the NADH decrease in absorbance rate with urease activity (no solvent extract addition), and A_i represents that rate after incubation with solvent extract. The reaction rate was taken from the linear range of the absorbance reading as the slope of the linear regression curve. The concentration of inhibitor required to diminish enzyme activity by 50% was calculated by plotting percent inhibition against the concentration of inhibitor. One hundred percent activity was expected to be determined in the absence of inhibitor. Each assay was carried out in triplicate.

4.2.3.6 Effect of pre-incubation time on the inhibition of urease activity

The effect of pre-incubation time on the inhibition of urease activity was determined by the pre-incubation of the enzyme with test solution (honey extract) at 10 minutes intervals for 1 hr prior to addition of substrate and all other assay components required for measuring urease activity. Comparisons of reaction rates in the presence of inhibitor with those for control reactions were used to calculate the degree of enzyme inhibition each time.

4.2.3.7 Determination of IC_{50}

The main objective of this work is to explore the inhibitory potential of honey extracts on the activity of urease. The inhibitory potential of an inhibitor is described either as the concentration of an inhibitor at which the enzyme activity falls to 50% of its original value or as the molar concentration of the compound required to inhibit 50% of the urease activity (IC_{50}). In order to determine the IC_{50} values (50% inhibition), honey extracts (inhibitors) were diluted in phosphate buffer to concentration ranging from 20% (v/v) to 100% (v/v).

4.2.3.8 Determination of the type of enzyme inhibition

Determination of the inhibition type is critical for identification of mechanism of inhibition and the sites of inhibitor binding. One method was applied to monitor the effect of inhibitor (solvent extracts) on both K_m and V_{max} values. This was done by plotting the reciprocal of the rate of the reactions against the reciprocal of the substrate concentration as Lineweaver-Burk plot. The Dixon plot for urease at different urea concentrations was also constructed.

4.2.4 Characterization of intracellular Urease of *H. pylori*

4.2.4.1 pH optimum

To establish the optimum pH for the partially purified urease, activity was determined at different pH values. Urease preparations were made in different buffers of pH varying from 2 - 11. The different buffers used at varying pH were: sodium citrate (pH 2 – 5, 0.1 M); potassium phosphate buffer (pH 6 – 8, 0.1 M) and bicarbonate buffer (pH 9 – 11, 0.1 M). The urease activity was determined as described in Chapter 2 (2.2.6.2).The determination of enzyme activity was done in triplicate at each pH.

4.2.4.2 Temperature optimum

The temperature profile for the partially purified urease was determined over a temperature range of 4 – 100°C at the pH optimum. The urease activity was determined as described in Chapter 2 (2.2.6.2). The determination of enzyme activity was done in triplicate at each temperature.

4.2.4.3 Thermal stability

To determine the optimal thermal stability of the urease, extracts were heated at 25, 45, and 65°C for 1 h at the pH optimum. Aliquots were withdrawn from each extract at 10 min intervals and assayed for urease activity. Samples were prepared in triplicate and residual enzyme activity was measured as described in Chapter 2 (2.2.6.2).

4.2.4.4 Estimation of kinetic parameters (K_m and V_{max})

Under all conditions described in this thesis the rate of urea hydrolysis was found to be linear with time or under steady state conditions. The kinetic properties of *H. pylori* intracellular urease were determined by varying the substrate (urea) concentration from 100 – 2400 mM. Urease activity was determined at each substrate concentration and Lineweaver-Burk double reciprocal plots were constructed showing relationship between substrate and enzyme activity.

4.3 RESULTS AND DISCUSSION

Kinetic studies were carried out to explore the catalytic activity of urease extracted from our *H. pylori* clinical isolate (369C) in the presence of urea and the potential inhibitors (honey extracts). The activity was compared against a commercial Jack bean urease (control) and the urease extracted from ATCC 43526 (reference strain).

4.3.1 Cytoplasmic urease linearity

The linear range of the intracellular urease was determined from assays in which the enzyme concentration was varied while keeping the substrate concentration constant at 1.8M (Figure 4.1). This assay was generated from duplicate assays in which the enzyme was varied from 0 to 1.0 mg ml^{-1} using a constant substrate concentration of 1.8 M, at 25^{o}C and pH 7.6. A plot of the resulting data (Figure 4.1) shows linearity up to 1 mg ml^{-1} in the concentration of urease. A urease concentration of 1 mg ml^{-1} was therefore selected for all subsequent assays, while the pH and temperature were kept constant at 7.6 and 25^{o}C, respectively.

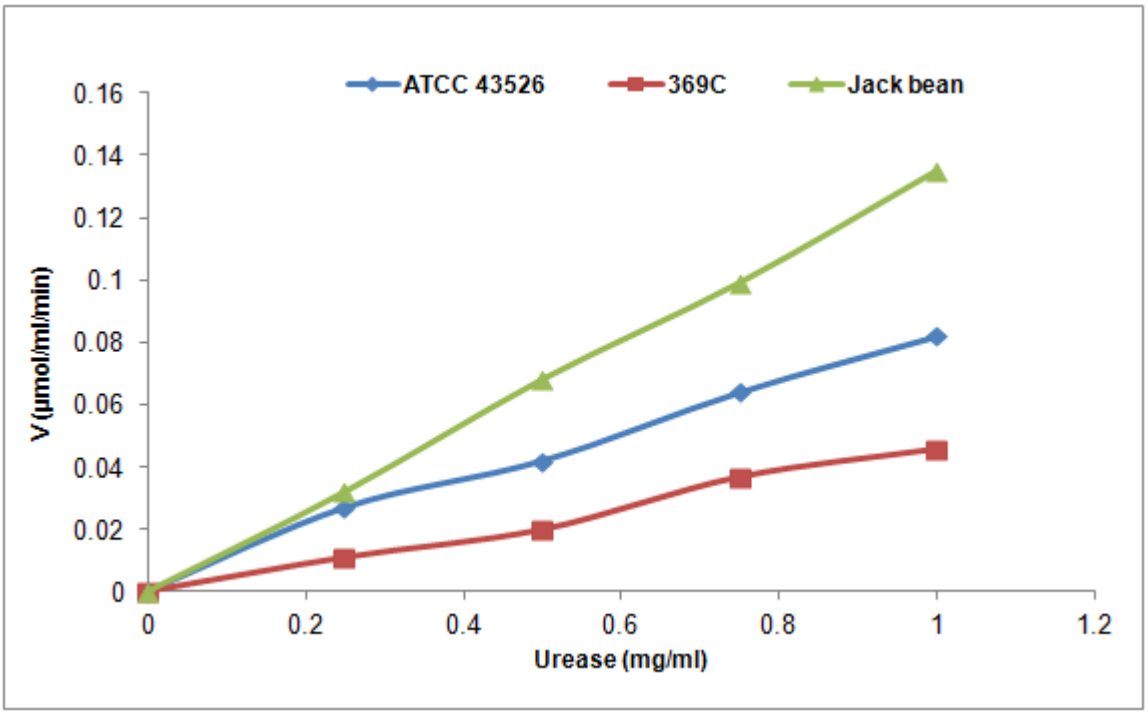


Figure 4.1 Plot of v0 versus urease concentration for determining the linearity dependence of urease. The data points represent the mean of triplicate assays.

4.3.2 Urease substrate dependence

To explore the substrate dependence of urease, assays were run with a series of substrate concentrations ranging from 10 mM – 2400 mM whilst keeping the urease concentration constant at 1 mg ml^{-1}. These assays were run in triplicate at constant temperature of 25^{o}C and a pH of 7.6.

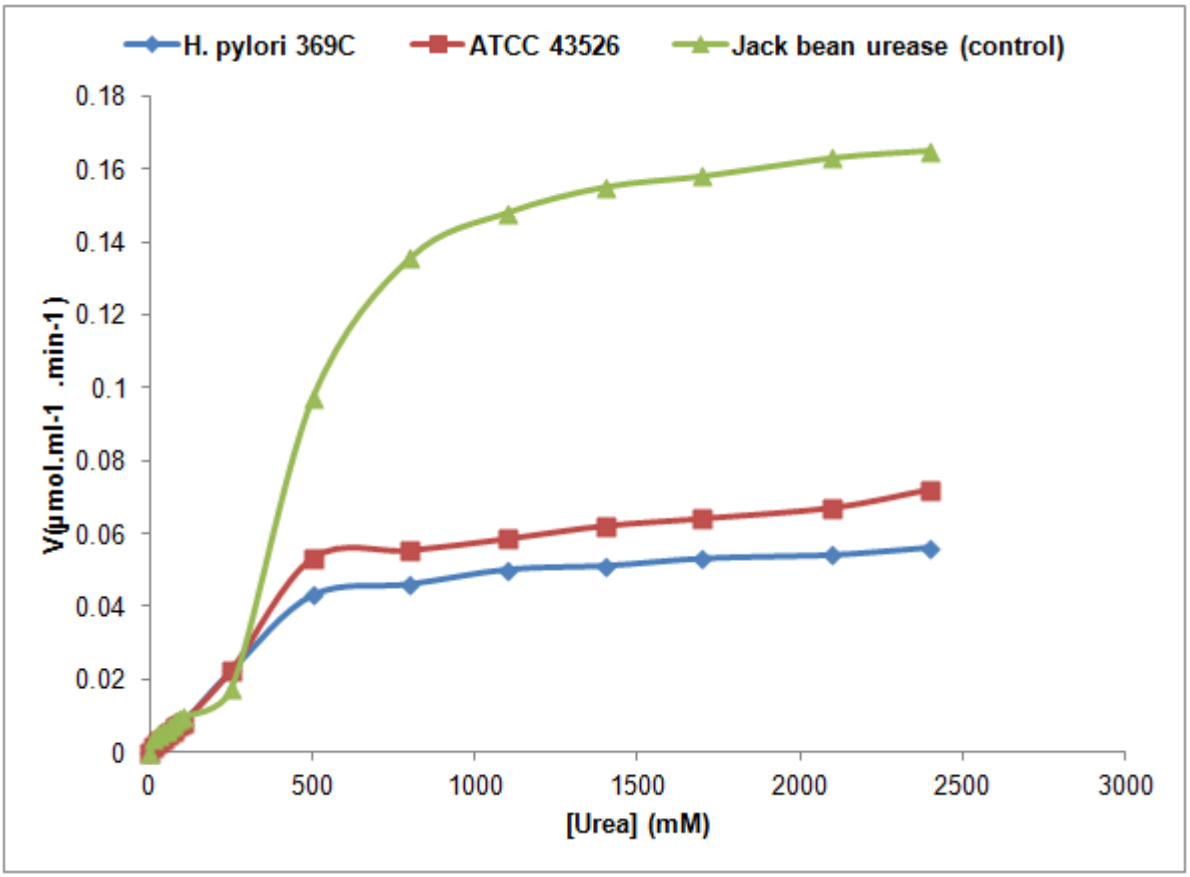


Figure 4.2 Michaelis Menten plot of *H. pylori* (369C) urease, *H. pylori* (ATCC 43526) urease and Jack bean urease. The data points represent the mean of triplicate assays.

