

THE DEVELOPMENT OF A SOLID PHASE SCREENING ASSAY
FOR THE DETECTION OF DIPEPTIDE SYNTHESIS

by

ELIZA YUE

B.S. (Agr.), The University of British Columbia, 1989

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Food Science)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

December 1995

© Eliza Yue, 1995

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Food Science

The University of British Columbia
Vancouver, Canada

Date February 5, 1996

ABSTRACT

A rapid screening assay was developed to detect formation of DNP-asn-leu dipeptides on a special type of microtiter plate, called the Nunc Immuno Module. Secondary amino groups, to which small molecules can be coupled, were grafted onto the surface of the plate. The carboxyl group on L-leucine molecules, activated in the presence of 0.1 M N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, were covalently coupled to these amino groups; 11.04 μg of L-Leucine was used in the coupling reaction. The reaction mixture, consisting of either commercial grade thermolysin or crude protease mixture isolated from *Bacillus subtilis* culture, DNP(dinitrophenyl)-L-asparagine and pH 6 sodium acetate buffer solution, was subsequently added to the wells. During incubation at 48°C, dipeptide formation occurred between the immobilized leucine and the free DNP-L-asparagine in the liquid phase. After washing off unreacted DNP-L-asparagine, the final product was detected by the addition of an antibody-peroxidase conjugate, which reacted specifically with the dinitrophenyl group attached to the amino group of the asparagine molecule. The amount of increase in optical density measured at 492 nm was proportional to the relative amount of dipeptides formed on the microtiter plate. Two-way analysis of variance showed that the effect of enzymatic treatment on the mean optical density measured at 492 nm was significant ($P < 0.01$).

TABLE OF CONTENTS

ABSTRACT.....	ii
TABLE OF CONTENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
ACKNOWLEDGEMENTS	ix
 INTRODUCTION	 1
LITERATURE REVIEW	5
I. Enzymatic peptide synthesis	5
a. Advantages and disadvantages.....	5
b. Examples of useful peptides synthesized by protease-catalysed reactions	6
c. Methods for detecting peptide formation ...	7
II. Proteases	9
a. Thermolysin	9
b. Neutral protease T	12
III. Immunoassay techniques	13
a. Radioimmunoassay	13
b. Advantages of enzyme linked immunosorbent assay	15
c. Principle of direct and indirect ELISA	15
d. Choice of enzyme and substrate	16
e. Microtiter plates	19
IV. <i>Bacillus subtilis</i>	21
a. Use of <i>Bacillus subtilis</i> as a host for production of heterologous proteins	21
b. Problems associated with using <i>B. subtilis</i> expression systems	23
 MATERIALS AND METHODS	 25
MATERIALS	25
METHODS	26
I. Thermolysin catalysed solid-phase synthesis of DNP-asn-leu dipeptide	26
a. Immobilization of DNP(Dinitrophenyl)- L-leucine or L-leucine to microtiter plates	26
b. Detection of immobilized amino acid or dipeptide	29
c. Solid-phase enzymatic DNP-asn-leu dipeptide synthesis	30

METHODS

II.	Enzymatic catalysed solid-phase synthesis of DNP-asn-leu dipeptide using crude enzyme mixture from <i>Bacillus subtilis</i> transformants	32
a.1.	Transformation of <i>B. subtilis</i> DB428 and WB600	32
a.2.	Confirmation of positive transformants by restriction enzyme digestion of plasmid DNA	33
a.3.	Preparation of crude proteases from <i>Bacillus subtilis</i> transformants	34
b.	Enzymatic solid-phase synthesis of DNP-Asn-Leu dipeptides using either concentrated or unconcentrated crude enzyme fraction from <i>Bacillus subtilis</i> (pNP22) transformants as catalysts	38
III.	Chemical assays	38
a.	Protease assay using casein as a substrate ..	38
b.	Protease assay using azocasein as a substrate	39
c.	Protein assay using BCA protein assay reagent	41
d.	SDS-gel electrophoresis of thermolysin and secreted crude proteases from transformed <i>B. subtilis</i> DB428 and WB600	42
IV.	Methods for statistical analysis	44
RESULTS AND DISCUSSIONS.....		45
I.	Immobilization of DNP-L-leucine to the solid phase	45
a.	Detection of immobilized leucine molecule ..	45
b.	Binding of DNP-L-leucine to conventional microtiter plate	48
c.	Non-covalent binding of DNP-L-leucine	48
d.	Quantification of the amount of DNP-L-leucine immobilized to the microtiter plate	49
II.	Thermolysin catalysed solid phase synthesis of DNP-Asn-Leu	50
a.	Selection of blocking agent	50
b.	Detection of the formation of DNP-Asn-Leu ..	51
c.	Effect of the concentration of DNP-L-asn on dipeptide formation	62
d.	The effect of reaction time on dipeptide synthesis	63
e.	The effect of thermolysin concentration on dipeptide synthesis	64
f.	Statistical Analysis	64

RESULTS AND DISCUSSIONS

III. DNP-Asn-Leu reactions catalysed by crude enzyme fraction secreted by bacterial culture	65
a. Use of <i>Bacillus subtilis</i> DB 428 and WB600 to produce enzyme mixture	67
b. Effect of varying the concentration of enzyme in the reaction mixture	70
c. Effect of varying the concentration of DNP-L-Asn	83
d. Comparison of peptide synthesis ratio for thermolysin and crude enzyme preparation ...	84
e. Statistical analysis	85

CONCLUSIONS	91
-------------------	----

REFERENCES	93
------------------	----

LIST OF TABLES

Table 1.	The optimal concentration of DNP-L-leucine for coupling on solid phase	46
Table 2.	The efficiency of various blocking agents	52
Table 3.	Reaction conditions for thermolysin-catalysed solid-phase synthesis of DNP-Asn-Leu	53
Table 4.	2-Way analysis of variance on optical density readings for five thermolysin catalysed Leu-Asn synthesis experiments	66
Table 5.	Extracellular protease activity from wild-type (168) and protease-deficient (DB428 and WB600) strains of <i>Bacillus subtilis</i>	68
Table 6.	Results of ultrafiltration of crude enzyme secreted by transformed <i>Bacillus subtilis</i> DB428.....	71
Table 7.	Reaction conditions for thermolysin and crude enzyme catalysed solid phase synthesis of DNP-Asn-Leu	72
Table 8.	Summary of the protease activity of thermolysin determined by utilizing two different substrates: Hammerstein casein and azocasein	73
Table 9.	2-Way analysis of variance on O.D. _{492nm} in micro-titer wells from five experiments of thermolysin or neutral protease T catalysed synthesis of Asn-Leu dipeptides	86

LIST OF FIGURES

Figure 1.	3-Dimensional structure of thermolysin	10
Figure 2.	Schematic representation of the active site of thermolysin with a polypeptide substrate bound ...	10
Figure 3.	Schematic chemical and physical configuration of the Covalink NH surface	27
Figure 4.	Covalent coupling of amino acid to Nunc Immuno Module	28
Figure 5a.	Restriction map of pNP22	35
Figure 5b.	Analysis of pNP22 recombinant plasmid with restriction endonucleases	36
Figure 6.	Standard curve for protease assay with Hammerstein casein used as a substrate	40
Figure 7.	Standard curve for BCA protein assay	43
Figure 8.	Immobilization of DNP-L-leucine to Nunc Immuno Module	47
Figure 9.	Exp. 1. Thermolysin catalysed synthesis of DNP-Asn-Leu	54
Figure 10.	Exp. 2. Thermolysin catalysed synthesis of DNP-Asn-Leu	55
Figure 11a.	Exp. 3a. Thermolysin catalysed synthesis of DNP-Asn-Leu	56
Figure 11b.	Exp. 3b. Thermolysin catalysed synthesis of DNP-Asn-Leu	57
Figure 11c.	Exp. 3c. Thermolysin catalysed synthesis of DNP-Asn-Leu	58
Figure 11d.	Exp. 3d. Thermolysin catalysed synthesis of DNP-Asn-Leu	59
Figure 12.	Exp. 4. Thermolysin catalysed synthesis of DNP-Asn-Leu	60
Figure 13.	Exp. 5. Thermolysin catalysed synthesis of DNP-Asn-Leu	61

Figure 14.	SDS-gel electrophoresis of thermolysin and crude proteases secreted by transformed <i>B. subtilis</i> DB428 and WB600	69
Figure 15.	Exp. 6. Thermolysin and crude enzyme catalysed synthesis of DNP-Asn-Leu	74
Figure 16a.	Exp. 7a. Crude enzyme catalysed synthesis of DNP-Asn-Leu	75
Figure 16b.	Exp. 7b. Crude enzyme catalysed synthesis of DNP-Asn-Leu	76
Figure 16c.	Exp. 7c. Crude enzyme catalysed synthesis of DNP-Asn-Leu	77
Figure 16d.	Exp. 7d. Crude enzyme catalysed synthesis of DNP-Asn-Leu	78
Figure 17.	Exp. 8. Crude enzyme catalysed synthesis of DNP-Asn-Leu	79
Figure 18.	Exp. 9. Thermolysin and crude enzyme catalysed synthesis of DNP-Asn-Leu	80
Figure 19.	Exp. 10. Thermolysin and crude enzyme catalysed synthesis of DNP-Asn-Leu	81
Figure 20.	Calculation of the maximum concentration of leucine immobilized on Nunc Immuno Module	88

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. S. Nakai, who supervised this study and provided advice throughout the course of this thesis. Appreciation is also extended to the members of the research committee Dr. B. Dill, Dr. B. Skura and Dr. R. MacGillivray for their guidance and constructive advice. Special thanks to Dr. E. Li-Chan and Dr. A. Matsuyama for their patience and for providing me with a great deal of technical assistance on my project.

I am also particularly grateful to my friend Grace Lee, who reviewed my thesis for me, and to Shirley Joe, who helped me to type part of my written report in spite of her very tight "work" schedule. Lastly, I wish to thank my grandmother for her invaluable encouragement and unconditional support.

INTRODUCTION

Various studies have shown that enzymes can be used to catalyse peptide bond formation by reverse proteolysis. The enzymatic method may be preferred over conventional chemical synthesis because the reactions can be performed stereospecifically and under very mild conditions. In addition, the functional groups of amino acid side chains do not require protection since proteases show specificity for the α -carboxyl or α -amino group at the reaction site (Sakina et al., 1988). The enzymatic method is especially suitable for the preparation of peptides useful in the food industry, such as aspartame, because there is less need for hazardous chemicals.

Modification of the physical and chemical properties of various enzymes is possible by the use of genetic engineering techniques. As an example, a tremendous amount of protein engineering research has been done on subtilisin, which is one of the most thoroughly characterized bacterial serine proteases secreted by a variety of *Bacillus* species. Some of the physical and chemical characteristics of subtilisin, including its thermostability, alkaline stability, as well as its ability to resist chemical oxidation, have been modified by using either site-directed mutagenesis or random mutagenesis techniques to introduce changes in the nucleotide sequence encoding the enzyme (Estell et al., 1985; Cunningham and Wells., 1987; Takagi et al., 1990; Takagi, 1993; Egmond et al., 1994; Heringa, 1995). Hence, it may also be possible to improve the reverse proteolysis property of an

enzyme by using similar genetic engineering methods.

Thermolysin, a thermostable proteolytic enzyme secreted by *Bacillus thermoproteolyticus*, has been intensively characterized: the primary and tertiary structures, the active site and substrate-binding site have been determined (Bigbee & Dahlquist, 1974; Matthews et al., 1972; Titani et al., 1972). Since many papers have been published on the use of thermolysin in promoting peptide bond formation, it may be worthwhile to attempt to further improve the peptide bond synthesis property of this enzyme by applying site-directed or random mutagenesis techniques. One of the original objectives of this thesis was to randomly mutagenize targeted regions of the *nprT* gene, from *Bacillus stearothermophilus*, which encodes a thermostable neutral protease T. Since the amino acid sequence of this protease is 85% homologous to that of thermolysin, the two proteases may have similar 3-dimensional structure as well as other physical characteristics (Takagi et al., 1985). However, in order to modify the reverse proteolysis property of neutral protease T, it is imperative to first develop a rapid and efficient screening system that is capable of identifying positive mutants, which produce enzymes with enhanced peptide bond synthesis activity, from a large number of transformants. This would require a simple assay system that is capable of detecting the presence of newly synthesized peptides. The two methods that are commonly used to detect enzymatically and chemically synthesized peptides include reverse-phase high performance liquid chromatography and thin layer

chromatography. However, neither method is suitable for rapid screening purposes.

The use of microtiter plates in an enzyme linked immunosorbent assay (ELISA) greatly facilitates the handling and washing of samples. When used with automated readers and multiple well washers, these plates allow large numbers of samples to be assayed within a relatively short time. Therefore, it would be advantageous if a similar kind of solid-phase assay system could be developed for the detection of dipeptides formed from enzyme-catalysed reverse proteolysis reactions. As in ELISA, one of the substrates (i.e. one of the amino acids) may be immobilized to the microtiter plate. The second substrate will then be added to the well along with the target enzyme. The second amino acid needs to be labelled for example, by another enzyme or by radioactive isotopes, so that it will provide a signal that is readily detectable. When a peptide bond is formed between the immobilized substrate and the free amino acid in the liquid phase, the resulting dipeptide will be bound to the well and can be easily detected by the label on the second amino acid. This type of solid-phase assay could provide a rapid screening system for positive mutants generated from mutagenesis experiments as the use of microtiter plates would make routine handling of a large number of bacterial cultures relatively easy. Therefore, the objective of this research was to develop a rapid and simple ELISA-like solid-phase assay for the detection of dipeptides formed from enzyme catalysed reactions. L-leucine and DNP-L-asparagine would be used

as the substrates for dipeptide synthesis. Initial synthesis experiments were to be catalysed by commercial grade thermolysin. Since thermolysin is commercially available in a concentrated form, its higher enzyme activity would likely ensure that some DNP-asn-leu would be formed in the microtiter wells. If the experiments using thermolysin were successful, the next step would be to use crude proteases, isolated from bacterial cultures, as catalysts. These data would be useful in determining if this rapid assay is sensitive enough for the purpose of screening crude unpurified mutant protease T enzymes secreted by bacterial cultures.

LITERATURE REVIEW

I. ENZYMATIC PEPTIDE SYNTHESIS

a. Advantages and Disadvantages

Proteases have been shown to be useful in catalysing peptide bond synthesis by reverse proteolysis (Cheng et al., 1988; Miranda and Tominaga, 1991; Morihara and Oka, 1980; Morihara and Oka, 1980; Morihara, 1987; Oka and Morihara, 1978; Sakina et al., 1988; Steinke et al., 1991). Enzymatic methods may be preferred over the conventional chemical methods of synthesizing useful peptides for various reasons. First of all, reactions can be performed stereospecifically. Therefore, purification of the final product should be easier and less costly since there is no need to remove undesired stereoisomers. Secondly, there is less need for expensive protection groups. Chemical synthesis requires the use of protecting groups on functional groups of amino acid side chains, which should not participate in the peptide bond formation (Bodanszky, 1988). In contrast, enzymatic synthesis of peptides can be performed without protection of these functional groups because proteases show specificity for the α -carboxyl or the α -amino group at the active site (Sakina et al., 1988). Thirdly, product yield may be higher with enzymatic synthesis mainly because purification of the final product should be less complicated than for the final product from conventional chemical production methods. Lastly, enzymatic reactions can be carried out under very mild conditions while chemical reactions often require high temperature and high pressure.

One of the major problems with using proteases in catalysing peptide synthesis is related to their narrow substrate specificity and the narrow range of conditions these enzymes can tolerate (Lars et al., 1991; Steinke et al., 1991). Often, very stringent temperature, pH, or ionic conditions are required in order for an enzyme to catalyse a reaction (Chen and Arnold, 1991). Another problem is related to the fact that some enzymes are fairly expensive (Moriyama, 1987). However, this problem may be overcome by the use of immobilized enzymes. Lastly, hydrolytic activities of proteases could lead to the formation of undesired by-products and to a decrease of product yield (Wong, 1992). Since water limits yield because of hydrolysis of the enzyme-substrate intermediate or of the final product, product yield may be enhanced significantly if the reaction is carried out in the presence of mixed or pure organic solvents instead of water (Chen et al., 1991). However, proteases are generally unstable in organic solvent, which quickly denatures and inactivates enzymes. For example, the half-life of subtilisin BPN' in anhydrous dimethylformamide is only about 20 minutes, which is hardly sufficient for large-scale production process (Wong, 1992). Protein engineering studies have been conducted by various research groups in an attempt to produce proteases with enhanced stability in organic solvents (Chen and Arnold, 1991; Martinez et al., 1992; Wong et al. 1990).

b. Examples of Useful Peptides Synthesized by Protease-Catalysed Reactions

Some proteases have been successfully used in enzyme-assisted

synthesis of useful peptides and peptide hormones. For example, thermolysin has been used to prepare the precursor of aspartame, which is then converted to aspartame by conventional chemical methods (Moriyama, 1987). Paul et al. (1988) demonstrated that aspartame could be directly synthesized from the coupling of L-aspartic acid to L-phenylalanine methyl ester in a reverse hydrolysis reaction catalysed by peptidase enzyme, which was isolated from a gram-positive coccus. Metenkephalin has been prepared by fragment condensation using papain (Moriyama, 1987). The δ -sleep inducing peptide was made in a series of reactions using only papain, except in a coupling step which was catalysed by α -chymotrypsin (Sakina et al., 1987). Industrial production of human insulin from swine is accomplished by digestion of porcine insulin with *Achromobacter* protease I, and then coupling with a threonine group using trypsin or *Achromobacter* protease I (Moriyama et al., 1986). This enzymatic synthesis reaction resulted in over 90% product yield while the yield of traditional chemical synthesis is much lower (about 10%).

c. Methods for Detecting Peptide Formation

The two methods that are commonly used for the detection and quantification of enzymatically and chemically synthesized peptides are reversed phase HPLC (high performance liquid-chromatography) and thin layer chromatography (TLC). For example, Moriyama et al. (1986) used a reversed-phase HPLC with a column of Nucleosil 5C₁₈ to quantitatively determine the semisynthesis of human insulin ester

Paul et al. (1988) detected formation of aspartame and its derivatives using reverse-phase HPLC with a μ Bondapack C18 column. Synthesis of Cbz-Phe-Leu-NH₂ was identified and product yield was determined by thin layer chromatography (Oka and Morihara, 1978). Formation of di- and tripeptide esters in reactions catalysed by thermolysin was detected by TLC using four different solvent systems for development (Miranda and Tominaga, 1991).

In reversed phase HPLC, proteins are separated according to their degree of hydrophobicity by interactions between the exposed nonpolar amino acid side chains on the protein and hydrophobic groups on the chromatographic matrix (Goheen and Stevens, 1985). Qualitative analysis is accomplished by either comparing the retention times or volumes of the synthesized peptides to the retention times of the standards, or by collecting the individual components as they emerge from the chromatograph and then identifying the components by other methods (Pearse, 1980). The coupling yield is usually calculated from the peak areas or peak heights corresponding to each component.

With thin-layer chromatography, the sample is spotted at the lower end of a glass, which has been precoated with a thin layer of adsorbent, such as alumina or silica gel. The solvent is then drawn upwards by capillary attraction. As the solvent moves past the sample the components of the sample selectively dissolve and are vertically displaced on the gel. The amount of movement of the different components on the gel is dependent upon the solvent flow

rate and selective retention of solutes by the stationary phase. The separated peptides and their starting materials are identified readily if colored. Otherwise, it may be necessary to detect fluorescence which is initiated with an ultraviolet lamp or "develop" colored compounds by spraying the separated spots with a colour-forming reagent (Pearse, 1980). For example, ninhydrin is used for producing color with amino acids containing spots on the plates. The quantity of each component can be determined by comparing the size of the migration spots of the separated peptides with those of the known standards. Alternatively, the colour intensities of the spots corresponding to the starting carboxyl components and the product can be measured using a TLC-scanner.

II. PROTEASES

a. Thermolysin

Thermolysin (EC 3.4.24.2) is an extracellular metallo-endopeptidase isolated from *Bacillus thermoproteolyticus*. It is most active at neutral pH. The enzyme has a molecular weight of 34.6 kDaltons and is made up of a single polypeptide chain of 316 amino acid residues, lacking thiol groups or disulphide bonds (Fontana, 1988). Thermolysin is one of the most well-characterized enzymes owing to extensive x-ray crystallographic and kinetic studies.

The 3-dimensional structure of thermolysin (Figure 1) has been elucidated and the mechanism for its peptide cleavage activity has been proposed based on crystallographic studies of various

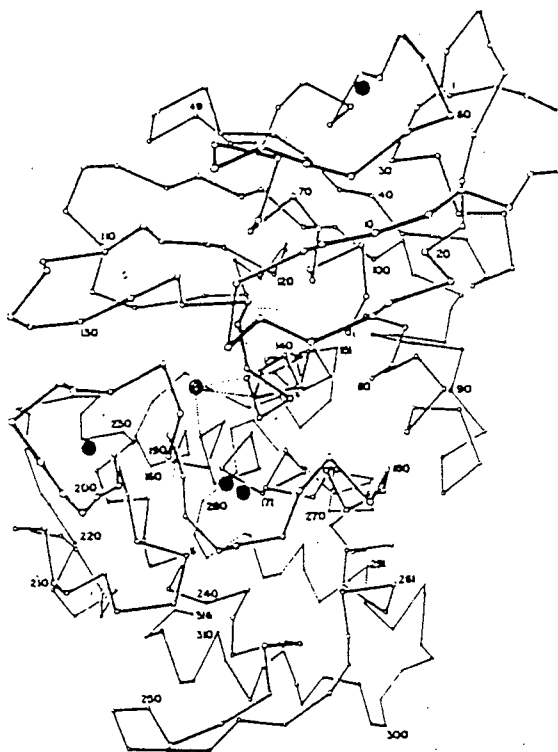


Figure 1. 3-Dimensional structure of thermolysin (Matthews et al., 1972)

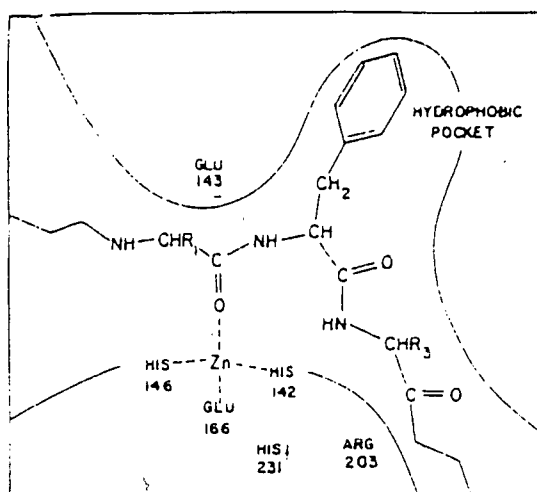


Figure 2. Schematic representation of the active site of thermolysin with a polypeptide substrate bound (Bigbee and Dahlquist, 1974)

thermolysin-inhibitor complexes (Monzingo and Matthews, 1982). The metalloenzyme normally contains one zinc ion and four calcium ions per molecule. The zinc ion has been shown to be essential to the catalytic activity of thermolysin, and is liganded at the active site of the enzyme by His 142, His 146, and Glu 166 (Figure 2). On the other hand, the calcium ions are not involved in catalysis but are thought to contribute to the thermal stability of the enzyme (Matthews et al., 1974). The presence of calcium ions is also important in stabilizing the thermolysin molecule against autolysis (Fontana, 1988). The active site of thermolysin contains at least four subsites, S_1 , S_2 , S_1' , and S_2' , which participate in binding the extended substrate (Tran et al., 1991) and are responsible for substrate specificity of the enzyme. The hydrophobic pocket to the right of the zinc is the primary recognition site (S_1'). Hydrophobic residues such as leucine, isoleucine and phenylalanine are preferred for binding at this position (Holden and Matthews, 1988). It is known that the carbonyl oxygen of the peptide bond to be cleaved is liganded to the zinc atom, displacing the water molecule that normally acts as the fourth ligand in the native thermolysin structure. Glu 143 and His 231 also appear to play a role in hydrolysis because they are the closest side chains to the scissile peptide bond. Other amino acid residues, such as Trp 115, Tyr 157, Asn 112, Ala 113 and Ala 118, in the active site region are thought to participate in the positioning of the substrate backbone in the active site by hydrogen bonding to the substrate main chain.

Several studies have demonstrated that thermolysin can be used to promote formation of peptide synthesis via a reverse hydrolysis reaction. For example, the enzyme has been used in enzymatic synthesis of asparagine-containing peptides, proline-containing tripeptides, cholecystokinin-octapeptide, and the precursor of aspartame (Miranda and Tominaga, 1991., Cheng et al., 1988, Sakina et al., 1988, Isowa et al., 1979). Although the mechanism involved in reverse proteolysis is not known, it has been proposed that, in the coupling reaction, the amino acid residues of the carboxyl and amino components used as starting materials occupy the same subsite in the active site of the enzyme as in hydrolysis of the corresponding product (Moriyama and Oka, 1980).

b. Neutral Protease T

Neutral protease T, an extracellular enzyme with a molecular weight of 34,579 daltons, is secreted by *Bacillus stearothermophilus* (Takagi et al., 1985). This enzyme possesses characteristics similar to thermolysin. For example, neutral protease T requires Zn^{2+} for its proteolytic activity and Ca^{2+} for its thermal stability. In fact, this enzyme has been found to be quite unstable in the absence of Ca^{2+} . For this reason, it has been suggested that the addition of Ca^{2+} to the culture medium is necessary whenever production of neutral protease is needed (Fuji et al., 1983). Neutral protease T is a thermostable enzyme which retains about 80% of its activity even after prolonged heating at 65°C for 30 minutes. Mutant neutral protease T with enhanced

thermostability and altered specific activity has been obtained by recombinant DNA technology (Takagi and Imanaka, 1989).

The gene for neutral protease T has been cloned and its nucleotide sequence has been determined (Takagi et al., 1985). The amino acid sequence of the extracellular form of this enzyme is highly homologous (85%) to that of thermolysin. The zinc binding site (His-142, His-146, and Glu-166) in thermolysin is also found in neutral protease T (His-145, His-149, and Glu-169). It is known that at least 5 amino acid residues participate in the catalytic reaction and positioning of the substrate backbone in the active site of thermolysin (Tran et al., 1991). These residues are found at homologous positions in neutral protease T from *B. stearothermophilus*. These facts suggest that thermolysin and neutral protease T may share similar 3-dimensional structures. The hydrolytic and reverse proteolytic activities of these two enzymes may be alike.

III. IMMUNOASSAY TECHNIQUES

a. Radioimmunoassay

Infectious diseases in humans were traditionally identified by the cultivation of the infecting agent in an in-vitro system or laboratory animal. However, the usefulness of this technique was severely limited by the fact that not all viruses that cause medically important diseases can be cultivated in tissue culture or animal systems (Yolken, 1982). Examples of these viral agents include rotavirus, hepatitis B virus, Norwalk virus, and Epstein-

Barr virus. Another major problem associated with the cultivation approach is that some viral agents require a long period of time to grow and the results are not useful for the treatment of ill patients. These problems are largely overcome through the development of rapid assays capable of directly detecting infectious agents in clinical specimens. The principle of most of these assays is based on the fact that most infectious agents can be detected by a specific antibody-antigen reaction, which can be completed and measured in a short period of time (Kemeny and Chantler, 1988).

Solid-phase radioimmunoassay used to be the most popular assay for detecting microbial antigens. Reasons for the widespread usage of radioimmunoassay are related to the fact that radioactivity can be detected with great sensitivity and that the antibody-antigen reaction can be measured objectively using generally available laboratory instrumentation (Yolken, 1982). On the other hand, the use of radioactive labels also has drawbacks. These include the short shelf-life of radioactive labels, which is generally limited to 2 months, the hazards involved in preparation and handling of radioactive isotopes, and the need for gamma counting instruments. Alternative non-radioactive immunoassay systems were therefore developed with the intention to overcome most of the disadvantages associated with radioimmunoassay. One of such systems that has attained wide spread usage is the enzyme linked immunosorbent assay (ELISA).

b. Advantages of Enzyme Linked Immunosorbent assay

ELISA methods have a number of advantages over radioimmunoassay. The labelled reagents used are stable and can be stored for prolonged periods of time without loss of activity (Kemeny and Chantler, 1988). The use of multiwell microtiter plates as the solid-phase instead of tubes greatly facilitates handling and washing of samples. Large numbers of samples can be assayed very rapidly when microtiter plates are used with automated readers and multiple well washers. A wider range of substrates and chromogens with higher sensitivity is available and a variety of enzyme-labelled antisera may now be purchased commercially which minimizes the need for preparing these reagents at the lab. Finally, ELISA has a higher potential sensitivity than radioimmunoassay because theoretically a molecule of enzyme can generate many molecules of product. The enzyme linked immunosorbent assay (ELISA) has been widely used for the detection of antibodies and antigens in human body fluids and veterinary diseases and in immunology (Kemeny and Chantler, 1988). Various important microbial antigens in human fluids that can be reliably detected by ELISA include rotavirus, hepatitis B virus, *Haemophilus influenzae* type b (Yolken, 1982).

c. Principle of Direct and Indirect ELISA

In the direct assay, an unlabelled antibody is bound to a solid phase by physical adsorption or by covalent linkage. Unbound antibody is removed by washing. A test sample is then added to the solid phase along with a buffer, which serves to insure that the

proper pH and ionic strength are available for the antigen-antibody reactions. The antibody coated solid phase will bind specific antigen present in the test sample during incubation. After removal of unbound material, enzyme-labelled antibody is added. This enzyme-antibody complex will react with antigen bound to the solid phase. When unbound enzyme labelled antibody is washed away, a substrate is added to the solid phase which will be converted into a coloured form by the enzyme adhered to the well. The amount of colour measured is proportional to the amount of antigen present in the test sample (Yolken, 1982).

In the indirect ELISA, the test sample is added to an antibody-coated solid-phase as in the direct system. Instead of adding an enzyme-labelled antibody to react with the bound antigen, an unlabelled antibody, which has been prepared in a different animal species than the antibody utilized for coating the solid phase, is added. Following removal of unreacted antibody, an enzyme-labelled antiglobulin directed against the second antibody is added. Substrate is then added and is detected as described in the previous paragraph. The main benefit for using an unlabelled second antibody from another animal source is that a single enzyme-labelled antisppecies globulin can be used for detection of a large number of different antigens while in the direct ELISA, a distinct antibody-enzyme conjugate is required for each antigen to be tested (Yolken, 1982).

d. Choice of enzyme and substrate

The three most commonly used enzymes in ELISA include

horseradish peroxidase, alkaline phosphatase, and β -D-galactosidase. The choice of enzyme frequently is dependent upon its purity, the sensitivity of its substrate detection, ease of conjugation of enzyme-antibody complex, and stability of conjugate (Kemeny and Chantler, 1988).

Horseradish peroxidase (HRP) is a relatively inexpensive and pure enzyme that also has a high turnover rate. The turnover rate of an enzyme marker greatly affects the sensitivity of an ELISA system. The major advantage for using this enzyme in ELISA is that peroxidase is a glycoprotein, whose carbohydrate portion provides a good binding site for antibody with minimal interference with the functionality of the enzyme (Wilson and Nakane, 1978). Antibody is linked to horseradish peroxidase by periodate oxidation, by sulphhydryl-maleimide conjugation, or the two-step glutaraldehyde reaction (Kemeny and Chantler, 1988). Another advantage of horseradish peroxidase is that various chromogens can be used with HRP substrate, hydrogen peroxide, to produce intense dark colour which can be easily measured spectrophotometrically. Common chromogens include 2,2-azino-di(3-ethylbenzothiazoline-6-sulphonate) (ABTS), orthophenylenediamine (OPD) and 3,3', 5,5'-tetramethylbenzidine hydrochloride (TMB). The last two are most sensitive in detecting low levels of enzyme (Kemeny and Challacombe, 1988). There are several drawbacks associated with the use of peroxidase in ELISA. First, the enzyme loses sensitivity if it becomes contaminated with microorganisms. Ordinarily, the addition of small amounts of antimicrobial agents

such as sodium azide and methanol would inhibit microbial growth. However, peroxidase is very sensitive to sodium azide and methanol. Secondly, most of the chromogens for peroxidase except TMB have been found to be carcinogenic or mutagenic. Lastly, it has been determined that peroxidase can be inactivated by polystyrene surfaces (Berkowitz and Webert, 1981). Fortunately, this problem can be overcome by pretreating polystyrene ELISA plates with Tween 20.

Unlike HRP, alkaline phosphatase is relatively stable during storage and is not sensitive to antimicrobial agents. This enzyme has a high turnover rate and is coupled to protein by two-step glutaraldehyde or sulphydryl/maleimide procedures. The substrate used for this enzyme is para-nitrophenyl phosphate, which is non-carcinogenic. The substrate reacts with alkaline phosphatase to produce a pale yellow which again can be measured spectrophotometrically. However, colour development is slower than with HRP substrates. Sensitivity may be improved by using fluorescent substrates or by using a cyclical enzyme-amplified system for alkaline phosphatase detection (Self, 1985). The major drawback of using alkaline phosphatase is that it is expensive because enzyme of high purity needs to be obtained from an animal source such as calf intestine (Volken, 1982).

β -galactosidase is not used as often as the other two enzymes mentioned above because it has a slower turnover rate. However, unlike HRP and alkaline phosphatase, β -galactosidase is not found in plasma or other body fluids and would be useful to use in

situations where endogenous enzyme activity cannot be removed. Coupling of this enzyme to proteins can be accomplished by one-step glutaraldehyde and maleimide procedures (Kemeny and Chantler, 1988). Chromogenic substrates such as p -nitrophenyl- β -D-galactosidase and fluorogenic substrates like 4-methylumbelliferyl- β -D-galactosidase have been used.

e. Microtiter Plates

Solid phase supports can be classified as low and high capacity systems. High capacity supports bind appreciably more proteins than low capacity supports, and are often made of agarose, Sephadex, cellulose, and nitrocellulose. On the other hand, low capacity solid phase matrices are often made from polystyrene, polyvinylchloride (PVC), nylon, and glass. Since the choice of solid phase matrix in ELISA is largely dependent on the ease of setting up and processing these assays, the convenience offered by microtiter plate format is probably the reason for the widespread popularity of ELISA in spite of the limited protein binding capacity of the microtiter plates.

The mechanism by which proteins bind to plastic surface is not well understood. However, it is believed that charge and hydrophobic interactions may be involved (Kemeny and Chantler, 1988). Proteins can also be covalently attached to ELISA plates by pretreating proteins with glutaraldehyde (Parsons, 1981), or with carbodiimide (Rotman and Delwel, 1983). The binding capacity of different plastic surfaces varies. PVC plates have been reported

to have greater binding capacity (De Savigny and Voller, 1980). Irradiated plates that bind more proteins than PVC plates are also available (Urbanek et al., 1985). Studies have shown that plates manufactured by different suppliers also have remarkably different protein binding capacities. For example, Urbanek et al. (1985) demonstrated that Nunc Immuno-1 had higher binding capacity for bee venom phospholipase A₂ than Dynatech Immunlon and Linbro microtiter plates. The stability of protein-plate interaction is also an important consideration because desorption of proteins from the microtiter plate during an assay could lead to false conclusions.

The rate and extent of protein binding to microtiter plates are dependent upon the type of protein and its concentration, time, pH, and temperature of incubation (Blake and Gould, 1984). The highest concentration of most proteins that would form a monolayer was reported to be 1 µg/mL (Cantarero et al., 1980). Addition of excessive amount of protein is wasteful as the additional protein is loosely bound to the other proteins and would be washed off during subsequent incubation and washing steps. A high concentration of proteins could also result in increased non-specific binding of proteins to the plastic surface. Non-specific binding of proteins to the microtiter plate could lead to undesired high background noise of the assay. One way to minimize this problem is to add non-ionic detergents such as Tween 20 (Berkowitz and Webert, 1981). Blocking proteins can also be added after coating to block any vacant binding sites. Common blocking proteins include gelatin (0.5 - 1%) and bovine serum albumin (1%)

(Kemeny and Chantler, 1988).

IV. *BACILLUS SUBTILIS*

- a. Use of *Bacillus subtilis* as a host for production of heterologous proteins

Recombinant DNA technology has made it possible to commercially produce useful enzymes and proteins from microorganisms, plants, and animals via cloning and expression. High yields of proteins may be achieved through insertion of multiple copies of a specific gene into a desired host organism and through development of high secretion systems. The choice of host organism may also affect the production levels of the desired protein (Pitcher, 1986). As an example, secretion of calf rennin or chymosin in bacteria is difficult to achieve. However, the secretion of properly processed prochymosin, a precursor of calf rennin, has been made possible through the use of a filamentous fungus (Pitcher, 1986).

Traditionally, *E. coli* and its plasmids and phages have been the main components of cloning and expression systems used in recombinant DNA studies. However, it is known that laboratory strains of *E. coli* are capable of exchanging genetic information with intestinal *E. coli*. In addition, the envelope of *E. coli* cell contains lipopolysaccharide, which causes endotoxin shock syndrome (Lovett and Ambulos, Jr., 1989). This makes it necessary to rigorously purify gene products from *E. coli* if these products are to be used as pharmaceuticals or in food applications. Another problem in using *E. coli* as expression system in commercial applications is that the organism expresses proteins

intracellularly, which complicates the process of protein recovery. As a result, alternative cloning and expression systems have been developed which have overcome some of the problems associated with the use of *E. coli*. The *Bacillus subtilis* system has emerged as the major prokaryotic alternative to cloning in *E. coli* (Lovett and Ambulos, Jr., 1989).

The major reason for commercial development of expression systems in *Bacillus subtilis* is related to its large secretion capacity. Unlike *E. coli*, *Bacillus subtilis* as well as many other strains of *Bacillus* are capable of directly secreting large quantities of a variety of enzymes into the growth medium. This greatly simplifies the recovery of the target protein since the secreted protein is in a relatively pure and soluble form in contrast to an insoluble intracellular product (Mountain, 1989). In addition, the expression system in *B. subtilis* allows accumulation of high levels of products in active forms, unlike the denatured proteins usually harvested from inclusion bodies that result from intracellular high-level expression and accumulation. The second reason which accounts for the increased commercial interest for making recombinant products in *B. subtilis* is related to the fact that well-established large scale fermentation and product recovery systems are available. Thirdly, unlike *E. coli*, *B. subtilis* is non-pathogenic and does not produce endotoxins. In fact, the organism has been granted GRAS (Generally Regarded As Safe) status in the United States. More importantly, the Food and Drug Administration gave GRAS status for the first time to an

heterologous enzyme (α -amylase from *Bacillus stearothermophilus*) produced in *B. subtilis* (Mountain, 1989).

b. Problems associated with using *B. subtilis* expression systems

The commercial use of *B. subtilis* as an expression system for heterologous proteins suffers from several drawbacks. Firstly, recombinant plasmids tend to be very unstable in this host organism. Both structural and segregational instability of recombinant plasmids have been observed in much greater frequency in *B. subtilis* than in *E. coli* (Mountain, 1989). Structural instability refers to deletion, insertion, or rearrangement of the cloned gene within a recombinant plasmid while segregational instability refers to the loss of the entire recombinant plasmid. One possible way of improving plasmid stability is to integrate multiple copies of the gene of interest into the chromosome of *B. subtilis*. As an example of such an approach, a *B. subtilis* carrying 2 copies of the α -amylase gene of *B. amyloliquefaciens* integrated at different locations in the chromosome produced as much amylase as a strain carrying the gene on a recombinant plasmid at 40 copies per cell (Kallio et al., 1987). Production of amylase appeared to be slower but production continued for a longer time for the integration strain.