4.3.3 Effect of pre-incubation time on the inhibition of urease activity

The results presented in Fig 4.5 and 4.6 shows that urease inhibition by honey extracts was independent of incubation time at the concentrations used. When urease extract was incubated with the test solutions (honey extracts) at 25^{o}C for 10 minute intervals for 1 hr, the decrease in activity was insignificant. When urease extract was incubated with a well-known inhibitor, acetohydroxamic acid, the decrease in activity with time was insignificant as well. Each point represents the mean ± S.D. of three experiments. Concentration of honey extract was 100% (v/v).

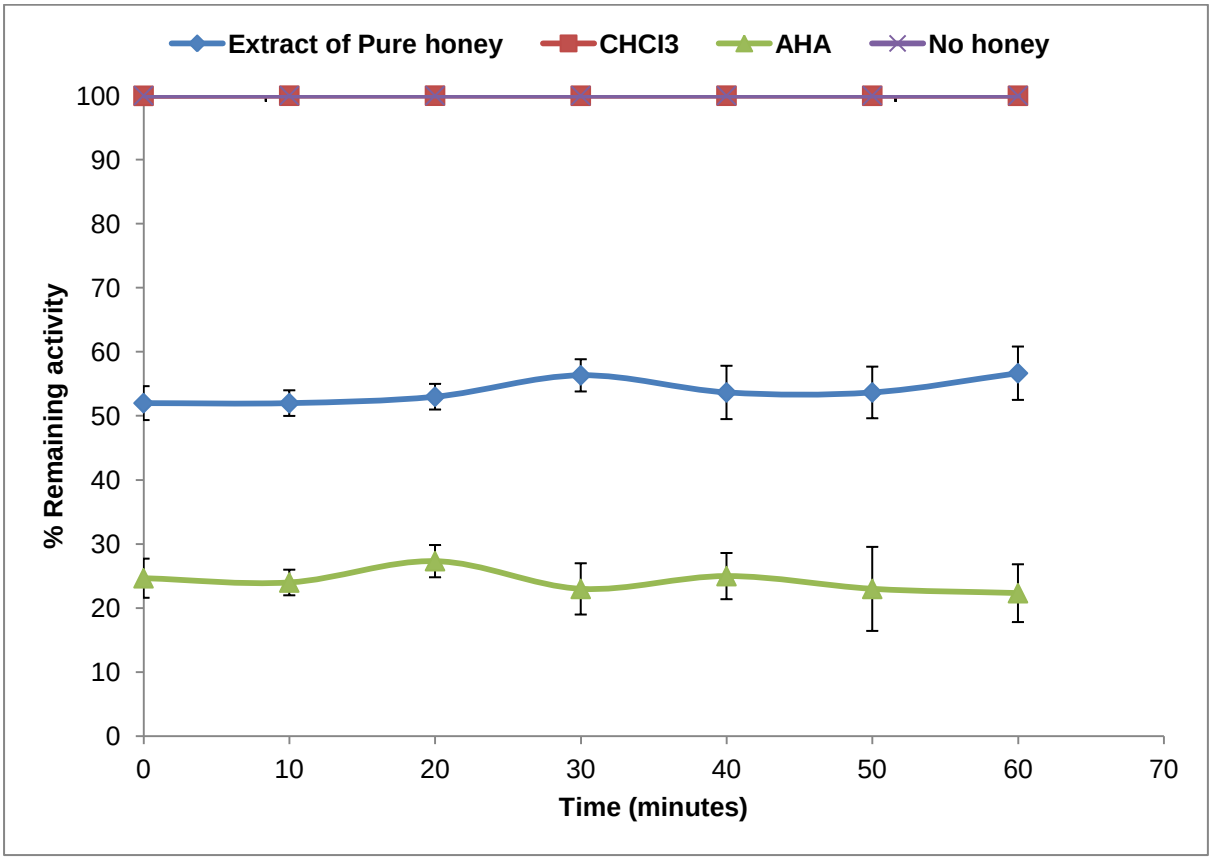


Figure 4.3 Time dependence of the inhibition of urease by chloroform extract of Pure honey. Negative controls were $CHCl_3$ and 369C urease (with no extract of Pure honey). Positive control was acetohydroxamic acid (AHA). Concentration of honey extract was 100% (v/v). Each point represents the mean ± S.D. of three experiments.

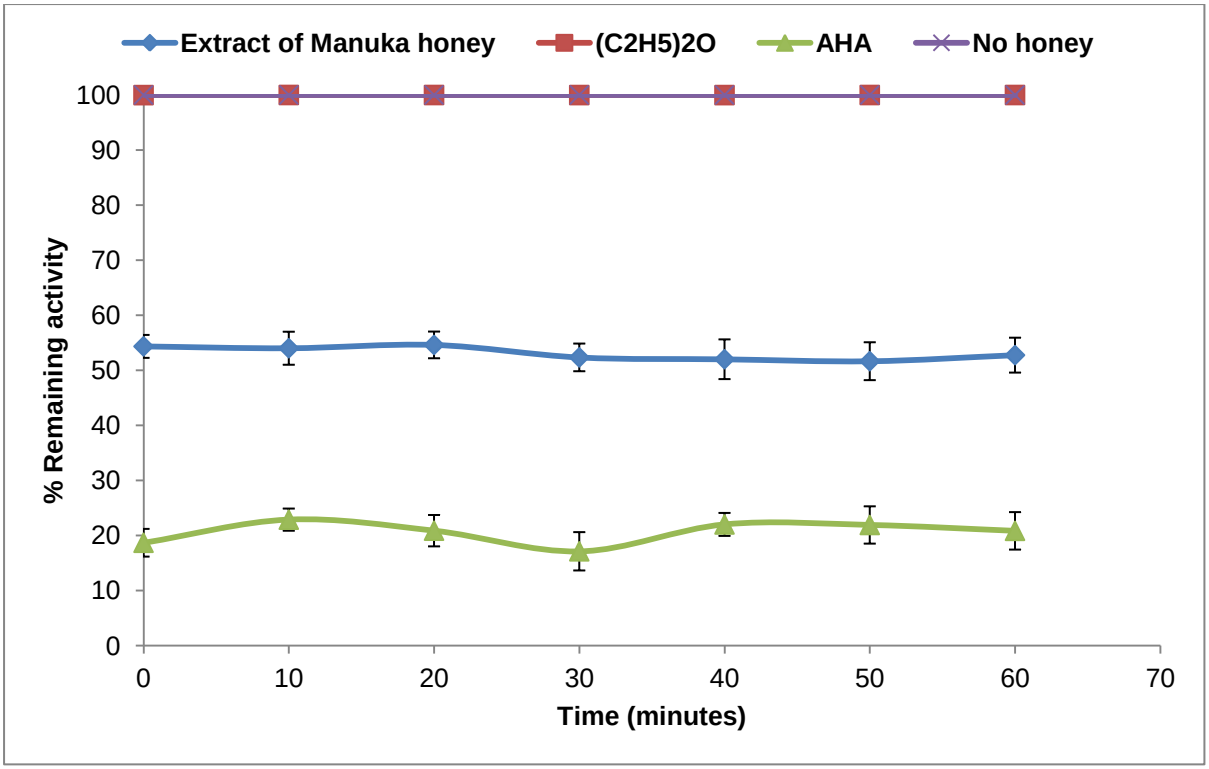


Figure 4.4 Time dependence of the inhibition of urease by diethyl-ether extract of Manuka honey. Negative controls were $(C_2H_5)_2O$ and 369C urease (with no extract of Manuka honey). Positive control was acetohydroxamic acid (AHA). Concentration of honey extract was 100% (v/v). Each point represents the mean ± S.D. of three experiments.

Usually, when the inhibitor binds the enzyme there is formation of covalent bonds. The formation of these bonds by the honey extracts however does not display any time dependency. The degree of inhibition does not seem to increase with time after the honey extracts interacts with the enzyme. This behaviour alone indicates that the honey extracts are unlikely to be irreversible inhibitors.

4.3.4 Effect of inhibitors

The two honey extracts were examined in a series of urease inhibition assays**.** All assays were run in triplicate at pH 7.6 and 25^{o}C. The effect of varying the concentration of these potential inhibitors on the activity of urease was explored. We observed that (Figure 4.7):

i. Both honey extracts possess some inhibitory effects on urease activity.

ii. Both extracts inhibited urease activities in a concentration dependent manner.