The second barrier hindering the development of efficient expression systems in *B. subtilis* is related to the tendency of the organism to produce high levels of extracellular proteases which degrade the secreted foreign protein. It is known that *B. subtilis*

has 6 extracellular proteases (Xu et al., 1991). Construction of protease deficient strains of host cells may help to alleviate such a problem. Construction of a double protease-deficient strain, DB 104, greatly improved the stability of the secreted foreign proteins (Kawamura and Doi, 1984). A strain of *Bacillus subtilis* deficient in six extracellular proteases was recently developed (Xu et al., 1991).

The third hurdle preventing wide application of *B. subtilis* as a host for the production of heterologous proteins is the lack of well-characterized, strong controllable promoters that are similar to the pL, pR, trp, lac, and tac promoters which have been shown to be useful in *E. coli* (Mountain, 1989). Various inducible vectors have been developed to overcome this problem. Some of these vectors use either the *E. coli* lac system (Le Grice, 1990) or the temperature sensitive repressor from λ (Breitling et al. 1990) or ϕ 105 (Osburne et al., 1984) to regulate expression of target foreign proteins while others are based on the regulatory region of *sacB*, a sucrose-inducible gene, that encodes for the extracellular enzyme, levansucrase (Xu et al, 1991).

MATERIALS AND METHODS

MATERIALS

Nunc Immuno Modules, CovaLink NH (cat. no. 478042), were obtained from Gibco Canada Inc., Burlington, ON. DNP(dinitrophenyl)-L-leucine, L-leucine, DNP-L-asparagine, L-asparagine, thermolysin (Protease Type X from *Bacillus thermoproteolyticus*), 1-ethyl-3-(dimethyl-aminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), dimethylsulfoxide, calcium chloride, casamino acid, kanamycin monosulfate, azocasein, and o-phenylenediamine (OPD) were purchased from Sigma Chemical Co., St. Louis, MO. Rabbit anti-DNP antibody horseradish peroxidase conjugate (Ra DNP-HRP) was obtained from Dakopatts, Glostrup, Denmark. N,N-dimethylformamide (DMF), 10 N sulphuric acid, disodium hydrogen carbonate, 30% hydrogen peroxide, sodium bicarbonate, sodium hydroxide, sodium acetate, calcium acetate, glucose, magnesium sulphate hexahydrate, EDTA, Hammerstein casein and trichloroacetic acid were obtained from BDH Canada, Toronto, ON. Broad range molecular weight standard was purchased from Biorad, Mississauga, ON. Tryptone, yeast extract, and nutrient agar were purchased from Difco, Detroit, MI. Carnation instant skim milk powder was purchased from a local retail store. Restriction enzymes and Hind III digest fragments were purchased from Boehringer Mannheim, Quebec, Canada. *Bacillus subtilis* DB428 and WB600 were provided by Dr. Sui-Lam Wong (University of Calgary, Alberta, Canada). Recombinant plasmid, pNP22, was provided by Dr. T. Imanaka (Osaka University, Osaka, Japan).

METHODS**I. THERMOLYSIN CATALYSED SOLID-PHASE SYNTHESIS OF DNP-ASN-LEU DIPEPTIDE**

- a. Immobilization of DNP(Dinitrophenyl)-L-leucine or L-leucine to microtiter plate

A special type of polystyrene microtiter plate, called Nunc Immuno Modules, was used as the solid phase on which leucine molecules were attached. These plates had special linker arms, called Covalink NH. These secondary amino groups were grafted on the polystyrene surface and served as bridgeheads for further covalent coupling. The amino groups were positioned at the end of a 2 nm long spacer arm; this made the NH-groups readily accessible for molecules present in the liquid phase (Figure 3). The linker arms permit covalent binding of even small molecules to the surface. It has been reported that tripeptides could be detected when bound to Covalink NH whereas the same peptides were only barely detectable when bound to normal microtiter plates without linker arms (Nunc InterMed Publication).

L-leucine molecules were covalently bonded to the solid phase via the carboxyl groups using water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as coupling agent in the presence of N-hydroxysuccinimide (NHS) (Sondergard-Andersen et al. 1990). The mechanism of this reaction is shown in Figure 4.

The method of Sondergard-Andersen et al. (1990) was used with a few modifications. A stock DNP-L-leucine or L-leucine solution was prepared by dissolving the amino acid either in dimethylsulfoxide (DMSO) and distilled water at 1.5 : 1 ratio or in

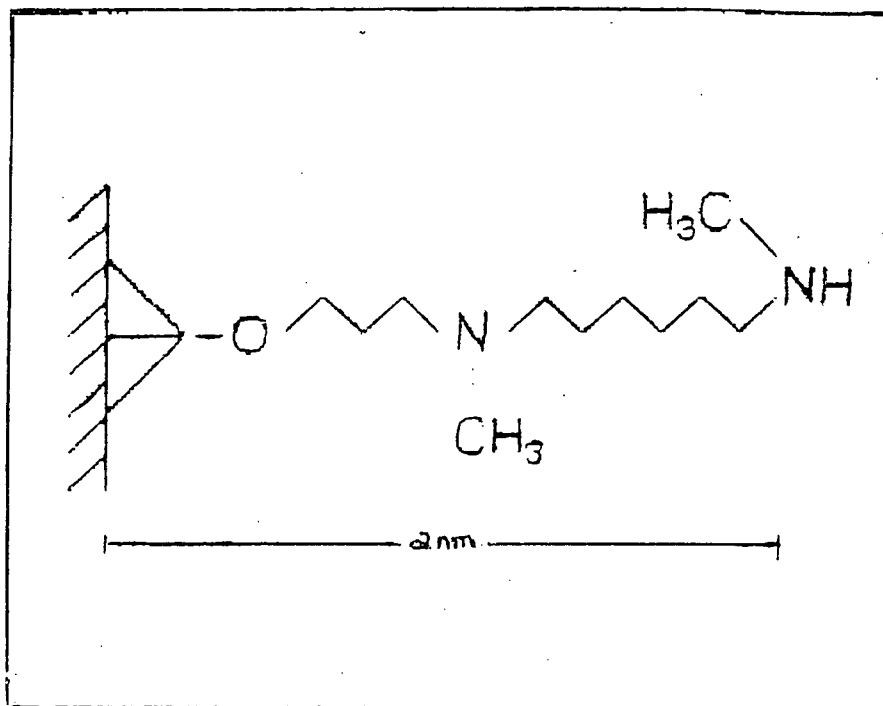


Figure 3. Schematic chemical and physical configuration of the CovaLink NH surface (Nunc InterMed publication)

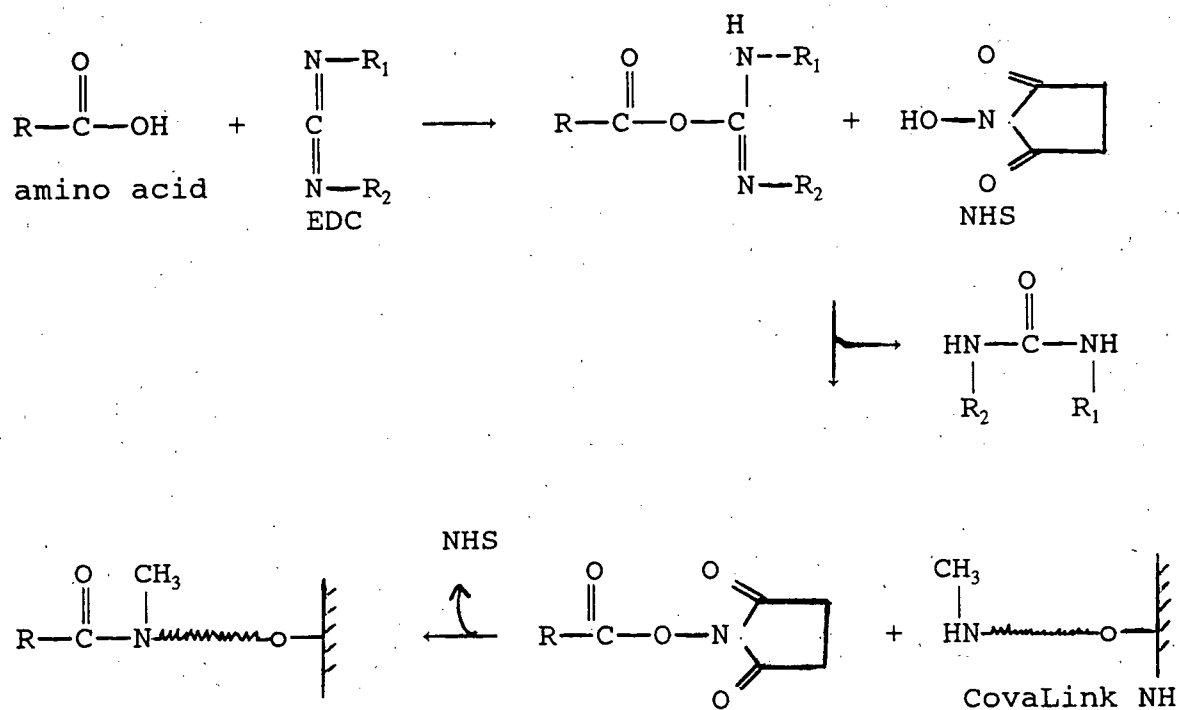


Figure 4. Covalent coupling of amino acid to CovaLink NH.
 EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
 NHS: N-hydroxysuccinimide

distilled water alone. DNP-L-leucine or L-leucine solution was added to an equal volume of 0.1 M NHS and 0.1 M EDC, which had been pre-dissolved in distilled deionized water. The mixture was thoroughly mixed and was then incubated at room temperature for 30 minutes to allow for the activation of the carboxyl groups on the DNP-L-leucine or L-leucine molecules. After incubation, the solution was diluted with ice cold 0.1 M carbonate buffer, pH 8.6. 100 μ L of the diluted mixture in different concentrations was added to designated wells in a Nunc Immuno Module. The plate was then incubated at 5°C for 30 minutes. All coupling reactions were carried out at 5°C in this thesis to minimize non-covalent binding of molecules to the CovaLink Module. After incubation, the wells were emptied and washed 3 times with 0.15 M phosphate buffered saline, pH 7.2, containing 0.05% (v/v) Tween 20. 0.15 M phosphate buffer saline was prepared by dissolving the following in 1 L of distilled water: 8 g NaCl, 0.2 g KCl, 1.15 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 g KH_2PO_4 ; pH was adjusted to 7.2 with either 5 M hydrochloric acid or 5 M sodium hydroxide. It should be noted that all buffers used in all the experiments in this thesis were previously autoclaved and cooled before use. Autoclaving was used as a precautionary measure against the possible inactivation of peroxidase by contaminating microorganisms, which could be present in buffer solutions.

b. Detection of Immobilized Amino Acid or Dipeptide

Rabbit anti-DNP antibody horseradish peroxidase conjugate stock (1.3 mg/mL) was diluted 500 times with 0.01 M phosphate

buffer (pH 7.2) containing 0.01% skim milk powder. 100 μ L of the diluted conjugate (2.6 μ g/mL) was pipetted to each microtiter well containing immobilized amino acid or dipeptide. The plate was incubated for 1 hour at room temperature with shaking at 110 rpm setting in a Psychrotherm Controlled Environment incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.). After incubation, the wells were emptied and were washed three times with 0.15 M phosphate buffered saline, pH 7.2. The buffer was kept in the wells for 15 minutes after the third wash in an attempt to remove the last trace of any unreacted antibody conjugate. 100 μ L of substrate solution (0.06% w/v o-phenylenediamine dihydrochloride, 0.015% w/v hydrogen peroxide in 0.5 M citrate-phosphate buffer, pH 5) was then added to each well. 0.5 M citrate-phosphate buffer was prepared by dissolving the following in 1 L of distilled water: 7.3 g citric acid, 11.86 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; pH was adjusted 5.0 with either 5 M hydrochloric acid or 5 M sodium hydroxide. The plate was left at room temperature for 10 minutes with shaking to allow for colour development. The reaction was then stopped by addition of 100 μ L 0.1 M sulphuric acid to each well. Optical density at 492 nm was measured using an ELISA titerplate reader, Easy Reader EAR 400 (SLT-Labinstruments, Salzburg, Austria).

c. Solid-phase Enzymatic DNP-Asn-Leu Dipeptide Synthesis

The reaction conditions selected for DNP-asn-leu synthesis were based on the method described by Miranda and Tominaga (1991)

with some modifications. The choice of substrates was largely based on the research done by Miranda and Tominaga (1991), who used thermolysin to catalyse the synthesis of Z-Asn-Leu-OEt. They reported that the yield of the final product was 62% when Z-Asn-OH and H-LeuOEt.HCl were used at equal molar concentration.

L-leucine, not labelled with dinitrophenyl, was coupled to CovaLink module as described in section I. a. 7.9 mg DNP-Asn was dissolved in 3.5 mL 0.2 M sodium acetate buffer, pH 6, with 50 mM calcium acetate, was adjusted to 6 using either 5 M sodium hydroxide or 5 M hydrochloric acid. Various volumes of DNP-Asn solution ranging from 1 to 20 μ L were pipetted to designated microtiter wells. One milligram of thermolysin was dissolved in 1 mL of the same sodium acetate buffer and 1, 5 or 10 μ L of the enzyme solution was pipetted into each of the wells. Then the final volume of each reaction mixture was made up to 100 μ L by adding sodium acetate buffer. The microtiter plate was incubated at 48°C with shaking at 110 rpm in a Psychrotherm Controlled Environment incubator shaker for various lengths of time with samples removed at fixed time intervals. After each incubation period, the peptide synthesis reaction was stopped by emptying the reactants from the wells. The wells were washed three times with 0.15 M phosphate buffer saline, pH 7.2, containing Tween 20. The plate was then blocked with 0.5% skim milk powder in 0.1 M phosphate buffer at room temperature for 45 minutes. Immobilized dipeptides (i.e. DNP-asn-leu) in the microtiter wells were detected using anti-DNP horseradish peroxidase conjugate as described in

section I.b.

II. ENZYME CATALYSED SOLID-PHASE SYNTHESIS OF DNP-ASN-LEU
DIPEPTIDE USING CRUDE ENZYME MIXTURE FROM *BACILLUS SUBTILIS*
TRANSFORMANTS

a.1. Transformation of *B. subtilis* DB428 and WB600

B. subtilis DB428 (He et al., 1992), a mutant strain deficient in 4 extracellular proteases, and *B. subtilis* WB600 (Xu et al. 1991), a mutant strain deficient in 6 extracellular proteases, were used as the host organism for plasmid, pNP22 (Takagi et al. 1985). This plasmid carries nprT gene (Takagi et al. 1985), which encodes for neutral protease T. The method used to transform DB428 and WB600 is a modified Spizizen's procedure (Personal communication with T. Imanaka). The procedure is as follows: 20 mL TFI (Transformation I) broth was inoculated with 1 mL of overnight host culture in a 250 mL Erlenmeyer flask. The cells were allowed to grow at 37°C for 3 to 4 hours with vigorous shaking until O.D._{660nm} reached about 0.56. A Magniwhirl water bath manufactured by Blue M Electric Company (Blue Island, IL) was used; shaker setting was at 8. After incubation, 4 mL of TFI culture was transferred into 36 mL TFII (Transformation II) broth in a 500 mL Erlenmeyer flask. The cells were grown at 37°C with vigorous shaking (shaker was set at 8) for 1.5 hours.

5 µL of plasmid DNA was dissolved in 95 µL TE buffer in a 16mmx150mm test tube. 1 mL of TFII culture was poured into test tube containing plasmid DNA; the mixture was shaken (shaker speed was set at 4) at 37°C for 30 minutes. The resulting bacterial cells were pelleted by centrifugation at 3000 x g for 10 minutes at

room temperature. The supernatant was discarded, and 3 mL of LB broth (1% tryptone, 0.5% yeast extract, 0.5 % NaCl) was added to the test tube. The culture was incubated at 37°C for at least 1 hour with gentle shaking in a Magniwhirl water bath; shaker setting was at 4. 100 μ L of bacterial cells was spread onto a casein agar plate containing 50 μ g/mL kanamycin. The casein plate was incubated overnight at 37°C. Plasmids were isolated from transformants that formed large halos on casein agar using the alkaline lysis method (Sambrook et al., 1989).

TFI broth consisted of 2 mL 10X Spizizen's salt solution, 0.2 mL 2% casamino acid, 1 mL amino acid solution (1 mg/mL of each of the following amino acids: glycine, alanine, valine, isoleucine, phenylalanine, tyrosine, tryptophan, cysteine, methionine, proline, aspartic acid, glutamic acid, histidine, lysine, arginine, threonine), 2 mL glucose (5%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2%), and 14.8 mL distilled water. 10 X Spizizen's salt solution was prepared by dissolving 20 g $(\text{NH}_4)_2\text{SO}_4$, 140 g K_2HPO_4 , 60 g KH_2PO_4 and 10 g $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ in 1 L of water. TFII broth consisted of 3.6 mL 10X Spizizen's salt solution, 0.18 mL 2% casamino acid, 0.18 mL amino acid solution, 3.6 mL glucose (5%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2%), and 28.44 mL distilled water. TE buffer consisted of 10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0).

a.2. Confirmation of postive transformants by restriction enzyme digestion of plasmid DNA

Positive transformants carrying pNP22 were confirmed by restriction enzyme digestion of plasmid DNA. The final volume of

each digestion mixture was 25 μ L, which consisted of 15 μ L of the plasmid DNA, 1 μ L of 10 x restriction enzyme buffer (supplied with restriction enzyme) for each enzyme, 1 μ L of restriction enzyme(s) and sterile water. The mixture was then incubated for 2 hours at 37°C without shaking. Digested DNA fragments were separated by electrophoresis through 0.7 to 0.8% agarose gel using Tris-Borate-EDTA (TBE buffer) according to the procedures described by Sambrook et al., 1989. 25 μ L of each of the digested DNA sample was then mixed with 5 μ L of the gel loading buffer; 5 μ L of the mixture was subsequently added to each slot in the agarose gel. The restriction map and agarose gel electrophoresis pattern of pNP22 recombinant plasmid are presented in Figure 5a and 5b.

10 x TBE gel loading buffer was prepared by dissolving 54 g Tris, 27.5 g boric acid and 20 mL 0.5 M EDTA (pH 8.0) in 1 L of distilled water. The gel loading buffer was made up of 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30 % glycerol in water.

a.3. Preparation of Crude Proteases from *Bacillus subtilis* transformants

Positive transformants carrying pNP 22 plasmids were inoculated into 8 mL of LB broth (containing 50 μ g/mL kanamycin and 5 mM CaCl_2) in a 250 mL Erlenmeyer flask. After 24 hours of incubation at 37°C with vigorous shaking in a Magniwhirl water bath (shaker setting was at 8), the culture was transferred into 500 mL of fresh LB broth in a 2 L Erlenmeyer flask. The culture was then reincubated for 24 hours with shaking in a Psychrotherm Controlled

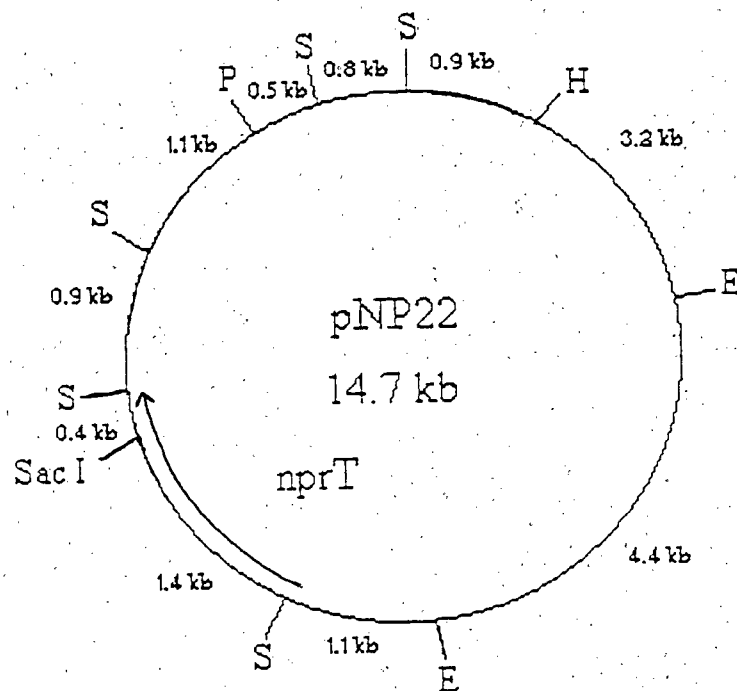


Figure 5a. Restriction map of pNP22.
S, *SalI*; H, *HindIII*; E, *EcoRI*; P, *PstI*.

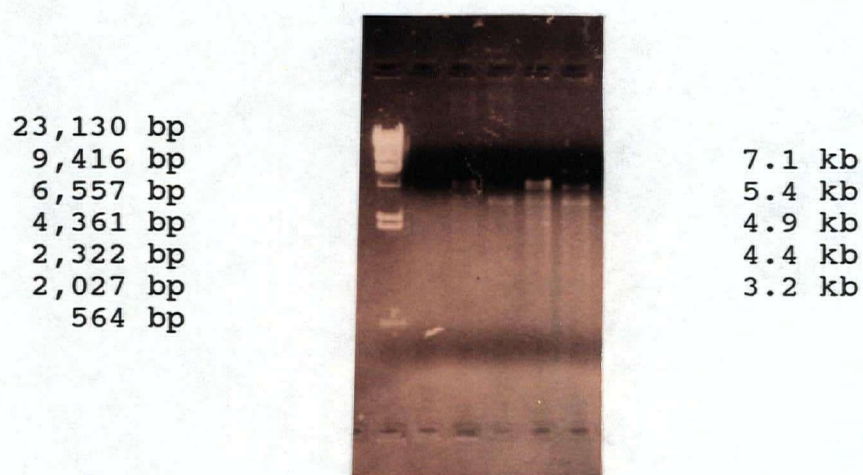


Figure 5b. Analysis of pNP22 recombinant plasmid with restriction endonucleases.
Lane 1. Molecular weight marker: λ -HindIII digest.
Lane 2. Blank lane.
Lane 3 and 5. pNP22 digested with *EcoRI* and *PstI*.
Lane 4 and 6. pNP22 digested with *EcoRI* and *HindIII*.

Environment incubator shaker at 160 rpm at 37°C. The 24 hour, 500 mL culture was centrifuged at 2900 x g for 10 minutes at 4°C to remove bacterial cells. The resulting supernatant containing secreted proteases was then frozen in liquid nitrogen and was stored at -16°C for later use.

Alternatively, the 500 mL culture was used to inoculate a Lab Pilot Fermentor CF3000 (Chemap AG., Switzerland) containing 10 L of fresh LB broth with 50 µg/mL kanamycin and 5 mM CaCl₂. The culture was incubated at 37°C with stirring at 500 rpm. The rate of aeration was at 4 L/min; there was no pH adjustment. After 24 hours of incubation, the bacterial cells were removed by centrifugation at 50,000 x g in a Sharples tubular bowl centrifuge Model T-1P (Alfa-Laval, Scarborough, ON). The resulting supernatant was either directly used to catalyze asn-leu synthesis on CovaLink modules, or was concentrated by using a 50 mL Amicon stirred cell (Amicon Corp., Lexington, MA) equipped with a 10,000 mol. weight cut off filter membrane, or a Millipore Pellicon Cassette Filter system (Millipore, Bedford, MA) using a membrane with 10,000 molecular weight cut-off. In the case of ultrafiltration, the retentate was subsequently diafiltered using the same ultrafiltration systems described above with 0.1 M Tris buffer, pH 7.2 with 5 mM CaCl₂ in order to remove the growth medium. The concentrated supernatant was then frozen using liquid nitrogen and was stored at -16°C until further use.

- b. Enzymatic solid-phase synthesis of DNP-Asn-Leu dipeptides using either concentrated or unconcentrated crude enzyme fraction from *Bacillus subtilis* (pNP22) transformants as catalysts

L-leucine was immobilized to Nunc Immuno Module as described in section I.a. DNP-L-asn solution was prepared as outlined in section I.c. Various volumes of crude unconcentrated or concentrated enzyme mixture, prepared as described in section II.a, ranging from 1 to 30 μ L, were pipetted into designated wells. The final volume per well was adjusted to 100 μ L using sodium acetate buffer, pH 6. The plate was incubated with shaking at 110 rpm in a Psychrotherm Controlled Environment incubator shaker at 48°C for various lengths of time with samples removed at fixed time intervals. The peptide synthesis was stopped simply by emptying the reactants from the wells. After washing 3 times with 0.15 M phosphate buffer saline (pH 7.2), the presence of immobilized DNP-asn-leu was detected according to procedures described in section I.b.

III. CHEMICAL ASSAYS

- a. Protease Assay Using Casein As A Substrate

Protease was assayed using a method described by Fuji et al. (1983) with the following modifications. The enzyme preparation was diluted with 50 mM Tris buffer (pH 7.5), which contained 5 mM calcium chloride. One mL of enzyme was then mixed with 1 mL of 50 mM Tris buffer (pH 7.5) containing 5 mM calcium chloride and 1% casein (Hammerstein). Calcium chloride was added to prevent autodigestion of the enzyme of interest. The mixture was incubated for 37°C for 15 minutes. Two mL of precipitating agent, made up

with 0.1 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid, was added to the mixture. After thorough mixing, the mixture was left at room temperature for 30 minutes followed by centrifugation at 2000 x g for 15 minutes. The supernatant was removed using a pasteur pipet. Absorbance of the supernatant at 275 nm was measured with a Shimadzu UV-Vis Recording Spectrophotometer UV-160, Kyoto, Japan. The reagent blank contained: 1 mL 50 mM Tris buffer with 5 mM calcium chloride, 1 mL 50 mM Tris buffer with 5 mM calcium chloride and 1% casein (Hammerstein), and 2 mL of precipitating agent. A calibration curve was made from a 1 mg / mL stock solution of tyrosine using a range of 1 - 200 μ g/mL (Figure 6). One unit of protease was defined as the quantity required to increase the absorbance at 275 nm by an equivalent of 1 μ g of tyrosine per minute at 37°C at pH 7.5.

b. Protease Assay Using Azocasein as a Substrate

The azocasein protease assay was modified from a procedure described by Ewings et al. (1984). Duplicates of reaction mixture were prepared by mixing the following reagents together:

- . 1.25 mL of 1.5% azocasein in 0.1 M Tris-HCl buffer (pH 7.5) with 5 mM calcium chloride; azocasein was prepared by boiling in the above Tris buffer for 5 minutes followed by filtration through Whatman #1 filter paper

- . 1.25 mL 0.1 M Tris-HCl buffer (pH 7.5) with 5 mM calcium chloride
- . 0.25 mL of diluted enzyme

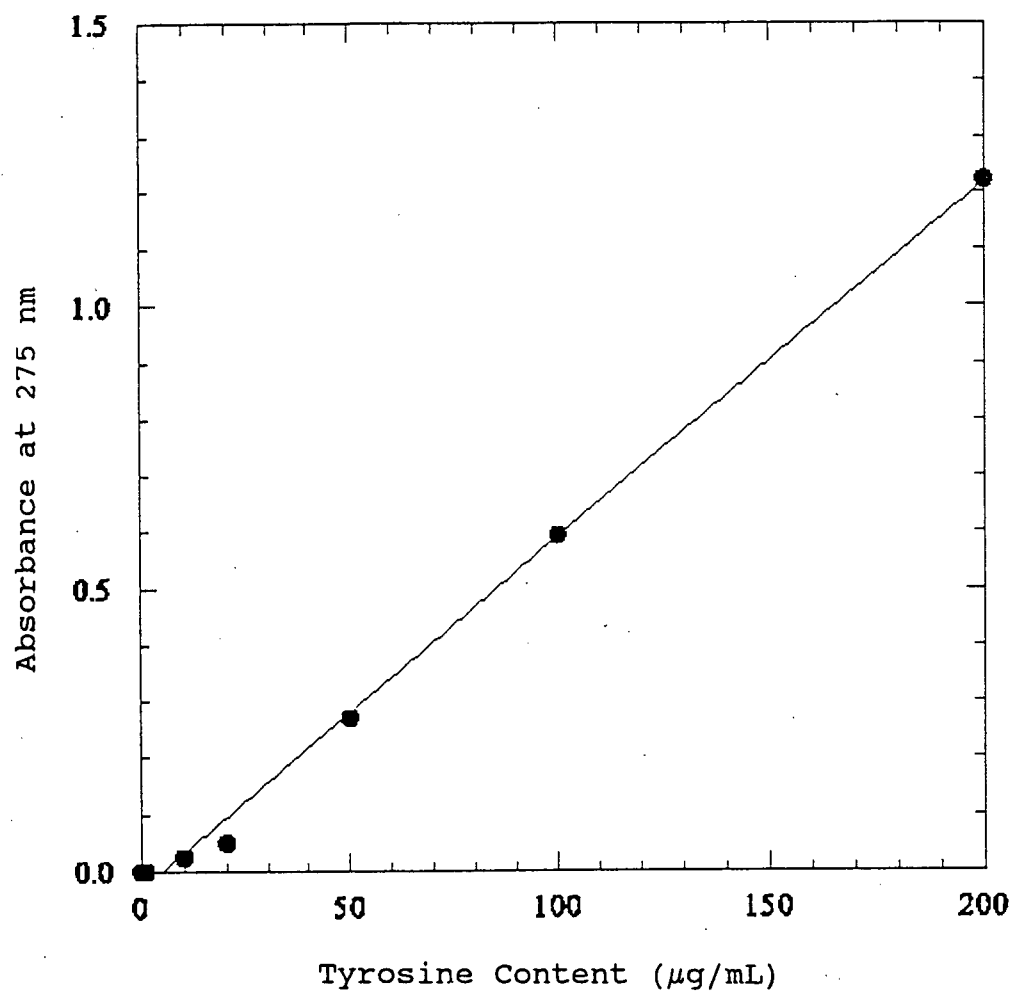


Figure 6. Standard curve for protease assay with Hammerstein casein used as a substrate.

Substrate blanks and sample blanks were used as controls. Substrate blank was prepared the same way as the reaction mixture except that 0.25 mL of diluted enzyme was replaced by 0.25 mL of Tris-HCl buffer. Sample blank was prepared the same way as the reaction mixture except that 2.5 mL 24% trichloroacetic acid (TCA) was added before the addition of each diluted enzyme solution at time 0.

All reaction mixtures were incubated at 48°C for 2 hours with shaking. Reaction was stopped by adding 2.5 mL of 24% TCA to each test tube. The samples were vortexed immediately and were placed into an ice bath for 15 minutes followed by centrifugation for 15 minutes at 3700 x g. The absorbance of the clear supernatant was measured at 366 nm using a Shimadzu UV-Vis Spectrophotometer.

One unit of protease activity

= the amount of enzyme required to produce an increase in absorbance at 366 nm of 0.01 per hour.

= $(\text{Reaction Mixture } t_{2 \text{ hr.}} - \text{sample blank } t_{0 \text{ hr.}} - \text{substrate blank } t_{2 \text{ hr.}}) / 0.01$

c. Protein Assay Using BCA Protein Assay Reagent

All reagents used were supplied in the BCA Protein Assay test kit (Pierce Chemical Company, Rockford, IL). The samples and reagent blanks were pipetted into the wells of microtiter plates for convenience. Fifteen μL of enzyme or protein preparation was added to each microtiter well containing 250 μL of BCA protein assay working reagent. The samples were left at room temperature for 2 hours in order for colour development to occur. At the end

of the incubation period, the optical density of the samples was measured at 620 nm using the Easy Reader EAR 400 plate reader. A calibration curve was prepared from a 1 mg/mL thermolysin stock solution using a range of 0 - 1000 μ g/mL (Figure 7).

d. SDS-Gel Electrophoresis of Thermolysin and Secreted Crude Proteases from Transformed *B. subtilis* DB428 and WB600

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a PhastSystemTM using PhastGel Gradient 8-25 and PhastGel SDS buffer strips (Pharmacia, Uppsala, Sweden). The buffer system in the gels was 0.112 M acetate and 0.112 M Tris pH 6.4. The buffer system in PhastGel SDS buffer strips was 0.20 M tricine, 0.20 M Tris and 0.55% SDS, pH 7.5.

Thermolysin sample was prepared as follows: 100 μ L of thermolysin, which was prepared by dissolving 1 mg of enzyme in 1 mL of 0.01 M phosphate buffer, pH 7.2, 100 μ L 0.01 M phosphate buffer, pH 7.2, 50 μ L 10% SDS and 12 μ L 0.05% bromophenol blue were mixed together. Samples of secreted crude proteases were prepared by combining 150 μ L of supernatants containing crude proteases, 50 μ L 0.01 M phosphate buffer, pH 7.2, 50 μ L 10% SDS and 12 μ L 0.05% bromophenol blue. All samples were heated for 5 minutes at 95°C in a hot water bath. 5 μ L of each of the prepared samples was applied to the gel. When separation was completed, the gel was silver stained according to PhastSystem Development Technique File NO.210 (Pharmacia).

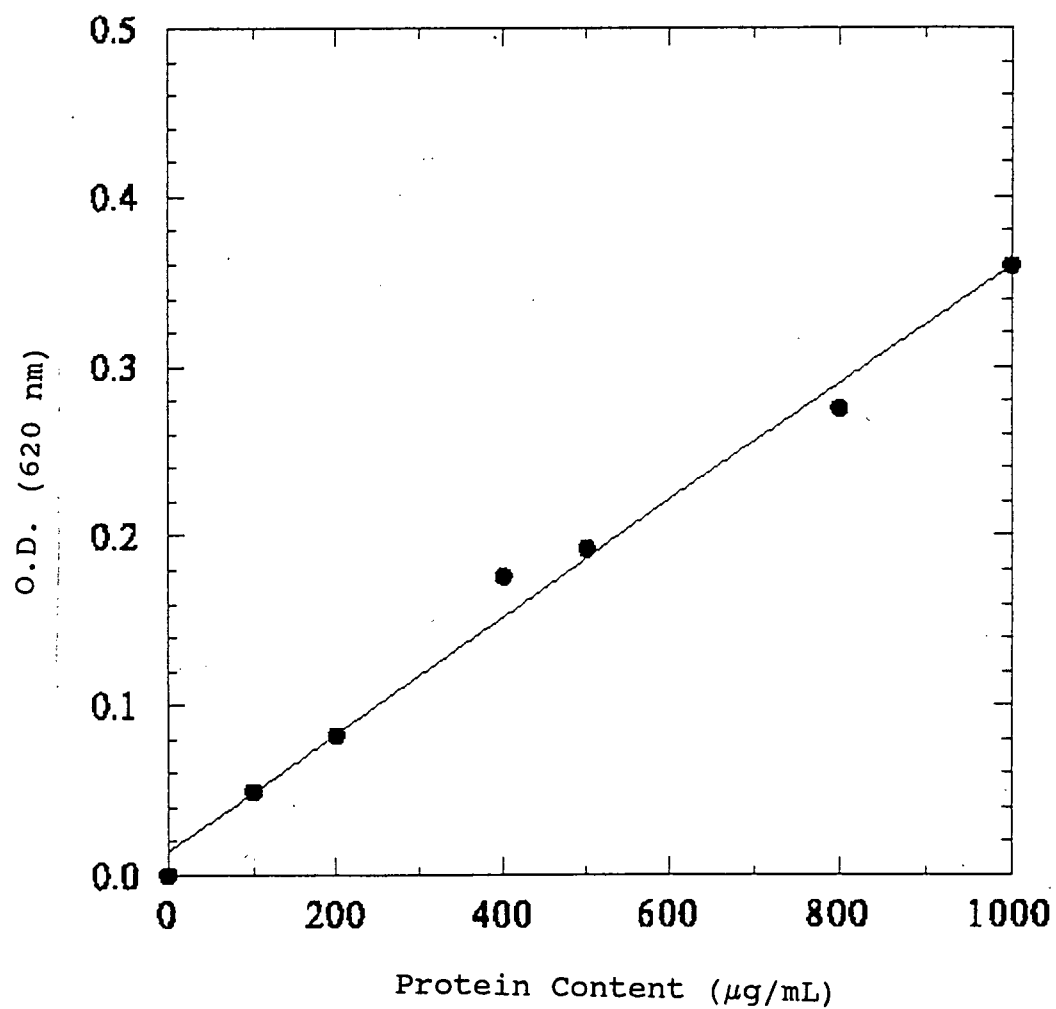


Figure 7. Standard curve for BCA protein assay (Pierce Chemical Company, Rockford, IL).

IV. Methods for Statistical Analysis

Systat Version 5.01 (Systat Inc., Evanston, IL) was the statistical package used for data analysis . The MGLH Module was used to compute 2-way analysis of variance (ANOVA). The effects of treatment and time were tested. A total of 3 or 4 replicates were measured for each treatment and time.

Linear regression analysis was performed to determine the best straight lines for the standard curves in the protease activity assay and the protein assay. The computation was carried out using Systat Version 5.01 using the Smooth=Linear Module.

RESULTS AND DISCUSSIONS

I. IMMOBILIZATION OF DNP-L-LEUCINE TO THE SOLID PHASE

a. Detection of Immobilized Leucine Molecules

Dinitrophenyl (DNP) labelled L-leucine molecules were used in order to determine if L-leucine molecules could be covalently coupled to the microtiter plate. Any immobilised leucine was detected by reacting with anti-DNP antibody, which was conjugated to horseradish peroxidase as described in section I.b of Materials and Methods.