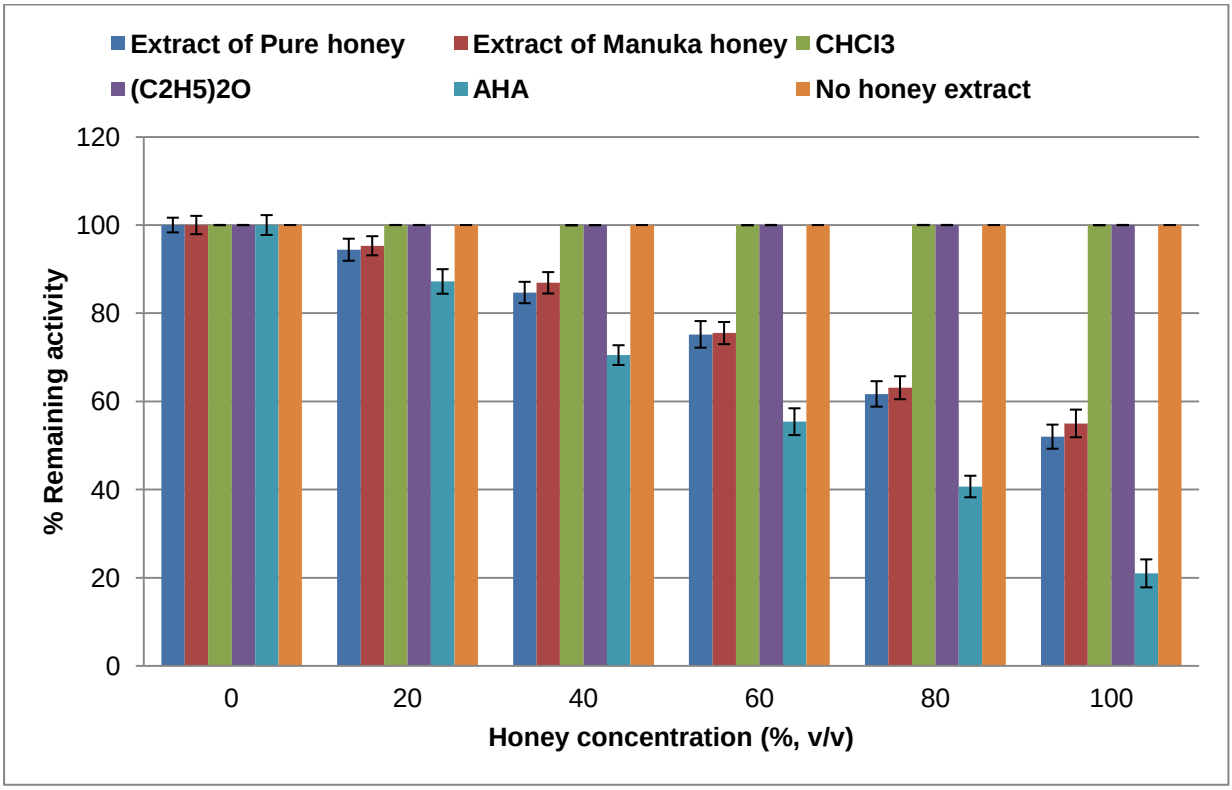


Figure 4.5 Concentration dependence of urease inhibition. Negative controls were $CHCl_3$, $(C_2H_5)_2O$ and 369C urease (no honey extract added). Positive control was acetohydroxamic acid (AHA). The data are the average of three experiments and bars indicate the standard deviations.

4.3.5 Inhibition activities against urease

Although enzymes that are purified are easier to work with than the crude preparations it should be remembered that in purifying the enzyme we are removing it from its natural environment. Since we are studying the urease in order to gain some understanding of how it is involved in the acid resistance mechanisms of *H. pylori* at a molecular level, it was important to keep the conditions as close as possible to the conditions within the cell. For these reasons we compared the inhibitory activities of the honey extracts against both the crude urease extract and the purified urease.

4.3.5.1 Inhibition of crude urease by extract of Pure Honey

The extract derived from Pure honey was screened for enzyme inhibition activities against crude urease forms (*H. pylori* ATCC and *H. pylori* 369C). Commercial Jack bean urease was used as a control.

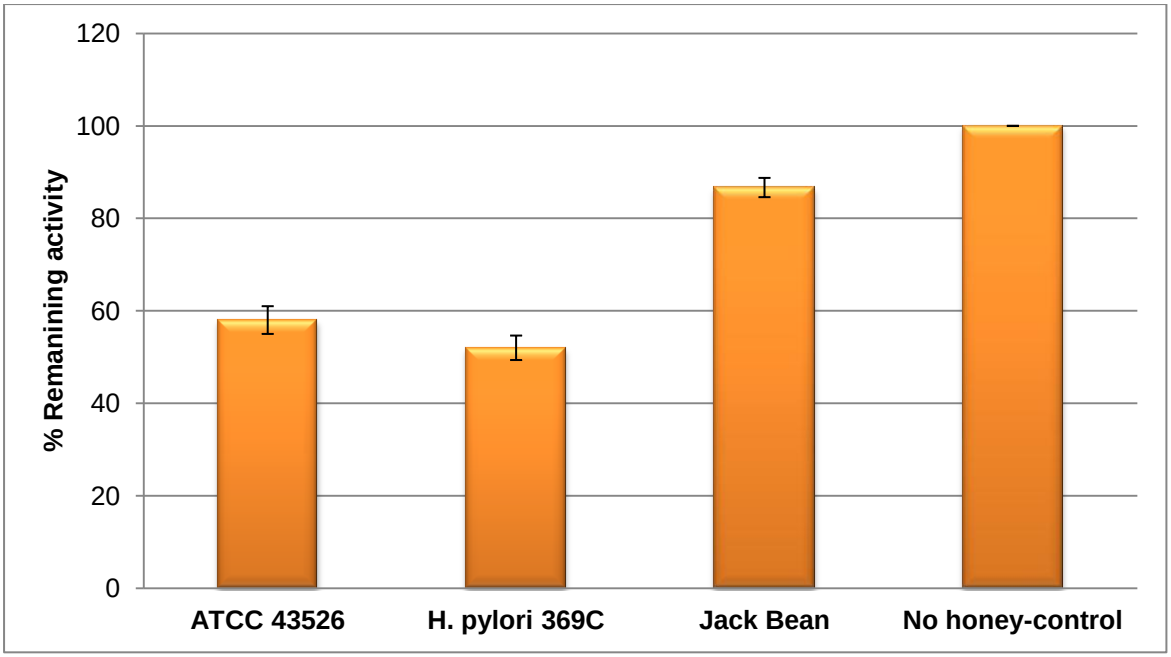


Figure 4.6 Effect of Pure Honey chloroform extract on crude urease activity. Concentration of honey extract was 100% (v/v). Jack bean urease and urease produced by *H. pylori* ATCC 43526 were used as controls. The data are the average of three experiments and bars indicate the standard deviations.

As shown in Figure 4.8, Pure Honey extract was found to be a relatively more potent inhibitor for *H. pylori* 369C urease (I=48%) than for *H. pylori* ATCC urease (I=42%). The extract of Pure honey was shown to be slightly active against purified Jack bean urease (control) giving 13% inhibition.

4.3.5.2 Inhibition of crude urease by extract of Manuka Honey

The extract derived from Manuka honey was screened for enzyme inhibition activities against crude urease forms (*H. pylori* ATCC and *H. pylori* 369C). Commercial Jack bean urease was used as a control.

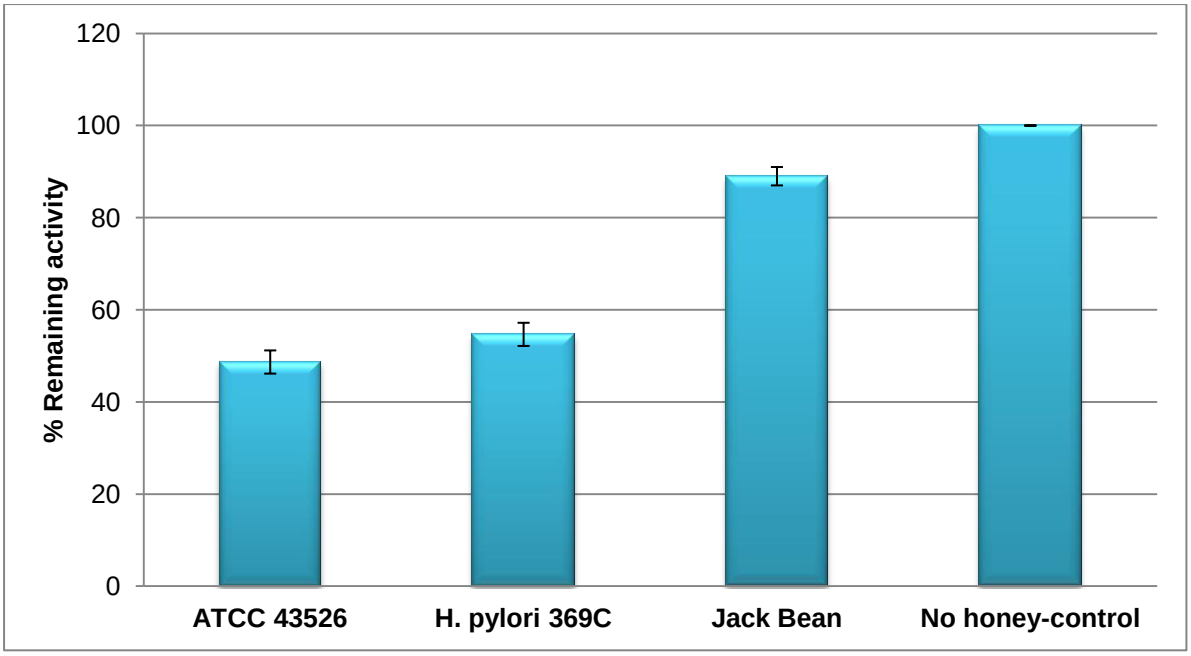


Figure 4.7 Effect of Manuka Honey diethyl-ether extract on crude urease activity. Concentration of honey extract was 100% (v/v). Jack bean urease and urease produced by *H. pylori* ATCC 43526 were used as controls. The data are the average of three experiments and bars indicate the standard deviations.