When the chromogenic orthophenylenediamine (OPD) was added to the reaction mixture along with the anti-DNP antibody horseradish peroxidase substrate (hydrogen peroxide), a soluble yellow to orange product was formed in the wells. The intensity of this colour, which was measured at 492 nm, would be expected to be directly proportional to the amount of leucine covalently coupled to the microtiter plate. DNP-L-leucine solutions with different concentrations were prepared and were immobilized to the solid phase with Covalink-NH₂ groups as outlined in the Materials and Methods section. DNP-L-leucine was dissolved either in distilled water or in a mixture of dimethylsulfoxide and water. The experimental data indicated that maximum absorbance at 492 nm occurred when the concentration of DNP-L-leucine in the microtiter well was at 25 µg/100 µL (Table 1, Figure 8); this was equivalent to 11.04 µg L-leucine/100 µL. In addition, absorbance was higher for DNP-L-leucine previously dissolved in dimethylsulfoxide-distilled water mixture. Such difference could be explained by the

Table 1. The optimal concentration of DNP-L-leucine for coupling on solid phase

Conc. of DNP-leucine ($\mu\text{g}/100 \mu\text{L}$)	Optical density at 492 nm for DNP-L-leucine dissolved in water*	Optical density at 492 nm for DNP-L-leucine dissolved in dimethylsulfoxide*	Optical density at 492 nm for DNP-L-leucine dissolved in dimethylsulfoxide+	Optical density at 492 nm for non-activated DNP-L-leucine in dimethylsulfoxide@
0.0	0.106 $\pm$ 0.014	0.103 $\pm$ 0.004	0.033 $\pm$ 0.003	0.026 $\pm$ 0.001
5.0	0.154 $\pm$ 0.029	0.500 $\pm$ 0.024	0.037 $\pm$ 0.003	0.040 $\pm$ 0.003
7.5	0.146 $\pm$ 0.021	0.589 $\pm$ 0.020	0.041 $\pm$ 0.005	0.031 $\pm$ 0.001
12.5	0.161 $\pm$ 0.016	0.531 $\pm$ 0.037	0.039 $\pm$ 0.002	0.036 $\pm$ 0.003
25.0	0.842 $\pm$ 0.066	1.216 $\pm$ 0.142	0.045 $\pm$ 0.003	0.039 $\pm$ 0.003
50.0	0.872 $\pm$ 0.054	0.986 $\pm$ 0.056	0.048 $\pm$ 0.006	0.037 $\pm$ 0.004
75.0	0.826 $\pm$ 0.062	0.902 $\pm$ 0.042	0.041 $\pm$ 0.005	0.042 $\pm$ 0.001

* Mean $\pm$ standard deviation of three duplicates.
DNP(dinitrophenyl)-L-leucine was activated by EDC and NHS.
Nunc Immuno Module was used as the solid phase.

+ Mean $\pm$ standard deviation of two duplicates.
DNP-L-leucine was activated by EDC and NHS.
Immunlon 2 polystyrene microtiter immunoassay plates without linker arms was used as the solid phase.

@ Mean $\pm$ standard deviation of three duplicates.
DNP-L-leucine was not activated by EDC and NHS.
Nunc Immuno Module was used as the solid phase.

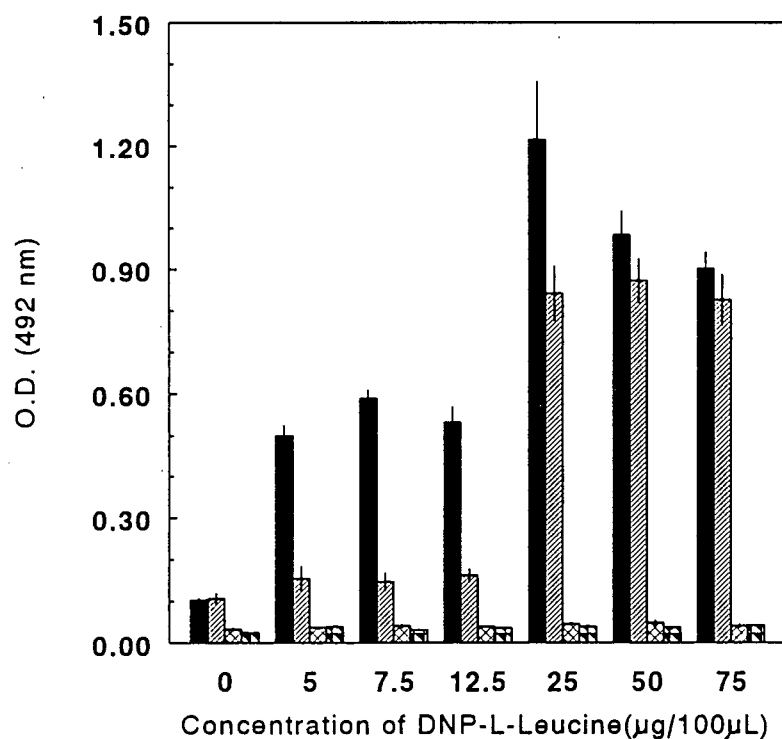


Figure 8. Immobilization of DNP-L-leucine to Nunc Immuno Module.

- DNP-L-leucine was dissolved in DMSO/water and was activated by EDC and NHS.
- DNP-L-leucine was dissolved in water and was activated by EDC and NHS.
- DNP-L-leucine was dissolved in DMSO/water and was activated by EDC and NHS. Immunolon 2 polystyrene microtiter plate instead of Nunc Immuno Module was used.
- DNP-L-leucine was dissolved in DMSO/water but was not activated by EDC and NHS.

fact that DNP-L-leucine was more soluble in the DMSO-water solvent than in water alone. Since the maximum binding of DNP-L-leucine molecules occurred at 25 $\mu\text{g}/100\ \mu\text{L}$, which was equivalent to 11.04 μg L-leucine/100 μL , as reflected by high absorbance readings, the concentration of L-leucine was kept at about 10 μg per well for all other experiments.

b. Binding of DNP-L-leucine to Conventional Microtiter Plate

To determine whether DNP-L-leucine would bind to ordinary microtiter plates without any secondary amino groups, an attempt was made to attach DNP-L-leucine to Immulon 2 polystyrene microtiter immunoassay plates (Dynatech Laboratories Inc., Chantilly, VA) following the method described in section I.a. of Materials and Methods. Figure 8 shows that only a very small amount of DNP-L-leucine would bind to a microtiter plate without any linker arms; this could be caused by the fact that smaller molecules do not adsorb very well to the polystyrene surface. Therefore, this type of solid phase was not as effective in binding small molecules as the Nunc Immuno Module.

c. Noncovalent Binding of DNP-L-leucine

To test whether leucine molecules could bind noncovalently to the Immuno Module, distilled water instead of EDC-NHS was added to DNP-L-leucine mixture during the activation step. Figure 8 showed that only a very low level of noncovalent binding occurred; this binding was probably due to passive adsorption of DNP-L-leucine to

the linker arms. But since optical density signal detected for EDC-NHS activated DNP-L-leucine molecules was much stronger than that detected for the unactivated molecules, it was evident that covalent attachment of DNP-L-leucine molecules had occurred on the microtiter plate.)

d. Quantification of the Amount of DNP-L-leucine Immobilized to the Microtiter Plate

It was unfortunate that the actual amount of DNP-L-leucine immobilized in microtiter wells could not be quantified as planned. Initially, it was assumed that the amount of leucine bound to the plate could be deduced from a calibration curve prepared from DNP-L-leucine solutions with a range from 0 - 100 $\mu\text{g/mL}$. In one experiment, DNP-leucine solution at various concentrations was pipetted into a normal microtiter plate so that the wells did not have any linker arms. When Ab-peroxidase and OPD substrate were subsequently added to the wells, it was anticipated that the intensity of colour produced in each well would be proportional to the amount of DNP-L-leucine present in that well; hence, the higher the concentration, the higher the intensity of colour. Unfortunately, all of the wells ended up having the same colour intensity regardless of what concentration of DNP-L-leucine was present. This was because the unreacted ab-peroxidase conjugate could not be removed from the reaction mixture, and it continued to react with the OPD substrate to produce a very intense red colour. The end result was that every well in the microtiter plate had the same reading, which were all off scale readings because of the

intense colour, regardless of how much DNP-leucine was present in the microtiter well. Although the actual amount of immobilized L-leucine could not be determined, the change in optical density at 492 nm was still indicative of the relative amounts of leucine that could be coupled to the plate. Thus, the higher the optical density reading, the greater was the amount of immobilized L-leucine.

II. THERMOLYSIN CATALYSED SOLID PHASE SYNTHESIS OF DNP-ASN-LEU

For solid phase dipeptide synthesis, L-leucine was the immobilized nucleophile while dinitrophenyl-L-asparagine (DNP-L-asn) was the free acyl donor. L-asparagine molecules that had been coupled with dinitrophenyl groups were used so that any dipeptide formed on the solid phase could be detected using AB-peroxidase conjugate and OPD. There was another reason for using dinitrophenyl labelled L-asparagine: labelling of the amino group on the L-asparagine molecule with dinitrophenyl effectively prevented formation of homopolymers of asparagine.

a. Selection of Blocking Agent

In order to minimize non-specific binding of Ab-peroxidase conjugate to the solid phase, unreacted sites left on the microtiter plate were blocked with a blocking agent. To test the effectiveness of various blocking agents on Immuno Modules, leucine molecules (without dinitrophenyl groups) were immobilized to the solid phase. Unreacted linker arms were blocked with blocking

agents. Antibody-peroxidase conjugate was then added to the microtiter wells. Theoretically, the antibody-peroxidase conjugate should not bind to the plate because there was no dinitrophenyl group for the antibody to react with. Therefore, detection of absorbance at 492 nm would be caused by non-specific binding of the conjugate to the plate. The intensity of absorbance would be indicative of the effectiveness of the blocking agent used. Various blocking agents were tested, and 0.5% skim milk powder solution was found to be the most effective (Table 2). Consequently, 0.5% skim milk powder was used as a blocking agent in all of the dipeptide synthesis experiments. An additional 0.05% (w/v) skim milk powder was also added to the antibody peroxidase complex to keep non-specific binding to a minimum.

b. Detection of the Formation of DNP-Asn-Leu

Initial experiments were focused on synthesis reactions catalyzed by commercial grade thermolysin. Some of the experimental data are shown in Figures 9, 10, 11 a to d, 12 and 13 with the reaction conditions of these experiments summarized in Table 3. The data clearly showed that there was an increase in optical density readings at 492 nm in those microtiter wells containing thermolysin when the plates were incubated for varying lengths of time. This suggested that some peptide synthesis activity had occurred in the microtiter wells. Unfortunately, it was not possible to quantify the amount of DNP-asn-leu formed in the microtiter wells for the same reason provided earlier in

Table 2. The efficiency of various blocking Agents

Type of blocking agent ⁺	Absorbance _{492nm} [*]
2% bovine serum albumin	0.125 ± 0.008
2% gelatin	1.714 ± 0.150
0.5% skim milk powder	0.018 ± 0.001

⁺ all blocking agents were dissolved in 0.01 M phosphate buffer, pH 7.2.

^{*} Values are the mean ± standard deviation of 6 readings.

Table 3. Reaction conditions¹ for thermolysin-catalysed solid-phase synthesis of DNP-Asn-Leu

Fig.	DNP-Asn per well (nmol)	Thermo- lysin per well (μ g)	Protease ² Activity per well (units)	Time of Incubation (Hr.)	pH	Temp. (°C)
9	7.56	50.2	38.8	6	6.5	48
10	7.56	50.2	38.8	6	6.0	48
11a	7.56	50.2	38.8	6	6.0	48
11b	37.84	50.2	38.8	6	6.0	48
11c	75.70	50.2	38.8	6	6.0	48
11d	151.40	50.2	38.8	6	6.0	48
12	7.56	10.0	7.8	2	6.0	48
13	7.56	10.0	7.8	2	6.0	48

¹ 76.3 nmol of L-leucine was used in all of the experiments.

² One unit of protease activity was defined as the quantity required to increase the absorbance of 1 μ g of tyrosine per min at 37°C.

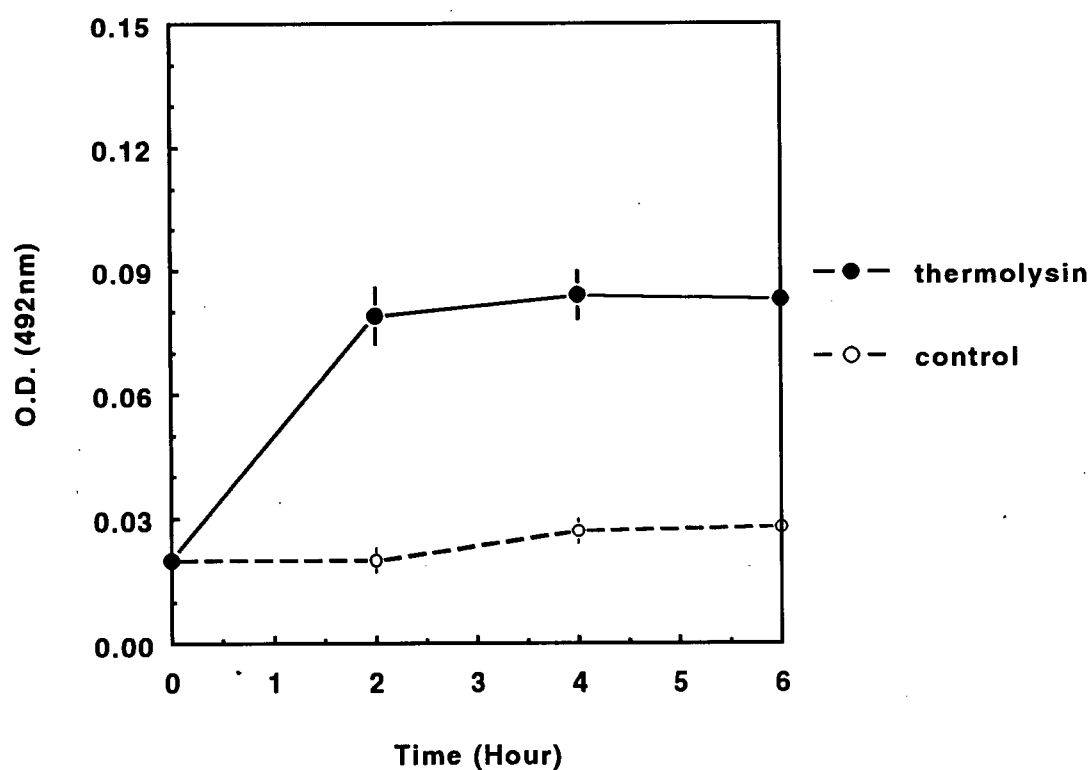


Figure 9. Exp. 1. Thermolysin catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6.5 and 50.2 μ g thermolysin with 38.8 units of protease activity* were added to the wells; thermolysin was not added to the control samples.

* Protease activity was defined as one unit of protease required to increase the absorbance of 1 μ g of tyrosine/min at 37°C.

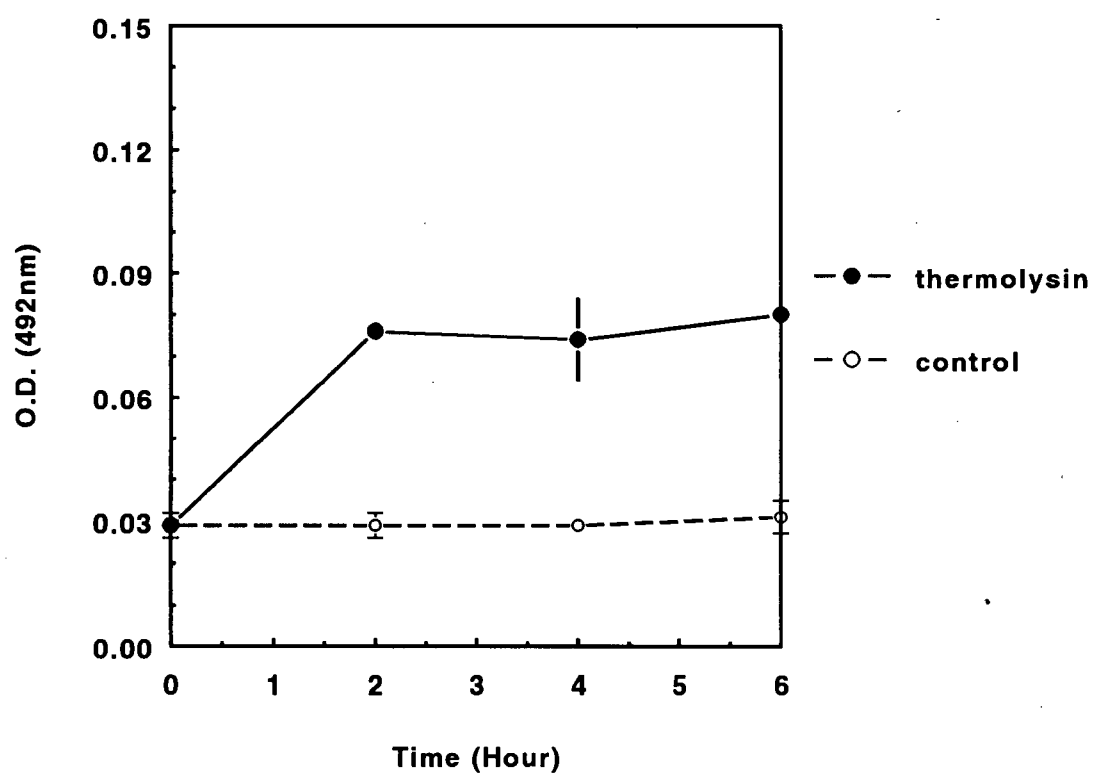


Figure 10. Exp. 2. Thermolysin catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6.0 and 50.2 μ g thermolysin with 38.8 units of protease activity* were added to the wells; thermolysin was not added to the control samples.

* Protease activity was defined as one unit of protease required to increase the absorbance of 1 μ g of tyrosine/min at 37°C.

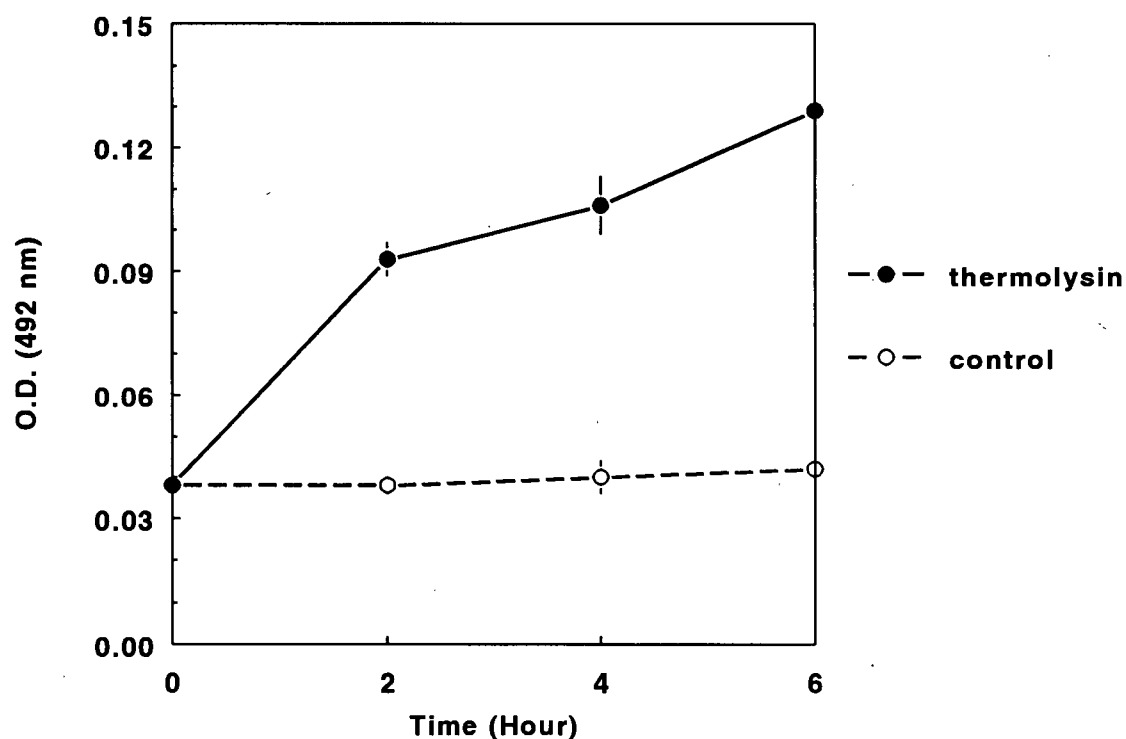


Figure 11a. Exp. 3a. Thermolysin catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6.0 and 50.2 μ g thermolysin with 38.8 units of protease activity* were added to the wells; thermolysin was not added to the control samples.

* Protease activity was defined as one unit of protease required to increase the absorbance of 1 μ g of tyrosine/min at 37°C.