Manuka Honey extract was found to be a comparatively more effectual inhibitor for *H. pylori* ATCC urease (I = 51%) than for *H. pylori* 369C urease (I = 45%). The extract of Manuka honey also exhibited slight inhibitory activity against purified Jack bean urease (control) giving 11% inhibition.

4.3.5.3 Inhibition of purified urease by extract of Pure Honey

The extract derived from Pure honey was also screened for enzyme inhibition activities against the urease forms (*H. pylori* ATCC and *H. pylori* 369C) obtained from Superdex 200 pg size exclusion chromatography. Commercial Jack bean urease was used as a control.

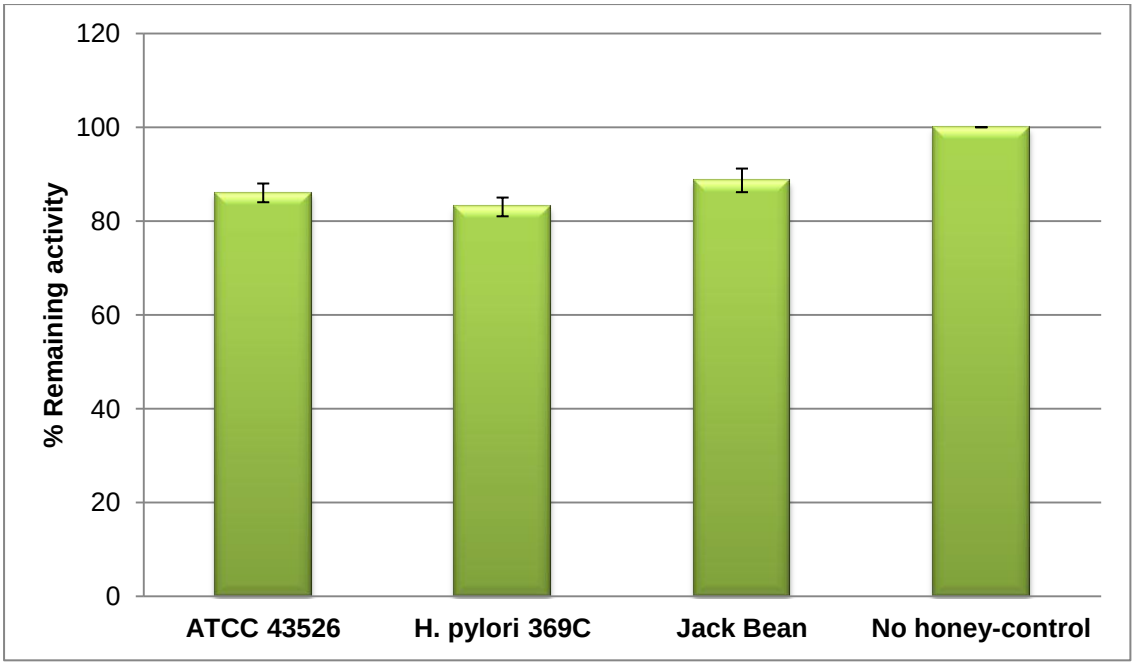


Figure 4.8 Effect of Pure Honey chloroform extract on purified urease activity. Concentration of honey extract was 100 % (v/v). Jack bean urease and urease produced by *H. pylori* ATCC 43526 were used as controls. The data are the average of three experiments and bars indicate the standard deviations.

The extract of Pure Honey was found to be a relatively weak inhibitor for purified forms of the enzymes. The extract shows 17% inhibition for *H. pylori* 369C urease and 14% inhibition for *H. pylori* ATCC urease (Figure 4.10). The extract was also a very weak inhibitor against the control enzyme, Jack Bean urease giving an inhibition percent of 11.3.

4.3.5.4 Inhibition of purified urease by extract of Manuka Honey

The extract derived from Manuka honey was also screened for enzyme inhibition activities against the urease forms (*H. pylori* ATCC and *H. pylori* 369C) obtained from Superdex 200 pg size exclusion chromatography. Commercial Jack bean urease was used as a control.

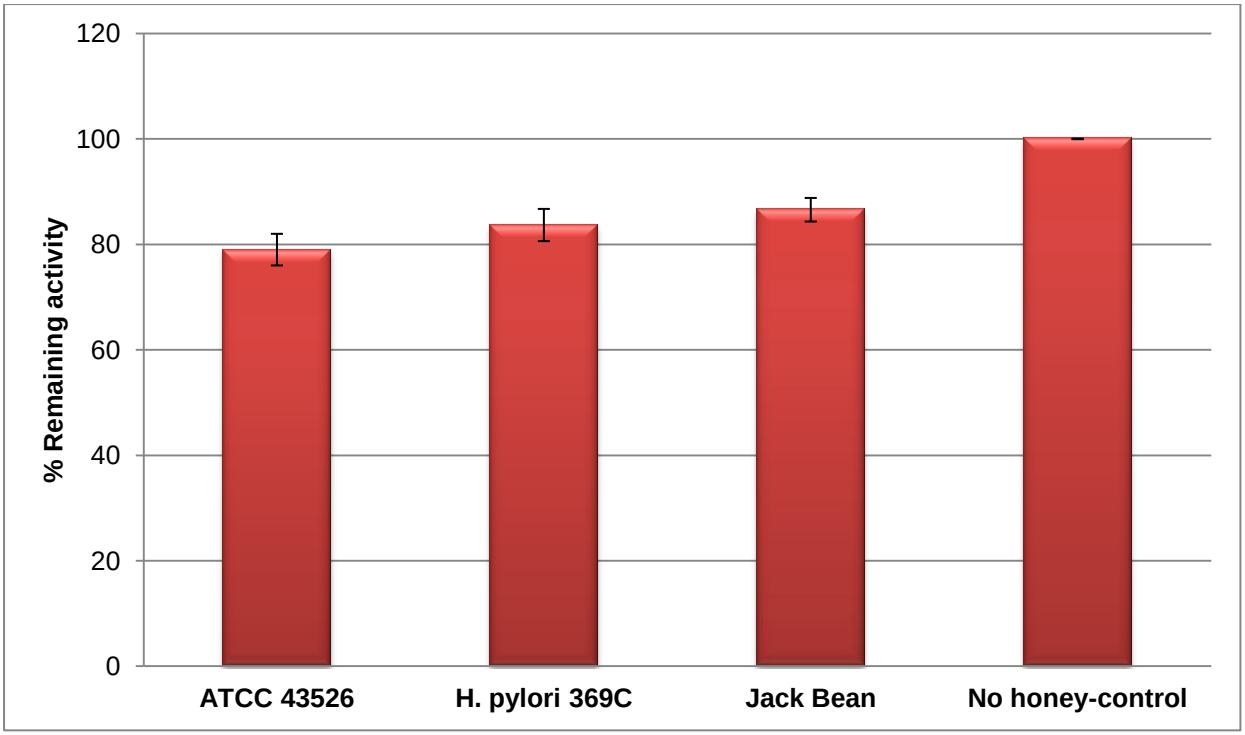


Figure 4.9 Effect of Manuka Honey diethyl-ether extract on purified urease activity. Concentration of honey extract was 100 % (v/v). Jack bean urease and urease produced by *H. pylori* ATCC 43526 were used as controls. The data are the average of three experiments and bars indicate the standard deviations.

The extract of Manuka Honey was also found to be a relatively weak inhibitor for both purified forms of the enzymes. The extract gives 16.3% inhibition for *H. pylori* 369C urease. The extract was shown to be slightly active against purified *H. pylori* ATCC urease giving 21% inhibition (Figure 4.11). However, the extract was also a very weak inhibitor against the control enzyme, Jack Bean urease giving an inhibition percent of 13.4.

4.3.5.5 Comparison of inhibitory activity in crude enzyme and purified enzyme

On comparing the inhibitory activities of the honey extracts against the crude urease extract and the purified urease we found some interesting observations. Both honey extracts gave higher inhibition percentages when tested with the crude forms of both ureases than with the purified forms of the enzymes (Figure 4.8, 4.8, 4.10 and 4.11).

The extracts of both honeys have no significant urease inhibitory activity when tested with the purified forms of all ureases (Figure 4.10 and 4.11). Higher inhibition percentages associated with crude forms of the enzymes may be due to several reasons. For instance, when the cell was disrupted, the resulting lysate might have had marked changes resulting in substantial changes in the concentrations of the various metabolites which made up the crude enzyme preparation.

Cellular reactions cannot be ruled out on sonicating the cells. For instance, on sonication of the cells, free radicals are generated in the cavitation bubbles which then react with other molecules. These substances and other impurities might be responsible for the inhibition of the enzyme activity that was observed, however, these high inhibition percentages may also indicate interesting and unusual properties of the enzyme itself. On the other hand, on sonication of the cells there are also several tightly associated enzymatic activities which may affect urease activity *in vitro* bringing about false inhibition.

Recommendations

From observations of this study it can safely be concluded that these two honeys exhibit antiurease activity *in vitro*. Since crude enzyme extracts more closely resembles the physiological conditions of the cell than the pure forms of the enzyme then the high inhibition percentages associated with crude enzyme forms are applicable. In addition, with crude extracts, the enzymes interact with many other substances that are undoubtedly subject to physiological control mechanisms which are lost if one is to purify the enzyme. As of such, in the purified urease, there are very few proteins that are associated with the enzyme structure. It is speculated that the purification procedures might have altered the properties of the enzyme in a way. It is therefore recommended further studies to investigate if the properties of the enzyme were altered in any way after the purification steps.