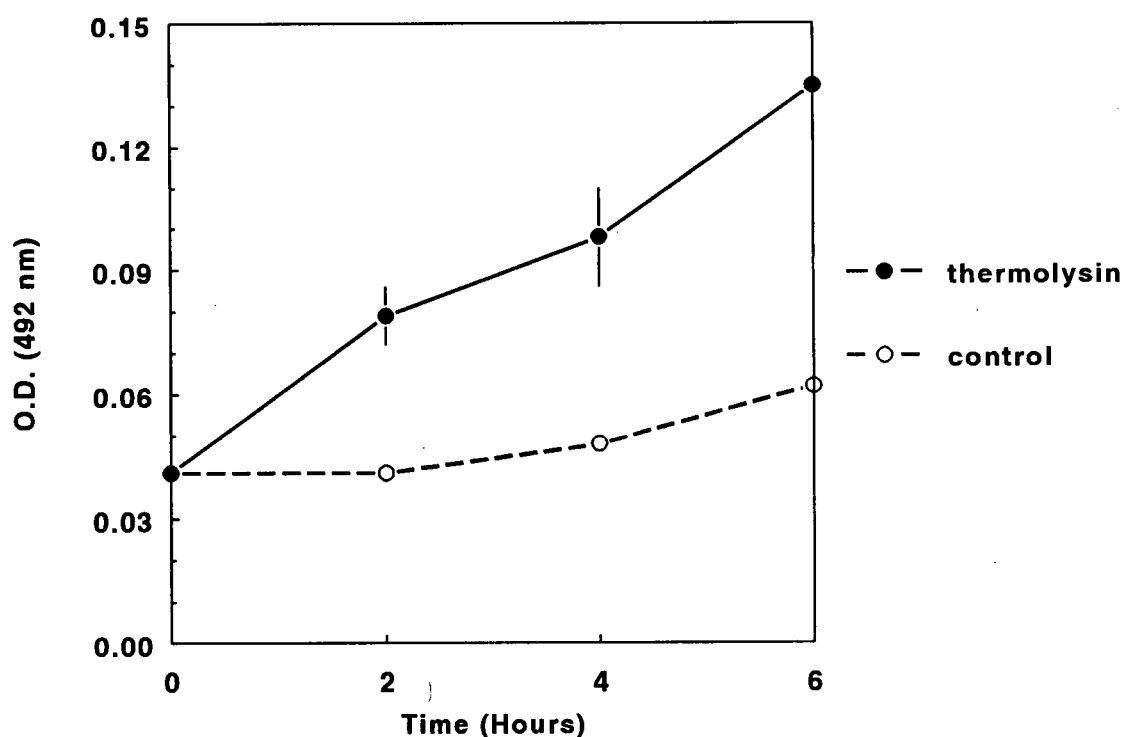


Figure 11b. Exp. 3b. Thermolysin catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6.0 and 50.2 μ g thermolysin with 38.8 units of protease activity* were added to the wells; thermolysin was not added to the control samples.

* Protease activity was defined as one unit of protease required to increase the absorbance of 1 μ g of tyrosine/min at 37°C.

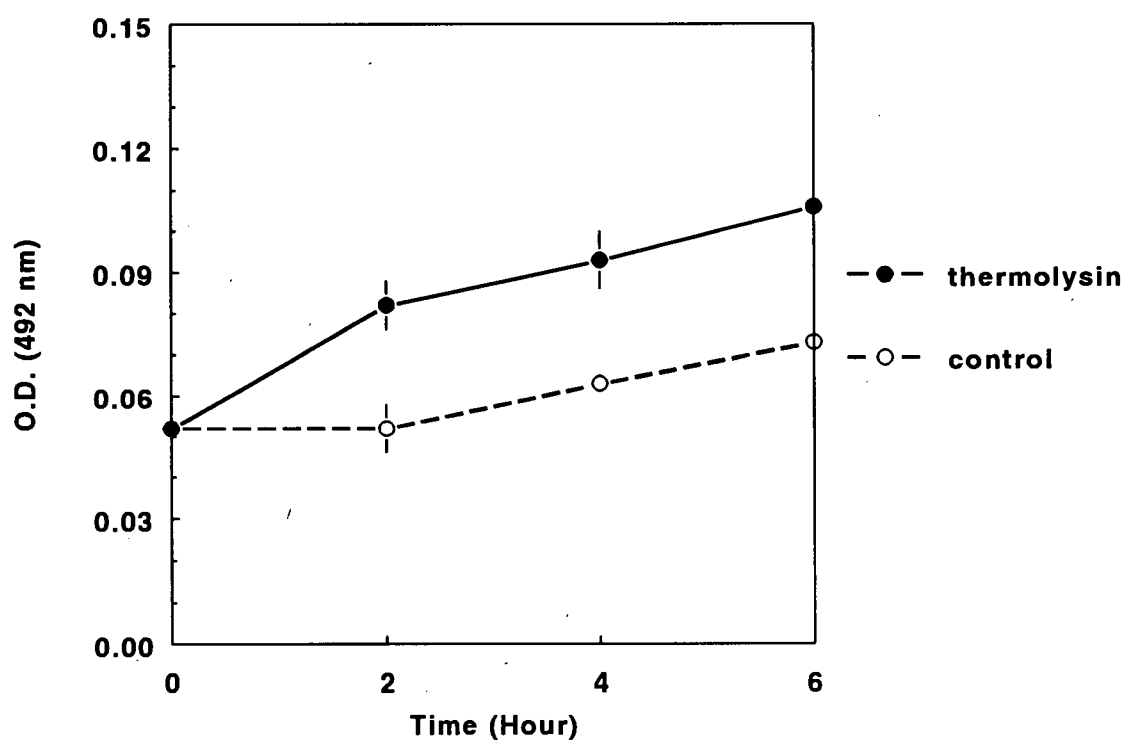


Figure 11c. Exp. 3c. Thermolysin catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6.0 and 50.2 μ g thermolysin with 38.8 units of protease activity* were added to the wells; thermolysin was not added to the control samples.

* Protease activity was defined as one unit of protease required to increase the absorbance of 1 μ g of tyrosine/min at 37°C.

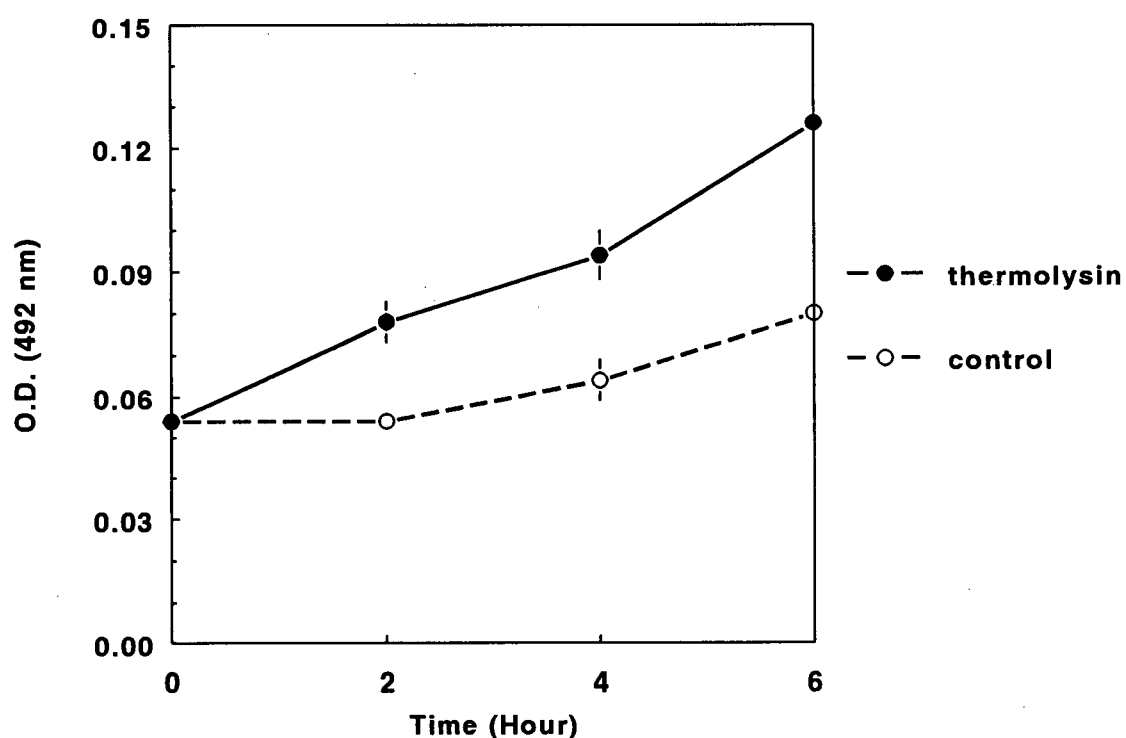


Figure 11d. Exp. 3d. Thermolysin catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6.0 and 50.2 μ g thermolysin with 38.8 units of protease activity* were added to the wells; thermolysin was not added to the control samples.

* Protease activity was defined as one unit of protease required to increase the absorbance of 1 μ g of tyrosine/min at 37°C.

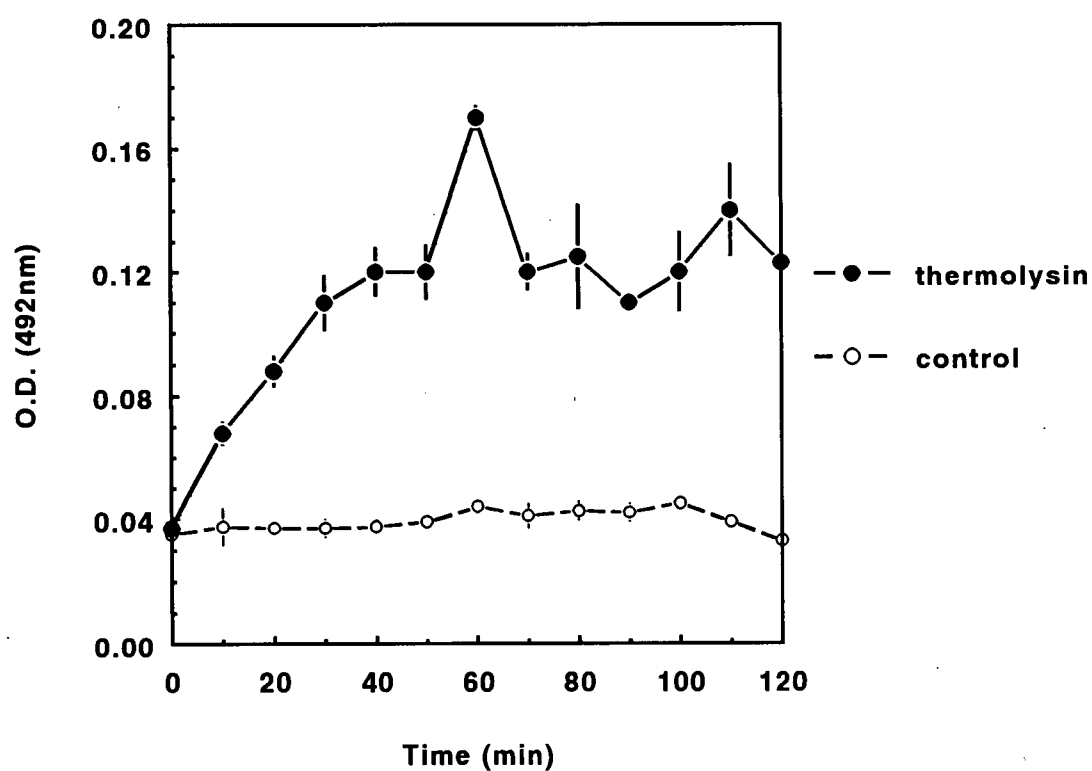


Figure 12. Exp. 4. Thermolysin catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6.0 and 10 μ g thermolysin with 7.8 units of protease activity* were added to the wells; thermolysin was not added to the control samples.

* Protease activity was defined as one unit of protease required to increase the absorbance of 1 μ g of tyrosine/min at 37°C.

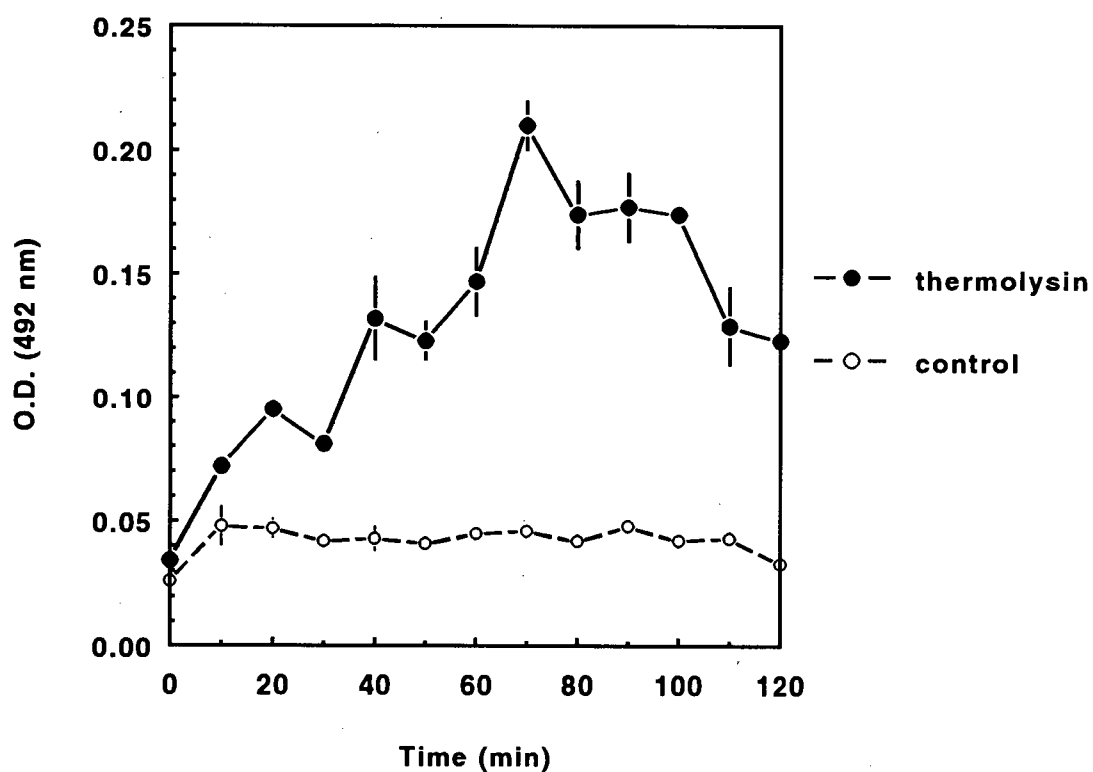


Figure 13. Exp. 5. Thermolysin catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6.0 and 10 μ g thermolysin with 7.8 units of protease activity* were added to the wells; thermolysin was not added to the control samples.

* Protease activity was defined as one unit of protease required to increase the absorbance of 1 μ g of tyrosine/min at 37°C.

section I.d of Results and Discussions. Nevertheless, the increase in optical density was indicative of the relative amount of dipeptide formed on the microtiter plate; in other words, the greater the increase, the greater was the yield of DNP-asn-leu. On the other hand, the control samples with the wells containing only buffer solution and DNP-L-asparagine had relatively constant optical density readings in most cases. The background readings observed in these wells was probably caused by either non-specific binding of DNP-L-asparagine, or Ab-peroxidase conjugate to the microtiter plates.

c. Effect of the Concentration of DNP-L-asn on Dipeptide Formation

Figures 11 a to d show the effect of varying the concentration of DNP-L-asparagine in the reaction mixture. The use of higher concentrations of DNP-L-asparagine did not seem to have any beneficial effect on dipeptide formation because there was no notable increase in the optical density readings in the test samples: the mean O.D._{492 nm} readings after 4 hours of incubation were 0.106, 0.098, 0.093 and 0.094 for the test groups containing 7.57 nmol, 37.84 nmol, 75.7 nmol and 151.4 nmol of DNP-L-asparagine respectively. In contrast, the use of higher concentration of DNP-L-asparagine resulted in an increase of non-specific binding of DNP-L-asparagine to the micro-titer plate as evidenced by the increased optical density readings over time in the control group with no thermolysin. Therefore, the amount of DNP-L-asparagine

added to each well was kept at 7.56 nmol for all other experiments.

d. The Effect of Reaction Time on Dipeptide Synthesis

Although Miranda and Tominaga (1991) reported that optimum reaction time for asn-leu synthesis reaction was 24 hours, experimental data with present study did not support the fact that longer reaction time was needed for higher yield except in experiment 3 (Figures 11a to 11d). In fact, when the microtiter plates were incubated for more than 8 hours at 48°C, evaporation of the reaction mixture actually occurred to varying degrees in the wells in spite that each plate was already covered with a plastic lid. Figures 9 and 10 show that optical density measurements remained constant from 2 to 6 hours of incubation; the same observation was made in three other similar experiments (data not presented). Thus, most of the reaction happened within the first 2 hours. Experiment 3 (Figures 11 a to d) was the only experiment in which the optical density readings continued to increase over time; the reason for this was not clear. Since longer reaction time did not improve the yield of dipeptides, the reaction time for subsequent experiments was reduced to 2 hours. As shown in Figures 12 and 13, the synthesis reaction proceeded rapidly within the first 40 to 60 minutes, and then started to either level off or decline. A drop in optical density readings could be attributed to concurrent enzymatic hydrolysis of already formed dipeptides.

e. Effect of Thermolysin Concentration on Dipeptide Synthesis

Dipeptide formation was not adversely affected when thermolysin concentration was reduced 5 fold from 50.2 μg to 10 μg per well (Figures 9 to 13). When the optical density readings recorded at the end of 2 hours of reaction period were compared, the readings measured from the wells containing the lower levels of thermolysin were actually slightly higher than those measured from microtiter wells containing 5 times more thermolysin: the mean optical density measurements (after 2 hours of incubation) for figures 12 and 13 was 0.12 while the average optical density measurements for figures 9, 10 and 11a was 0.09. It was possible that the presence of excessive amount of enzyme actually promoted more hydrolysis of the formed dipeptides, which in turn resulted in slightly lower optical density measurements.

f. Statistical Analysis

Statistical analysis was performed on the data for experiments 1 to 5 in order to determine whether or not there was a difference in the mean optical density measurements at 492 nm between the test group with thermolysin and the control group without any enzyme. The statistical analysis chosen was a two-factor analysis of variance (or 2-way ANOVA). The effect of time and treatments (i.e. with and without thermolysin), and their interactions on the mean optical density readings measured at 492 nm were tested and the

results are summarized in Table 4. The calculated F ratios revealed that there was significant difference ($P < 0.01$) in O.D._{492 nm} due to the different treatments in all of the experiments. This clearly indicated that the mean optical density measurements were not the same for the control groups and the test groups (with enzyme treatment). Since the change in optical density readings at 492 nm in the wells was related to the relative amount of dipeptides formed, one can therefore conclude that the effect of treatment was significant on the amounts of DNP-asn-leu formed in the microtiter wells. Hence, the experimental data and subsequent statistical analyses proved that it was possible to use the proposed rapid screening method to detect formation DNP-asn-leu dipeptides on a solid-phase when commercial grade thermolysin was used as a catalyst.