4.3.6 Determination of IC_{50}

Pure Honey exhibits an IC_{50} value greater than 100% (v/v) as shown in Figures 4.8 and 4.10. Manuka Honey exhibits an IC_{50} value greater than 100% (v/v) for *H. pylori* 369C urease (Figure 4.9 and Figure 4.11) whereas the extract inhibited activity of urease isolated from ATCC 43526 strain by 51% (Figure 4.9) In order to establish the type of inhibition involved, Lineweaver-Burk was constructed using the concentrations of the extract that gave up to about 50% residual enzyme activity. In this study Manuka honey extract (100%, v/v) inhibited activity of urease isolated from ATCC 43526 strain by 51% thus it was used to determine the type of enzyme inhibition involved.

4.3.7 Determination of the type of enzyme inhibition

The nature of inhibition of urease activity by extract of Manuka honey was predicted from Lineweaver-Burk (Figure 4.12) and Dixon plots (Figure 4.13). Lineweaver-Burk plot indicated several possibilities for the type of inhibition. As of such we decided to ignore the values for bigger dilutions since chances of error are high with such dilutions. On ignoring such dilutions, the Lineweaver-Burk plot now indicated a urease inhibition that seemed to be mixed inhibition. However, the Dixon plot confirmed the Manuka honey extract to be a competitive inhibitor.

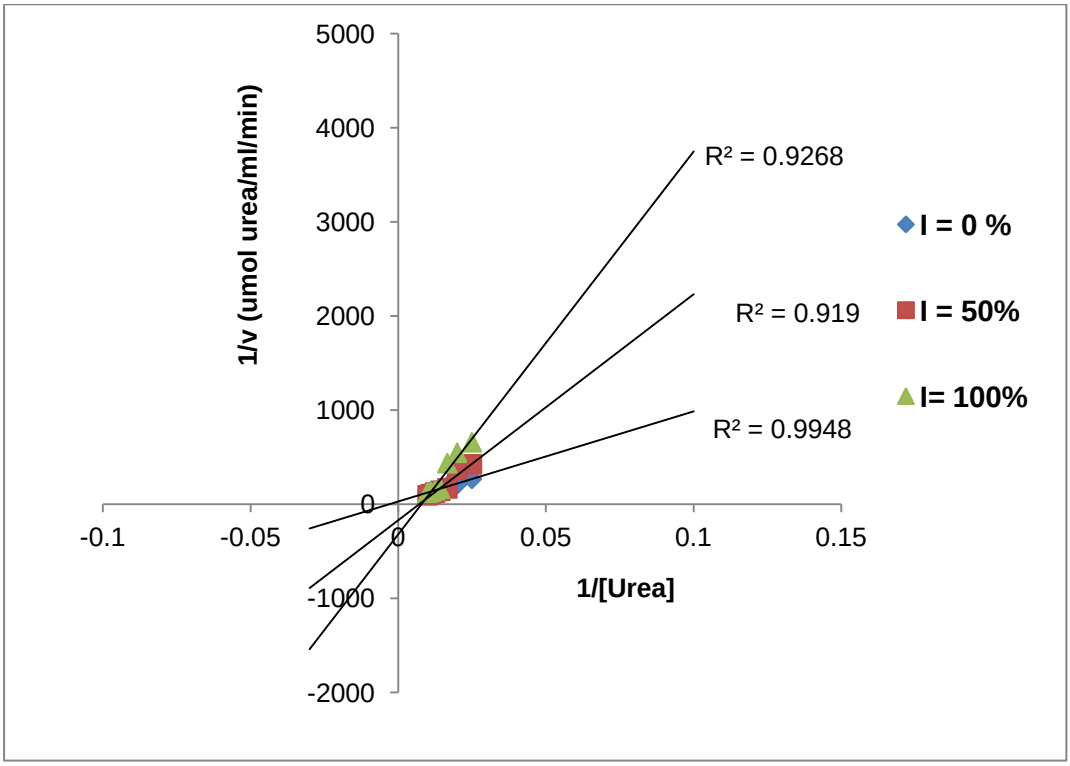


Figure 4.10 Lineweaver-Burk plot representing reciprocal of initial velocity versus reciprocal of urea concentration in the absence and presence of Manuka honey extract (50% (v/v) and 100% (v/v)) at pH7.6. The data are the average of three experiments.

The Dixon plot indicated a different nature of urease inhibition by the extract of Manuka honey. The plot reveals the inhibition to be competitive, a behavior where the substrate and the inhibitor compete for the enzymatic site binding. This characteristic of competitive inhibitors is shown by the lines converging above the x axis (Figure 4.13), thus the honey extract is a competitive inhibitor. Therefore, it is speculated that there are some honey components that probably share structural resemblances with the substrate urea.

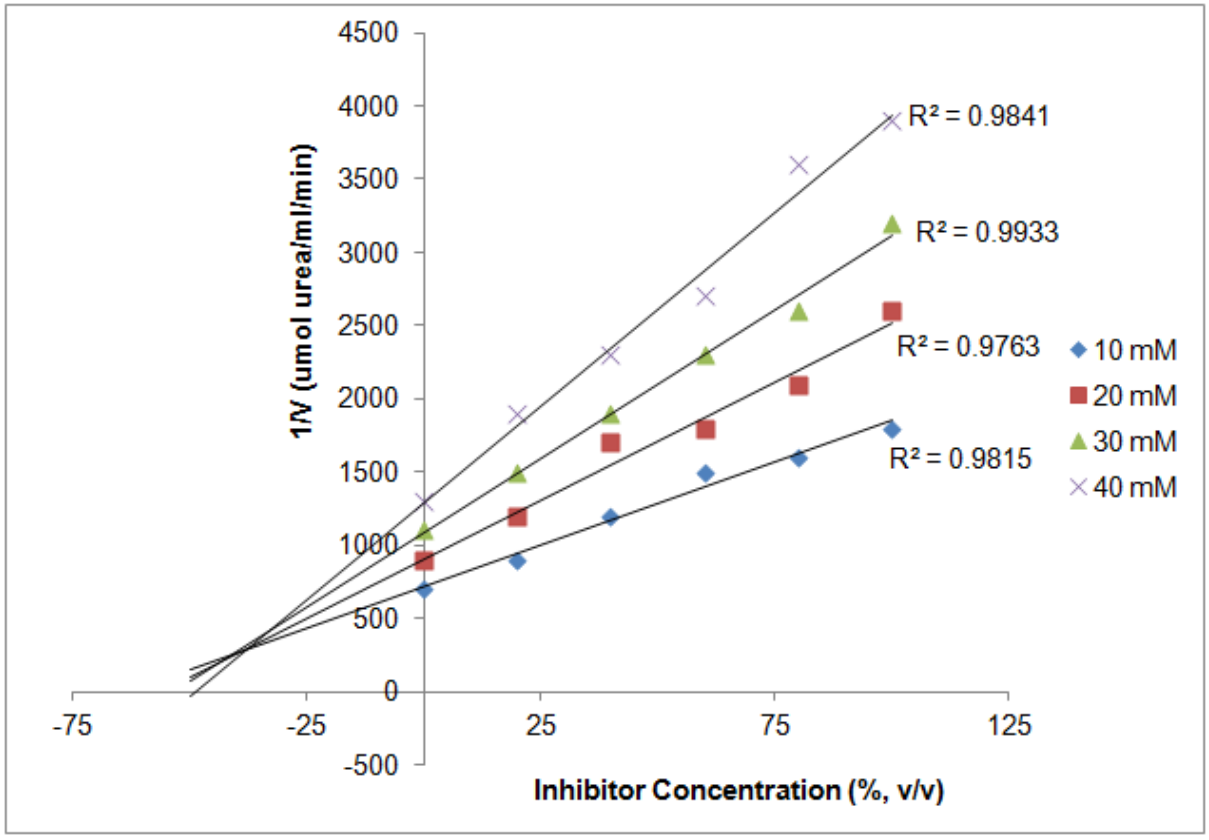


Figure 4.11 Dixon plot for ATCC 43526 urease at four urea concentrations in the absence and presence of Manuka honey extract at pH 7.6. The data are the average of three experiments.

4.3.8 Characterization studies

Since urease serves as the basis of detection of *H. pylori* in gastric biopsies and represents an important virulence factor, biochemical characteristics of the enzyme were determined.

4.3.8.1 pH profile

The activity of most enzymes strongly depends on the pH of the medium; therefore it was important to optimize the pH of urease. Since urease has been found in diverse species and in diverse environmental conditions the pH profile were explored using different buffers over a range of 4 – 10.

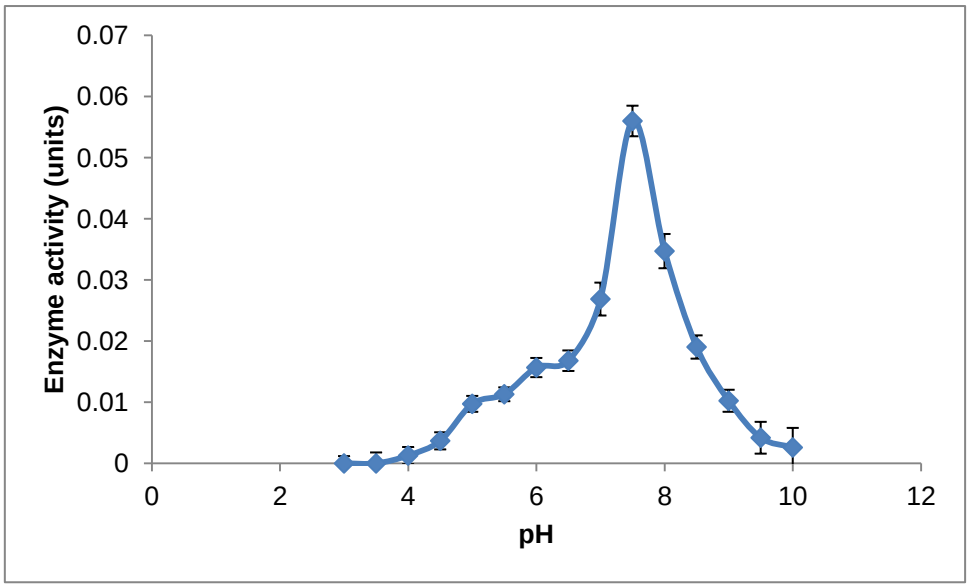


Figure 4.12 pH optimum of *H. pylori* 369C urease. The data are the average of three experiments and bars indicate standard deviations.

The pH optimum of urease was found to be 7.5 (Figure 4.14). This finding is in agreement with previous observations of pH optima by other investigators who reported it to be 8.2 (Mobley *et al*., 1988) and 7.4 (Sigh *et al*., 1990).

4.3.8.2 Temperature profile

The optimal temperature for urease at pH 7.5 was 40°C (Figure 4.15). This finding is similar to previous observations of temperature optima of other ureases; 45°C (Mobley *et al*., 1988) and 43°C (Mobley *et al*., 2001) from *Campylobacter pylori* and *H. pylori* respectively. The temperature profile represents a typical shape which is typical of enzyme kinetics whereby the rate of enzymatic reaction increases as temperature increases. However, at higher temperatures there is disruption of the enzyme structure and subsequent denaturation of the enzyme.

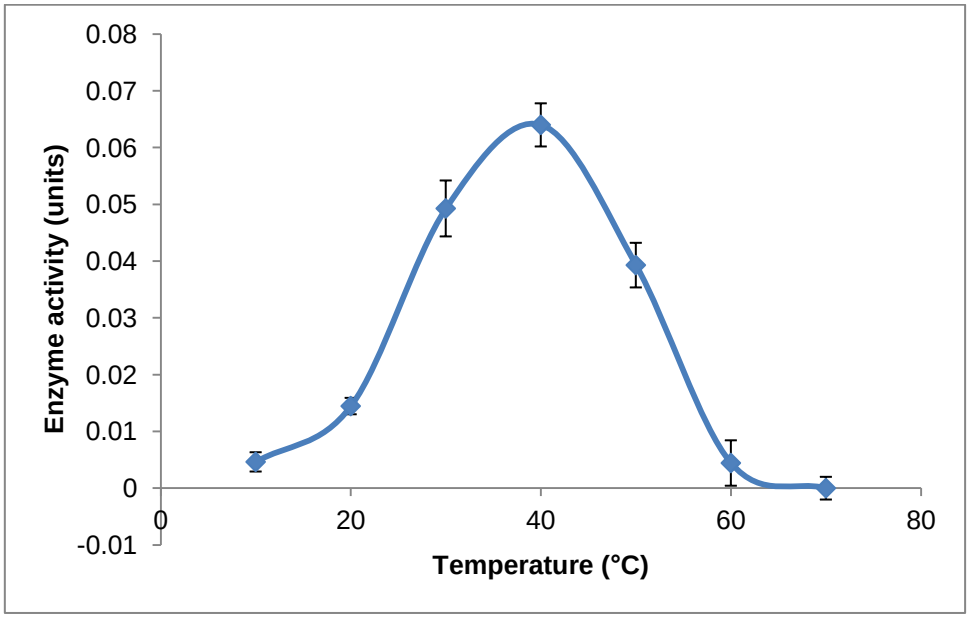


Figure 4.13 Temperature optimum of *H. pylori* 369C urease. The data are the average of three experiments and bars indicate standard deviations.

4.3.8.3 Thermal stability

Thermostability study was performed and exhibited that the urease in the crude extract was highly stable until to a temperature of about 40 ^{o}C (Fig. 4.16).

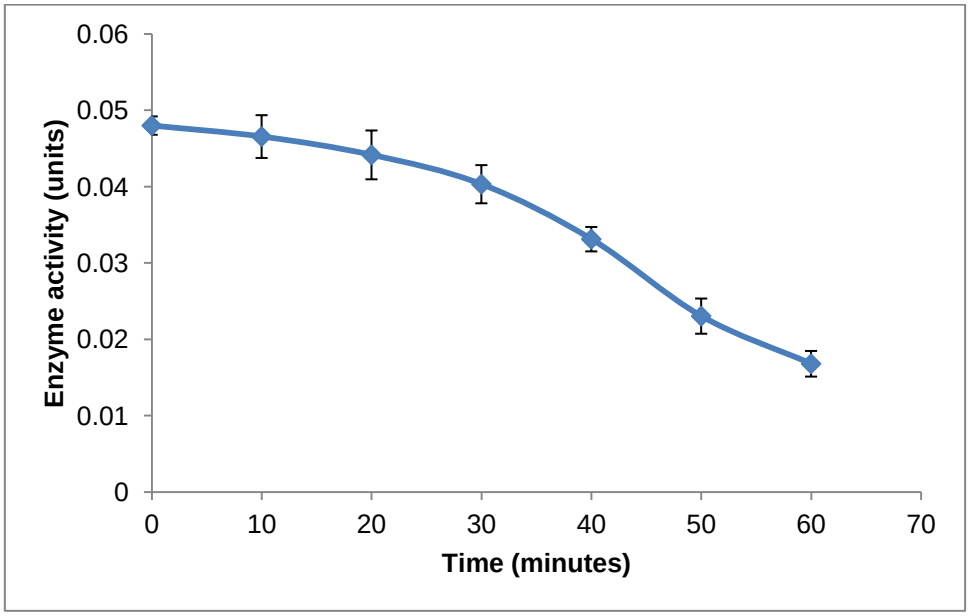


Figure 4.14 Thermal stability profile of *H. pylori* 369C urease. The data are the average of three experiments and bars indicate standard deviations.

When the temperature rose above this the urease was highly unstable that are no factors in the crude extract that we had thought would stabilize the enzyme against thermal shock.

4.3.9 Estimation of kinetic parameters (K_m and V_{max})

The Michaelis-Menten plots clearly shows that at low substrate concentrations the rate of reaction increases more or less linearly with the substrate concentration while, at higher substrate concentrations the reaction rate becomes constant. The Michaelis constant, K_m, can be obtained by interpolation since it is the substrate concentration at which the reaction rate is half of its maximum value ($V_{max}/2$), which is the reason why K_m is also called the half-saturation value.

However, it is difficult to specify the exact substrate concentration corresponding to K_m from this hyperbolic plot. For this reason, Lineweaver-Burk plots were used because they allow accurate measurements of the values of both K_m and V_{max} even at higher substrate concentrations.

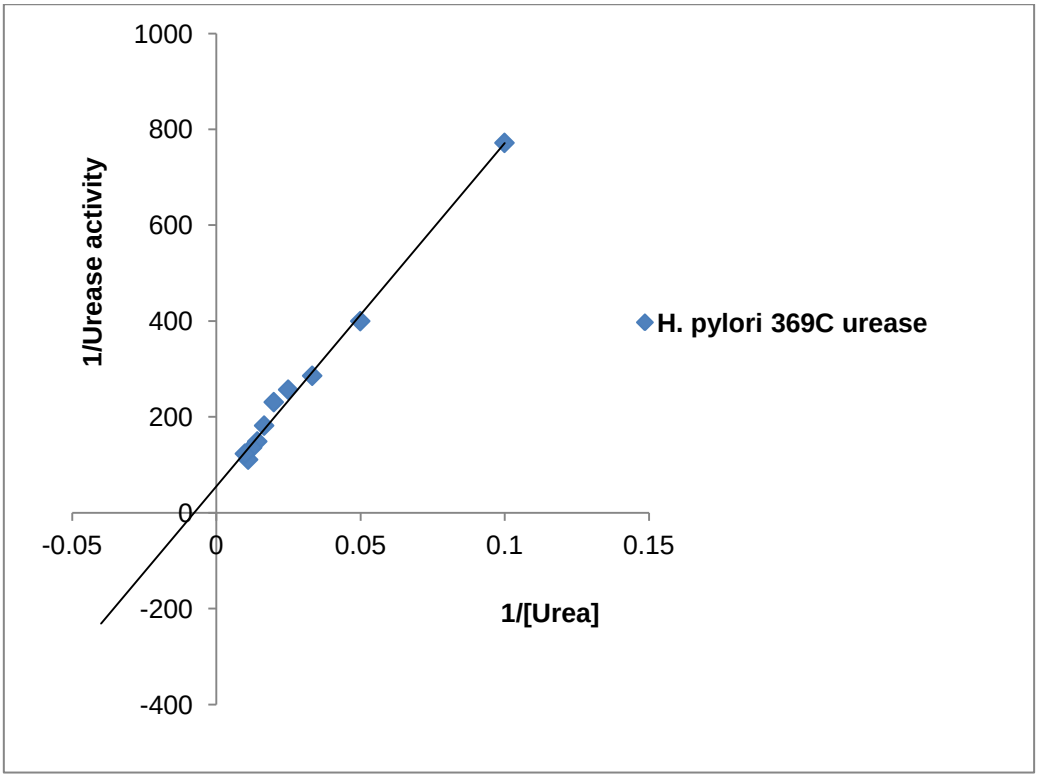


Figure 4.15 Lineweaver-Burk plot of *H. pylori* 369C urease. The data are the average of three experiments.