III. DNP-ASN-LEU REACTIONS CATALYSED BY CRUDE ENZYME FRACTION SECRETED BY BACTERIAL CULTURE

The rapid solid-phase screening assay was developed with the intention to detect mutant enzymes with enhanced reverse proteolysis property. These enzymes would be produced by bacterial cells harbouring a mutagenized nprT gene. The nprT gene from *Bacillus stearothermophilus* encodes for a thermolysin-like neutral protease. Since any secreted enzymes from bacterial cultures would exist in a less concentrated form than commercial grade thermolysin, it was therefore important to determine whether such crude secreted enzyme fractions with lower enzymatic activity were

Table 4. 2-Way analysis of variance on optical density readings for five thermolysin catalysed Leu-Asn synthesis experiments

Fig.	Exp.	Source of Variation	DF	F ratio
9 ^b	1	Time	3	104.4**
		Treatment ^a	1	660.7**
		Time x Treatment	3	73.66**
		Error	24	
10 ^c	2	Time	3	36.02**
		Treatment ^a	1	308.4**
		Time x Treatment	3	17.42**
		Error	16	
11a ^b	3 a	Time	3	144.9**
		Treatment ^a	1	986.9**
		Time x Treatment	3	123.9**
		Error	24	
11b ^b	3 b	Time	3	53.90**
		Treatment ^a	1	154.1**
		Time x Treatment	3	22.42**
		Error	24	
11c ^b	3 c	Time	3	28.06**
		Treatment ^a	1	52.44**
		Time x Treatment	3	6.270
		Error	24	
11d ^b	3 d	Time	3	102.0**
		Treatment ^a	1	146.4**
		Time x Treatment	3	22.45**
		Error	24	
12 ^b	4	Time	12	55.49**
		Treatment ^a	1	3125**
		Time x Treatment	12	44.52**
		Error	78	
13 ^b	5	Time	12	64.71**
		Treatment ^a	1	2538**
		Time x Treatment	12	54.35**
		Error	78	

^a Treatment 1 = Control sample (DNP-asparagine + buffer).
Treatment 2 = Test sample (DNP-asparagine + buffer + thermolysin).

^b 4 wells per time-treatment combination.

^c 3 wells per time-treatment combination.

** Significant at $P < 0.01$.

capable of promoting dipeptide formation on a solid phase. To test this hypothesis, crude enzyme fractions were isolated from *Bacillus subtilis* DB428 and *Bacillus subtilis* WB600, which had been transformed with the npr T gene.

a. Use of *Bacillus subtilis* DB428 and WB600 to Produce Enzyme Mixture

The mutant strains of *Bacillus subtilis* DB428 and WB600 were chosen as the host organisms for 2 reasons. First, *Bacillus subtilis* organisms produce enzyme extracellularly, so secreted enzyme(s) could be easily recovered from the growth medium. Second, DB428 and WB600 are protease deficient strains which produce little background enzymatic activity. Hence, any enzyme released into the growth medium by this transformed organism would likely be the products encoded by the npr T gene. The background extracellular protease activity of the wild type *Bacillus subtilis* (strain 168) as well as those of the protease deficient DB428 and WB600 are summarized in Table 5.

Transformed *Bacillus subtilis* DB428(pNP22) and WB600(pNP22) were prepared as outlined in Materials and Methods (section II. a.1). The collected supernatants with enzymatic activity were directly used to catalyse dipeptide synthesis or were subsequently concentrated by ultrafiltration and the retentate was diafiltered to remove low molecular weight impurities from the growth medium. Washed retentate was used to catalyse DNP-aspartyl-leucyl dipeptide synthesis on the Immuno Modules. Figure 14 shows the SDS-gel electrophoresis patterns of commercial grade thermolysin as well as

Table 5. Extracellular protease activity from wild-type (168) and protease-deficient (DB428 and WB600) strains of *Bacillus subtilis*

Strain	Protease Activity ¹ at 24 hr. (unit/0.25 mL supernatant) ²	% Protease Activity
168	772	100.0
DB428	17	2.2
WB600	11	1.4

¹ Data was the average values of the supernatant of two separate cultures, which had been incubated for 24 hours at 37°C.

² 1 unit of activity on azocasein = change in absorbance at 366 nm of 0.01/2 hours

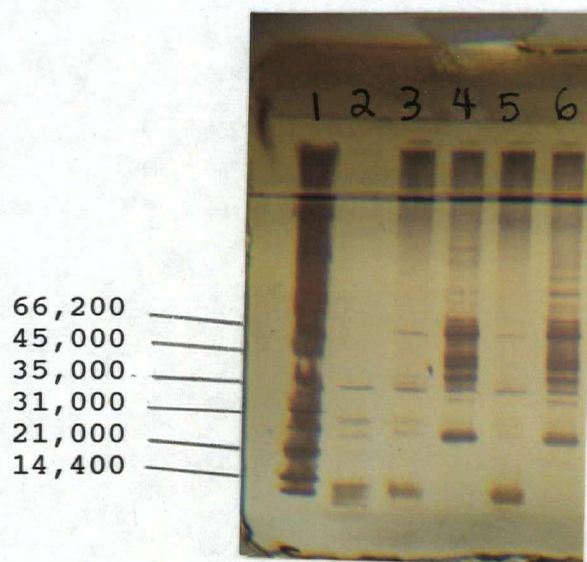


Figure 14. SDS-gel electrophoresis of thermolysin and crude proteases secreted by transformed *B. subtilis* DB428 and WB600. The gel was silver stained.

- Lane 1. Molecular weight marker
- Lane 2. Commercial grade thermolysin
- Lane 3. *B. subtilis* WB600(pNP22)
- Lane 4. *B. subtilis* WB600
- Lane 5. *B. subtilis* DB428(pNP22)
- Lane 6. *B. subtilis* DB428

of the crude proteases secreted by the transformed *B. subtilis* DB428 and WB600.

Table 6 summarizes the results of some of the ultrafiltration experiments which involved supernatants recovered from transformed *B. subtilis* DB428 cultures. The higher ultrafiltration factors obtained when Millipore Pellicon cassette filter system was used demonstrated that this system was a more efficient concentration method than Amicon stirred cell system. This could be related to the fact that only one hour of filtration was required when Pellicon cassette was used. On the other hand, 4 hours of filtration was needed when Amicon stirred cell was used. Therefore, it was possible that autodigestion of the secreted proteases was more extensive when longer filtration time was needed.

b. Effect of Varying the Concentration of Enzyme in the Reaction Mixture

The experimental data for crude enzyme catalysed synthesis reactions have been summarized in Figure 15 to Figure 19 while the reaction conditions for these experiments are recorded in Table 7. A different protease assay, which utilized azocasein as a substrate, was used to determine the protease activity of both thermolysin and crude enzyme preparations because difficulty was experienced in dissolving Hammerstein casein in the previous assay method. The results obtained from determining the protease activity of thermolysin using both Hammerstein casein and azocasein as substrates are summarized in Table 8.

Table 6. Results of ultrafiltration of crude enzyme secreted by transformed *Bacillus subtilis* DB428

Vol. of Supernatant	Vol. of Retentate	Protease Activity* (U/0.25mL) of Supernatant	Protease Activity* (U/0.25mL) of Retentate	Protease Activity* (U/0.25mL) of Filtrate	U.F. ¹ Factor	U.F. ² Factor
³ 30 mL	4.5 mL	725	3270	0	4.5	6.7
⁴ 8.7 L	0.87 L	583	5960	0	10.2	10.0

* 1 unit (U) of protease activity = change in absorbance at 366 nm of 0.01/2 hours (units of activity on azocasein).

$$^1 \text{ Ultrafiltration (U.F.) factor} = \frac{\text{protease activity per 0.25 mL retentate}}{\text{protease activity per 0.25 mL supernatant}}$$

$$^2 \text{ Ultrafiltration (U.F.) factor} = \frac{\text{volume of retentate}}{\text{volume of supernatant}}$$

³ Supernatant was concentrated by Amicon stirred cell.

⁴ Supernatant was concentrated by Millipore Pellicon cassette filter.

Table 7. Reaction conditions¹ for thermolysin and crude enzyme catalysed solid phase synthesis of DNP-Asn-Leu

Fig.	Exp.	DNP-Asn per well (nmol)	Thermo- lysin per well (μ g)	Crude ² enzyme per well (ug)	Protease Activity per well ³ (units)	Peptide Synthesis Ratio ⁴ at 1 Hour of Incubation
15	6	7.56	50.2	- 5.89 7.90	3105 32 44	- - -
16a	7a	7.56	- - -	6.52 ⁵ 13.04 ⁵ 19.56 ⁵	42 84 126	- - -
16b	7b.	37.84	-	19.56 ⁵	126	-
16c	7c.	75.70	-	19.56 ⁵	126	-
16d	7d.	151.40	-	19.56 ⁵	126	-
17	8	7.56	10.0 -	- 17.36 ⁶	621 477	3.7×10^{-5} 1.5×10^{-4}
18	9	7.56	10.0 -	- 17.36 ⁶	621 477	1.1×10^{-4} 1.4×10^{-4}
19	10	7.56	10.0 - -	- 5.27 ⁶ 26.04 ⁶	621 80 715	3.4×10^{-5} 1.4×10^{-4} 7.9×10^{-4}

¹ 76.3 nmol of L-leucine was used in all of the experiments. All dipeptide synthesis reactions were carried out at pH 6. Incubation time for experiments 6 and 7 was 6 hours. Incubation time for experiments 8 to 10 was 2 hours.

² Protein content was determined by BCA method.

³ 1 unit of activity on azocasein = change in absorbance at 366 nm of 0.01 during 2 hrs. at 48°C

$$\frac{\text{O.D.}_{492\text{nm}} \text{ of test sample} - \text{O.D.}_{492\text{nm}} \text{ of control}}{\text{Units of protease activity per well}}$$

⁴ Peptide synthesis ratio = $\frac{\text{Peptide synthesis ratio}}{\text{Units of protease activity per well}}$

⁵ Supernatant was concentrated by Amicon stirred cell.

Table 8. Summary of the protease activity of thermolysin determined by utilizing two different substrates: Hammerstein casein and azocasein

Amount of Thermolysin (nmol)	Units of Protease Activity by Assay #1 ^a	Units of Protease Activity by Assay # 2 ^b
0.29	7.8	621
1.45	38.8	3105

^a Hammerstein casein was used in assay # 1.

^b Azocasein was used in assay # 2.

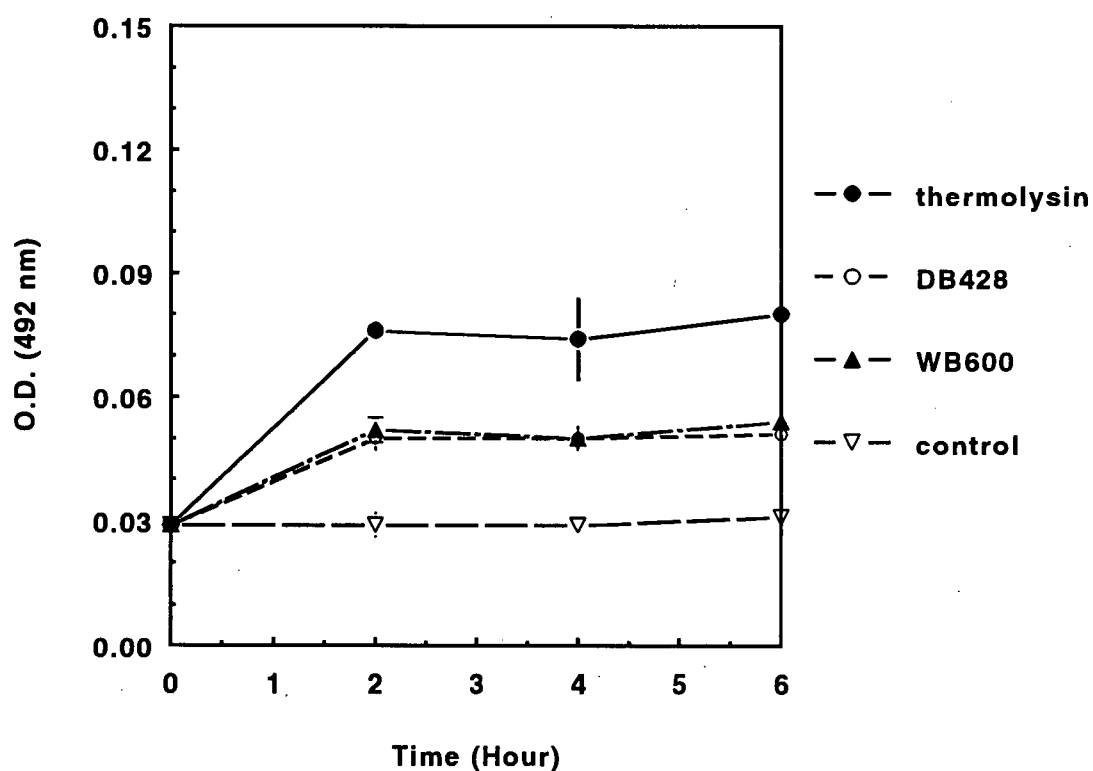


Figure 15. Exp. 6. Thermolysin and crude enzyme catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6 were added to the microtiter wells. The following enzymes were added to designated wells:

- 50.2 μg thermolysin with 3105 units of protease activity*
- 5.89 μg proteases secreted by *B. subtilis* DB428 with 32 units of protease activity*
- 7.89 μg proteases secreted by *B. subtilis* WB600 with 44 units of protease activity*

No enzyme was added to the control samples.

* 1 unit of protease activity on azocasein = change in absorbance at 366 nm of 0.01/2 hours

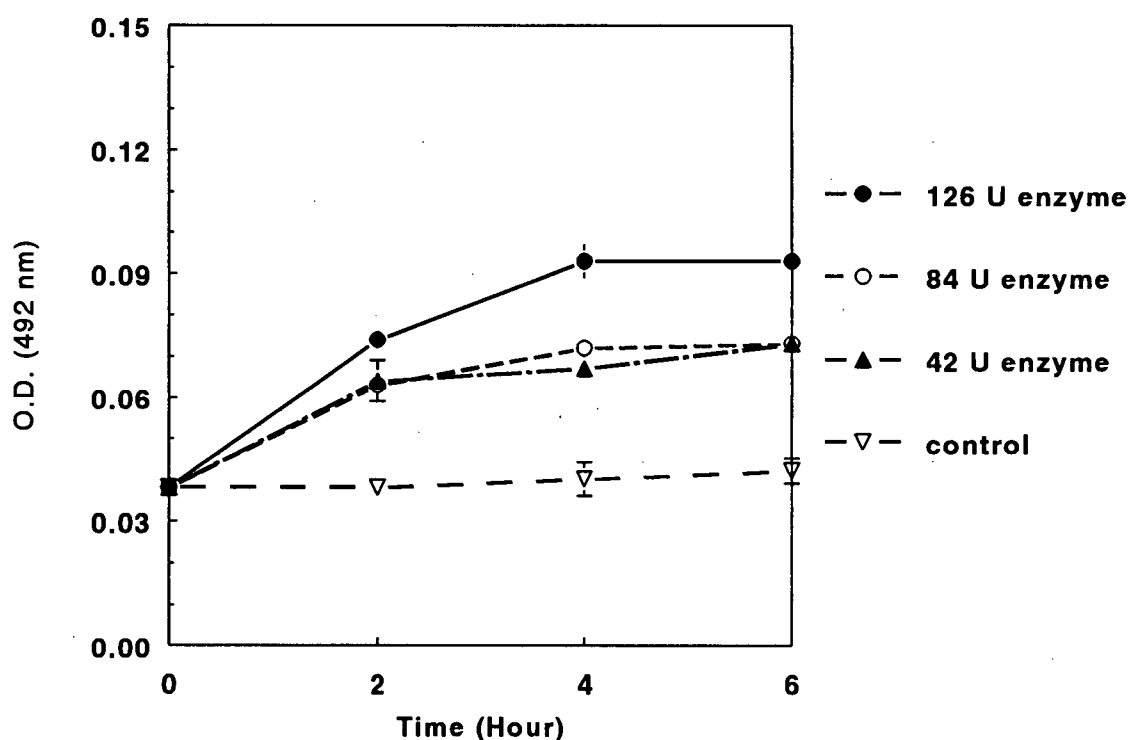


Figure 16a. Exp. 7a. Crude enzyme catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6 and the following amount of crude proteases, secreted by transformed *B. subtilis* DB428, was added to designated wells:

- a. 6.52 μg with 42 units of protease activity*
 - b. 13.04 μg with 84 units of protease activity*
 - c. 19.56 μg with 126 units of protease activity*
- No enzyme was added to the control samples.

* 1 unit of protease activity on azocasein = change in absorbance at 366 nm of 0.01/2 hours

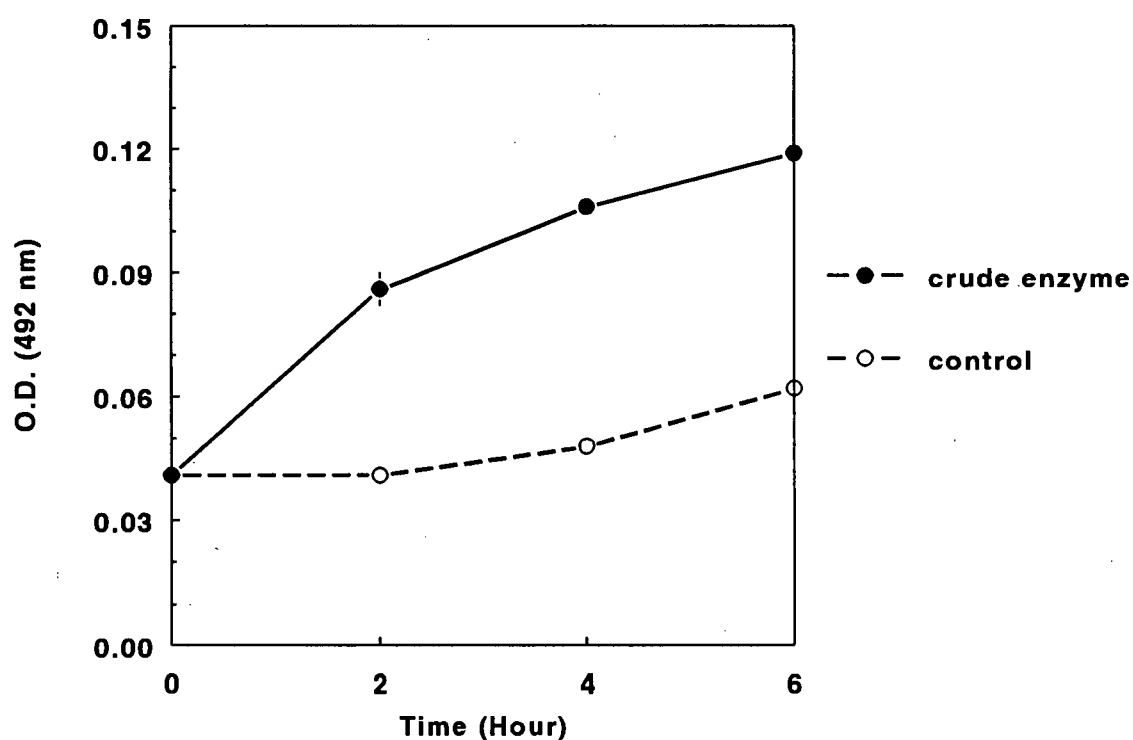


Figure 16b. Exp. 7b. Crude enzyme catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 37.84 nmol DNP-L-asparagine at pH 6 and 6.52 μ g of crude proteases secreted by transformed *B. subtilis* DB428, with 126 units of protease activity*, was added to designated wells; enzyme was not added to the control samples.