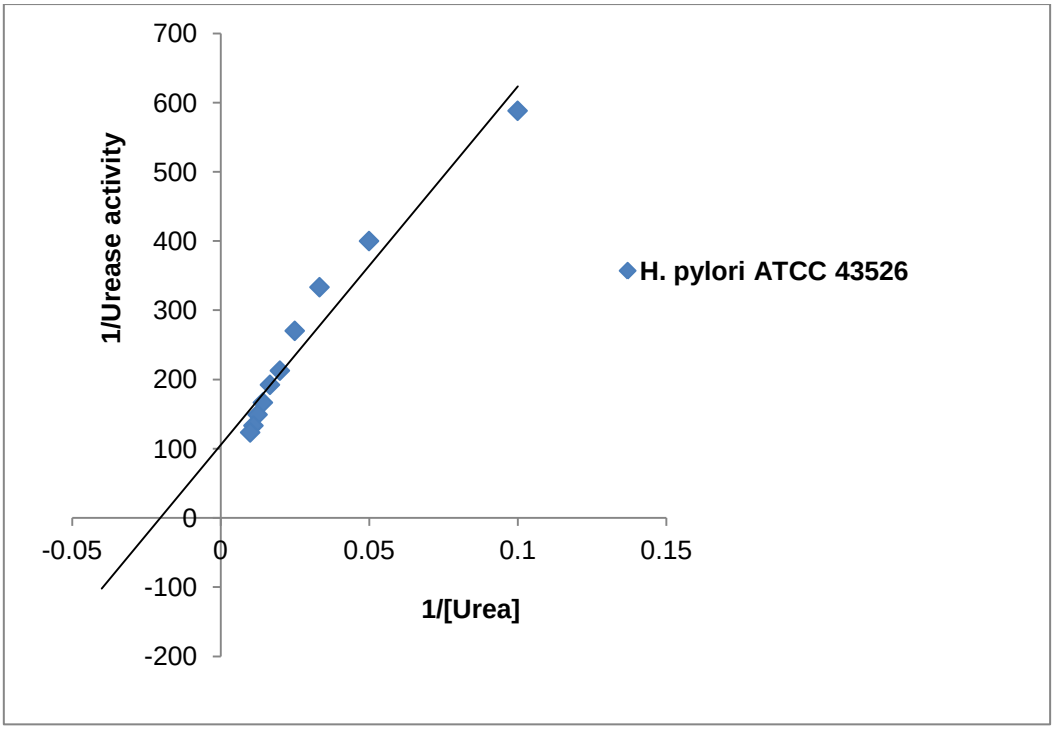


Figure 4.16 Lineweaver-Burk plot of *H. pylori* ATCC 43526 urease. The data are the average of three experiments.

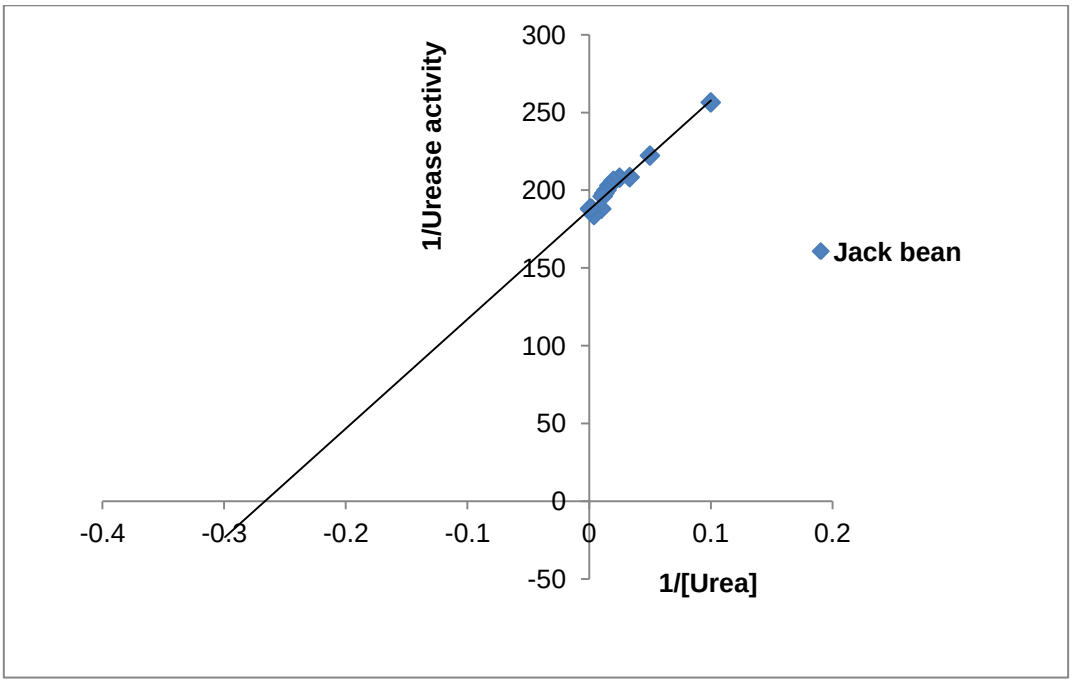


Figure 4.17 Lineweaver-Burk plot of Jack bean urease (control). The data are the average of three experiments.

From these plots, the following K_m and V_{max} were found:

Table 4.1 Kinetic parameters of urease.

Enzyme	**K_m (mM)**	**V_{max} (µmol/min/ml)**	**Literature values**	**References**
Jack bean urease	3.43	190.41	2.9 -3.6	Blakeley *et al*.1969 Dixon *et al*. 1980 Krajewska *et al*.1999
H. pylori ATCC 43526 urease	40.07	98.25	0.2 – 0.8	Mobley *et* al. 1988 Hu and Mobley (1990) Dunn *et* al. 1990
H. pylori 369C urease	61.11	45.32		

In this study, crude ureases were tested for kinetic properties. Jack bean urease, a commercial purified enzyme was used as a control. Generally, K_m values can be obtained from crude cell extracts. In this study, we found that the urea K_m values of purified *H. pylori* ureases from literature did not agree with the values observed for crude cell extracts. A possible explanation for this is that some buffers interfere with urease activity and therefore leads to inaccurate K_m determinations. Thus, different values can be reported as the K_m values vary with buffer, pH and assay conditions.

We also reported different K_m values for the two strains of *H. pylori*. This observation indicates that different strains of the same species possess different K_m values (Table 4.1).

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Helicobacter pylori infects about half of the world's population (Go, 2002). Infection by this bacterium is the main cause of various gastric diseases such as gastritis, gastric lymphoma, peptic ulcers, and stomach cancer (Go, 2002). The fact that the organism is ubiquitous worldwide, responsible for significant morbidity and mortality, and is intricate to eradicate makes it a prime target for novel therapeutic approaches. However, the advancement of successful eradication therapies based on typical antibiotics has shown to be very problematic seeing that though treatment regimens, have confirmed rational levels of eradication, the occurrence of strains resistant to these antibiotics is a serious cause of concern. The regimens have also put up substantial problems such as adverse side effects and poor compliance amongst patients (Ndip *et al*., 2008). In view of these challenges and other obstacles that plague attempts to eliminate *H. pylori* infection, novel therapeutic regimens are essential and urease inhibitors may play a role in these future therapies.

To date, several urease inhibitors have been discovered. Although most of them exhibit great pharmoco dynamic and pharmaco kinetic properties they have not been used for therapeutic purposes because of their adverse effects and toxic effect *in vivo* (Xiao *et al*., 2007; Von Kreybig *et al*., 1968). For these reasons, much attention now focuses on exploring the novel urease inhibitory activities of natural products because of their low toxicity and good bioavailability.

Natural products like honey have been used since primeval times in folk medicine all over the world due to its many biological properties such as antitumor, antioxidant, antimicrobial and anti-inflammatory among others (Meda *et al*., 2005).

In light of this research, honey has been found to inhibit *Helicobacter pylori* growth *in vitro*. The mechanism by which honey inhibits this bacterium's growth has not been fully elucidated. Since urease is one of the major virulence factors in the pathogenesis of this bacterium, investigations on the effect of honey solvent extracts on the enzyme activity were carried out. This current study investigated the potential of honey to inhibit urease activity of *H. pylori*.

It can be concluded from this study that, the chloroform extract of "Pure honey" showed stronger inhibition of urease activity than the diethyl ether extract of "Manuka honey". Further studies are needed to isolate and identify the active compounds from the solvent extracts of these honeys. The sonication conditions which gave the best extraction method of urease from H pylori were found to be at low amplitude (20W) and a time five minutes. Urease was found to be both surface-associated and cytoplasmic. After identifying and optimizing the extraction of both forms of the enzyme, a time course survey was carried out to determine optimal time at which highest enzyme production occurs. Maximum cytoplasmic urease activity was found to occur after 72 hr whereas maximum extracellular urease activities were found to occur after 96 hr.

Following this, solvent extracts of two honeys from different geographical locations were tested for their inhibitory effects on partially purified intracellular ureases. We observed that both honey extracts possess some inhibitory effects on urease activity. The effects of varying concentrations of these extracts on the activity of urease at pH 7.6 and 25^{o}C was then explored. It was found that both extracts inhibited urease activities in a concentration dependent fashion. The two honey extracts were also examined in a series of urease inhibition assays to explore the effect of pre-incubation time on the inhibition of urease activity.

It was found that enzyme inhibition was independent of incubation time.'Pure Honey' extract showed to be a more potent inhibitor for 369C urease (I = 48%) than for ATCC urease (I = 42%) and the control, Jack bean urease (I = 13.3 %). Whereas, Manuka Honey extract showed to be a fairly more potent inhibitor for ATCC urease (I = 51.3%) than for 369C urease (I = 45 %) and the control, Jack bean urease (I = 11%). The kinetic data indicates that these extracts inhibit urease in a non-competitive manner. The inhibitory activities of these two honeys from different geographical locations were thus almost similar. These honeys did not display any uniqueness toward the inhibition activities.

After isolation it was necessary to purify and characterize the urease enzyme and two strategies were used and compared. The purification was done for ureases from both strains (*H. pylori* 369C and the reference strain, *H. pylori* ATCC 43526). The 369C urease concentrated by freeze drying had one peak containing urease activity on Superdex 200 pg with a specific activity of 203.7 µmol of urea hydrolysed/min/mg and a purification fold of 14.3. The ATCCC 43526 urease concentrated by ammonium sulphate precipitation also had one peak containing urease activity on Superdex 200 pg with a specific activity of 264.7 µmol of urea hydrolysed/min/mg and a purification fold of 5.66.

Analysis of the fractions from both strains by a 12% SDS-PAGE showed three distinct bands of molecular sizes which are in the same magnitude as other ureases purified from H pylori. Characterisation studies also showed that *H. pylori* 369C urease operates optimally at a pH of 7.5 and temperature of 40°C. Kinetic parameters K_m and V_{max} for substrate urea were found to be 61.11 mM and 45.32 µmol/min/ml respectively.

The overall objective of the present study was to inhibit urease activity using a urease extract, however, experiments conducted using the crude urease were not able to give IC_{50} at concentrations below 100% (v/v). The most likely explanation could be that the solvent honey extracts were not pure enough to effectively bring about inhibition. This problem can be overcome by isolation of pure compounds from the active fractions. From the important results of this study, it can be suggested that the screening against urease using urease inhibitory assay is a useful tool to guide the separation and purification process of the active urease inhibitor compounds from honeys from different geographic locations. The honey varieties are important and promising natural resources for the discovery of novel bioactive compounds such as natural urease inhibitors which have the potential to become safe and powerful anti-*H. pylori* drugs.

Future work involves

1. Establish further *H. pylori* urease purification with high yield and purity.
2. The chromatographic analysis of the pure compounds from the active fractions.
3. Establish the enzymatic mechanism for the inhibition of urease by the pure compounds from honey extracts.

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APPENDICES

Appendix A: Buffers

A1: Phosphate - buffered saline (pH 7.4)

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
Deionized water	800ml

Adjust pH to 7.4 with HCl/NaOH before filling up to 1litre with deionized water. Store at 4 ^oC.

A2: Potassium phosphate buffer (0.1M, pH 7.6)

1M K_2HPO_4	86.6 ml
1M KH_2PO_4	13.4 ml
Deionized water	900ml

Adjust pH to 7.6 with HCl/NaOH before filling up to 1litre with deionized water. Store at 4 ^oC.

A3: Na-citrate buffer (0.1M, pH 5.5)

0.1M Citric acid	4.7 volumes
0.1 M Na_3 citrate	15.4 volumes

A4: Sodium bicarbonate (1M)

Na_2CO_3	132g
Deionized water	900ml

This was made up to 1 litre with deionized water.

A5: Gel-permeation buffer (50mM NaH_2PO_4, 150mM NaCl, 1 mM EDTA); pH 7)

NaH_2PO_4	5.999g
NaCl	8.766g
EDTA	0.372g
Deionized water	900ml

Adjust pH to 7 with NaOH before filling up to 1litre with deionized water. Store at 4 ^oC.

A6: Urease screening buffer [(3 mM NaH_2PO_4, 110 mM urea, 7 µg ml^{-1} phenol red), pH 7.6]

NaH_2PO_4	0.360g
Urea	6.607g
Phenol red	0.007g
Deionized water	900ml

Adjust pH to 7.6 with NaOH before filling up to 1litre with deionized water. Store at 4 ^oC.

Appendix B: **Skirrow's antibiotics preparation**

The *antibiotic* supplement was prepared per vial. To rehydrate the contents of the vial 2 *ml* of sterile *distilled water was added followed by* mixing well to dissolve*.* Aseptically the contents of the vial were added to 500 ml of sterile, molten Columbia Agar with 7% v/v defibrinated horse blood.

Appendix C: **Method of protein determination - Bradford assay (Bradford, 1976)**

C1: Protein stock solution (2mg/ml)

Dissolve 0.02g of Bovine serum albumin (BSA) in 10 ml of ddH2O.

C2: Protein standard curve

Different protein concentrations were prepared from the BSA stock solution (2mg/ml) as shown in Table A1. A protein standard curve was generated by reading the absorbance of these different concentrations at 595nm (Figure I). For the test sample, 3ml Bradford reagent was added to 0.1ml diluted sample and the absorbance read at 595nm.

Table C1: Preparation of protein standard curve

Tube	Protein concentration (mg/ml)	BSA Stock solution (2 mg/ml) (ml)	Buffer(ml)	Bradford reagent(ml)
1	0	0	1	3
2	0.25	0.125	0.875	3
3	0.50	0.250	0.750	3
4	0.75	0.375	0.625	3
5	1.00	0.500	0.500	3
6	1.25	0.625	0.375	3
7	1.50	0.750	0.250	3

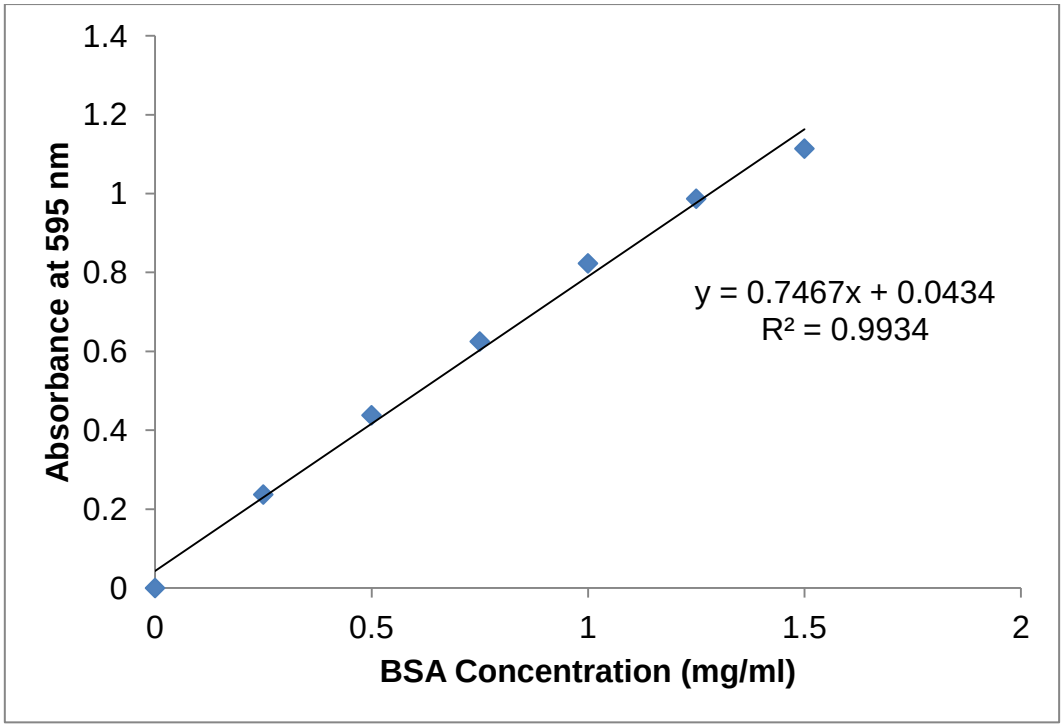

Figure I Protein Standard Curve.

<u>Appendix D</u>: Methods used for purification and partial characterization of *Helicobacter pylori* urease

D1 SDS-PAGE

Solution A: Acryl amide stock solution (30% acryl amide, 0.8% bis-acryl amide). Weigh 29.2g acryl amide and 0.8g bis-acryl amide, and then dissolve in 100ml de-ionized water and stir until acryl amide powder is completely dissolved. Store in the dark at 4 °C.

Solution B: 10% (w/v) SDS – Dissolve 10g SDS in 90 ml water with gentle stirring and bring to 100 ml with deionized water.

Solution C: 4x Separating Gel buffer – Add 75ml of 2M Tris-HCl buffer (pH 8.8) and 4ml of 10% SDS and then make up the volume to 100ml with deionized water.

Solution D: 4X Stacking Gel buffer – Add 50ml of 1M Tris-HCl buffer (pH 6.8) and 4ml 10% SDS, and make up the volume to 100ml with deionised water.

Solution E: 10% Ammonium per sulphate solution (APS) - Dissolve 0.5g ammonium persulfate in 5ml deionised water.

Solution F: Electrophoresis buffer – Add together 3g Tris base, 14.4g glycine and 1g SDS. Dissolve all three in 1 litre of deionised water.

Solution G: 5× Sample buffer – Add together 0.6ml 1M Tris-HCl buffer (pH 6.8), 5ml 50% glycerol, 2ml 10% SDS, 0.5ml 2-mercaptoethanol, 1ml 1% bromophenol blue, and make up the volume to 10ml with deionised water.

Solution H: Coomassie blue staining solution – Dissolve 1.0g Coomassie Blue R250 in a mixture of 450ml methanol, 450ml deionised water and 100ml glacial acetic acid.

Solution I: Coomassie Gel Destain solution – Add together 100ml methanol, 100ml glacial acetic acid and 800ml de-ionized water.

Table D1 Recipe for Resolving Gel

Solutions	**Volume (µl)**
DDI H_2O	3400
30% Stock Acryl amide	4000
1.5M Tris- HCl pH 8.8	2500
10% (w/v) SDS	100
10% Ammonium per Sulphate	50
TEMED	5

Table D2 Recipe for stacking gel

Solutions	**Volume (µl)**
DDI H_2O	6100
30% Stock Acryl amide	1300
0.5M Tris- HCl pH 6.8	2500
10% (w/v) SDS	100
10% Ammonium per Sulphate	50
TEMED	10

<u>Appendix E</u>. Calculation of the number of grams of ammonium sulphate (g) to add to 1 litre sample to give desired concentration (per cent saturation)

$$g = \frac{533\ (S2 - S1)}{100 - 0.3\ S2}$$

S1: Starting concentration

S2: Final concentration