* 1 unit of protease activity on azocasein = change in absorbance at 366 nm of 0.01/2 hours

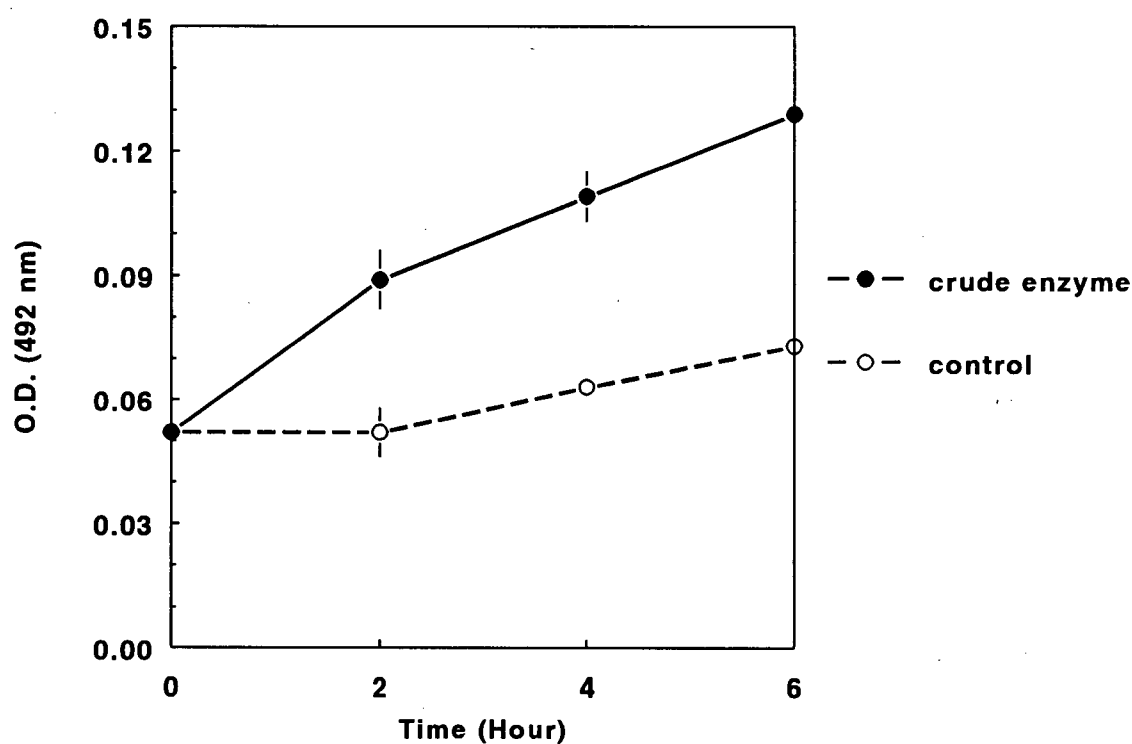


Figure 16c. Exp. 7c. Crude enzyme catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 75.7 nmol DNP-L-asparagine at pH 6 and 6.52 μ g of crude proteases secreted by transformed *B. subtilis* DB428, with 126 units of protease activity*, was added to designated wells; enzyme was not added to the control samples.

* 1 unit of protease activity on azocasein = change in absorbance at 366 nm of 0.01/2 hours

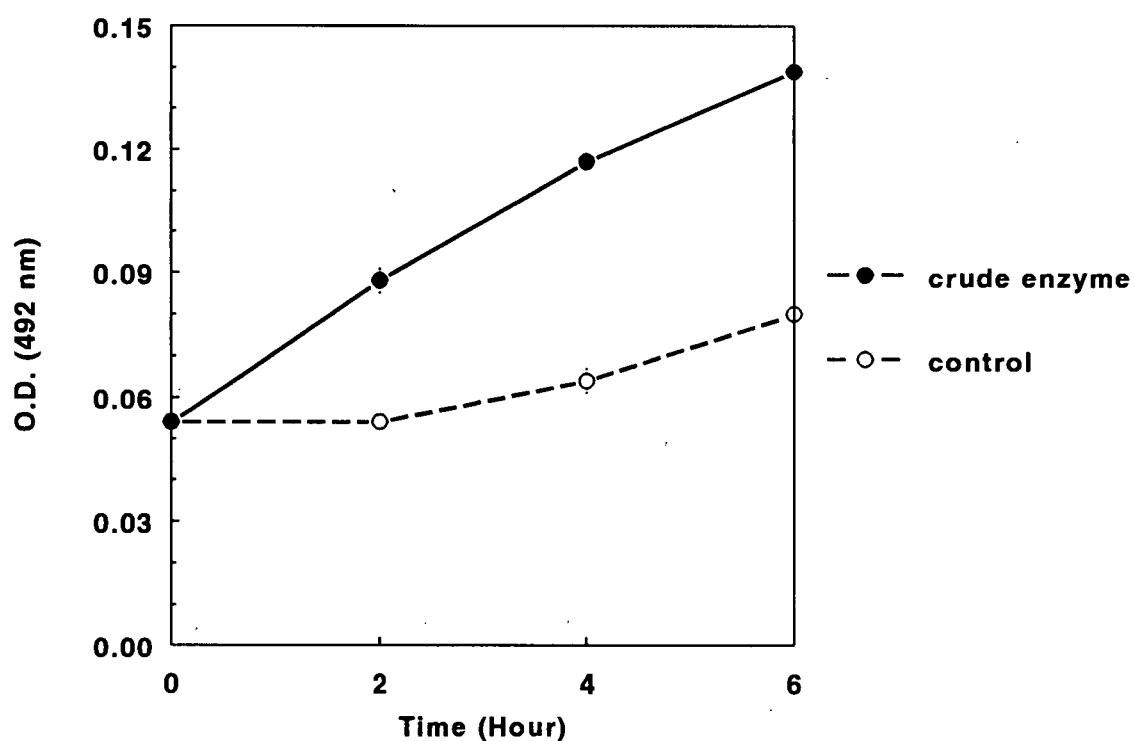


Figure 16d. Exp. 7d. Crude enzyme catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 151.4 nmol DNP-L-asparagine at pH 6 and 6.52 μ g of crude proteases secreted by transformed *B. subtilis* DB428, with 126 units of protease activity*, was added to designated wells; enzyme was not added to the control samples.

* 1 unit of protease activity on azocasein = change in absorbance at 366 nm of 0.01/2 hours

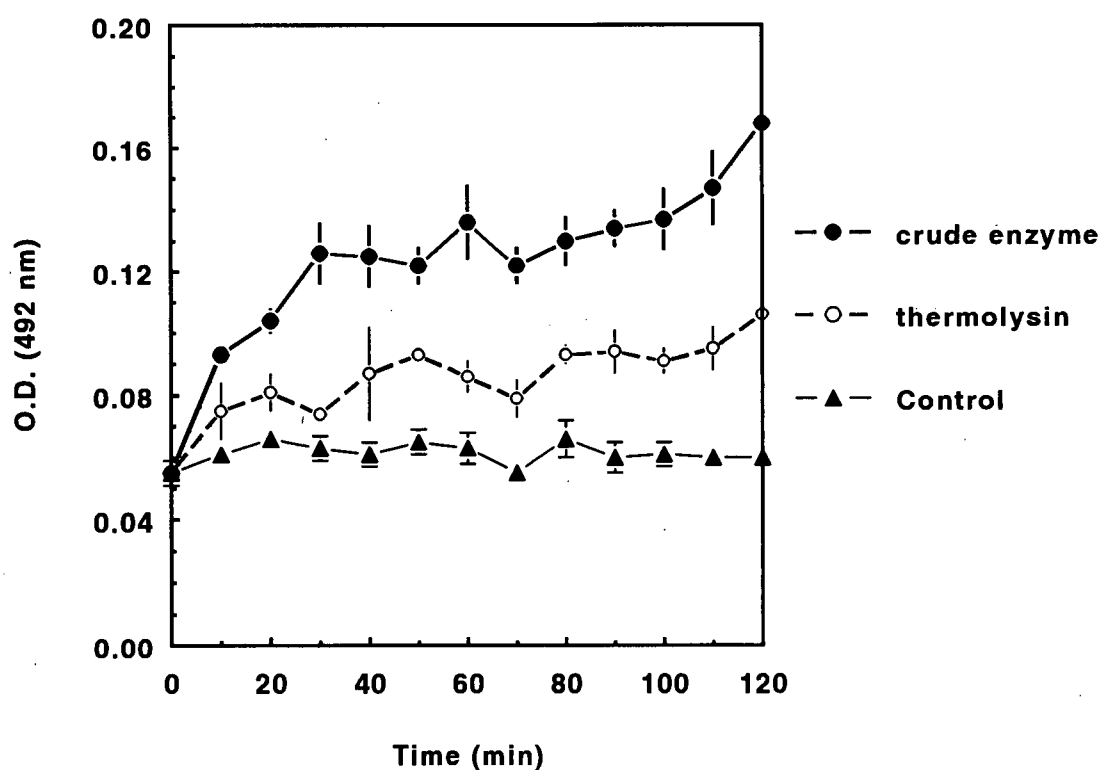


Figure 17. Exp. 8. Thermolysin and crude enzyme catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6 and either 10 μ g thermolysin with 621 units of protease activity* or 17.36 μ g crude proteases secreted by transformed *B. subtilis* DB428, with 477 units of protease activity*, was added to designated wells; enzyme was not added to the control samples.

* 1 unit of protease activity on azocasein = change in absorbance at 366 nm of 0.01/2 hours

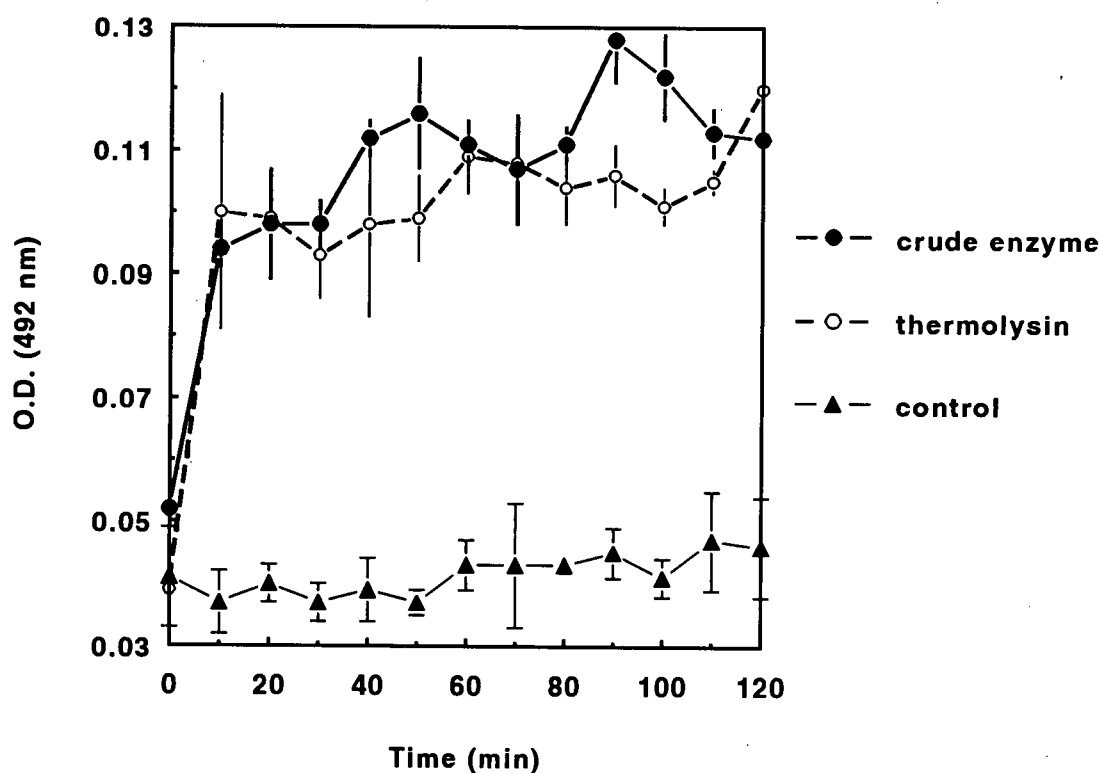


Figure 18. Exp. 9. Thermolysin and crude enzyme catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6 and either 10 μ g thermolysin with 621 units of protease activity* or 17.36 μ g crude proteases secreted by transformed *B. subtilis* DB428, with 477 units of protease activity*, was added to designated wells; enzyme was not added to the control samples.

* 1 unit of protease activity on azocasein = change in absorbance at 366 nm of 0.01/2 hours

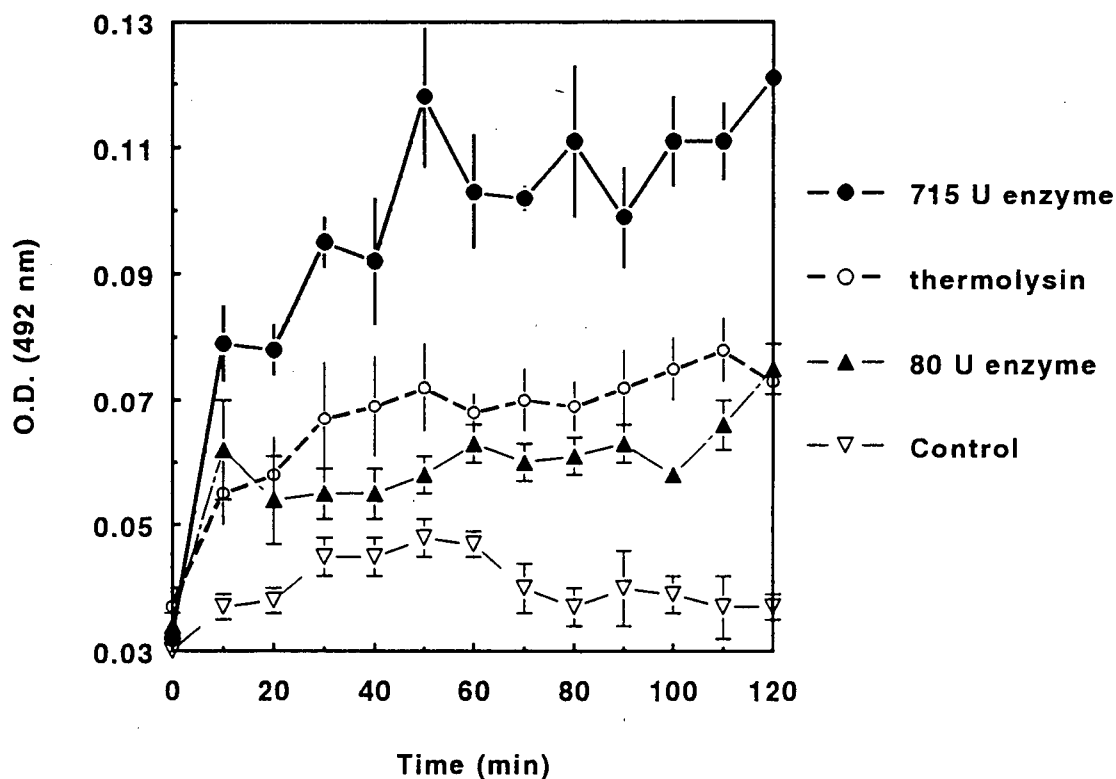


Figure 19. Exp. 10. Thermolysin and crude enzyme catalysed synthesis of DNP-Asn-Leu. 76.3 nmol L-leucine, 7.56 nmol DNP-L-asparagine at pH 6. The following amount of thermolysin and crude proteases secreted by transformed *B. subtilis* DB428 were added to designated wells:

- a. 10 μg thermolysin with 621 units of protease activity*
- b. 5.27 μg crude proteases with 80 units of protease activity*
- c. 26.04 μg crude proteases with 715 units of protease activity*

No enzyme was added to the control samples.

* 1 unit of protease activity on azocasein = change in absorbance at 366 nm of 0.01/2 hours

Figure 15 shows that optical density readings for samples which contained either thermolysin or crude proteases secreted by either transformed *B. subtilis* DB428 or WB600 increased quickly and then leveled off. On the other hand, the optical density readings for the control samples, which consisted of only buffer solution and DNP-L-asn, remained relatively constant for the entire incubation period. The optical density readings measured in microtiter wells containing crude proteases were notably lower than those wells containing thermolysin. This could be due to the fact that there was only a minute amount (5 to 8 μ g) of crude enzyme present in the wells, and that the enzyme had lower protease activity than thermolysin. Since the optical density readings measured at 492 nm were similar in those wells containing either proteases secreted by transformed *B. subtilis* DB428 or WB600, the proteases produced by only one type of transformant (*B. subtilis* DB428) were used to synthesize dipeptides in subsequent experiments. Figure 16a showed that as enzyme activity was increased from 42 U to 126 U per well, dipeptide formation reaction was increased slightly as well: the mean O.D._{492nm} readings after 4 hours of incubation was 0.093, 0.064, 0.064 when protease activity of crude enzyme was 126, 84 and 42 units, respectively. Higher optical density readings were observed again as the protease activity of the crude enzyme was increased to 477 to 715 units per well (Figures 17 to 19); hence, there appeared to be a positive relationship between DNP-asn-leu formation activity on the solid phase and the protease activity of the crude enzymes. On the

other hand, when thermolysin concentration was increased 5 fold to 50 $\mu\text{g nmole}$, there was an increase in protease activity but the formation of dipeptides was not improved. There was probably an optimum concentration of enzyme which resulted in maximum DNP-asn-leu formation. If the concentration of the target enzyme was increased beyond this level, the unreacted or excess enzyme might actually interfere with peptide bond formation, or might increase the concurrent breakdown of the synthesized dipeptides. Aggregation of enzyme molecules could also occur when the concentration of enzyme was too high.

c. Effect of Varying the Concentration of DNP-L-Asn

The effect of increasing the concentration of DNP-L-asn in the reaction mixture containing crude enzyme was summarized in Figures 16 a to d. The optical density readings for the control samples continued to increase during the entire incubation period; similar findings were observed in the thermolysin catalysed reaction. The observed increase in the optical density readings over time in the test samples containing crude enzyme was also caused by an increase in the non-specific binding of DNP-L-asn to the microtiter plate rather than by an actual increase in DNP-asn-leu formation. This was supported by the fact that the difference in O.D._{492nm} readings between the test samples and the control samples at any point of incubation was similar regardless of the concentration of DNP-L-asn added to the wells. For example, the difference in O.D._{492 nm} at 6 hours between the test group and the control group was as follows:

0.051, 0.060, 0.056 and 0.059 while the concentration of DNP-L-asn used was 7.57 nmol, 37.87 nmol, 75.7 nmol and 151.4 nmol, respectively.

d. Comparison of Peptide Synthesis Ratio for Thermolysin and Crude Enzyme Preparation

A peptide synthesis ratio (Table 7) was calculated for three separate dipeptide synthesis experiments catalysed by thermolysin and crude enzyme fraction, and was defined as the change in optical density readings at the end of 1 hour of incubation divided by the units of protease activity per well. The use of these ratios permitted direct comparison of the relative reverse proteolytic efficiencies of the crude enzyme preparation and commercial grade thermolysin. For experiment 9, the peptide synthesis ratio for thermolysin and the crude enzyme fraction was found to be 1.1×10^{-4} and 1.4×10^{-4} , respectively. This showed that the crude enzyme(s) secreted by transformed *Bacillus subtilis* DB428 performed at least as well as, if not better, than commercial grade thermolysin in catalysing the formation of peptide bonds. In experiment 10, when the protease activity of the crude enzyme was increased from 80 units to 715 units per well, the peptide synthesis ratio increased about 5 fold (Table 7). Thermolysin performed surprisingly poorly in experiments 8 and 10 as the increase in optical density readings was not as much as those in experiment 4, 5 and 9. The reason for this was not clear but could be related to increased hydrolysis of the synthesized product by thermolysin.

f. Statistical Analysis

Statistical analyses were performed for experiments 6, 7A, 8, 9 and 10 to determine if there was a difference in the mean optical density measurements at 492 nm between the test group with enzyme, and the control group without enzyme. Two-factor analysis of variance (or 2-way ANOVA) was again used. The effect of time, treatments (i.e. with and without thermolysin, or with and without crude enzyme fraction), and their interactions on the increase in optical density readings measured at 492 nm were tested. Table 9 summarizes the results of the statistical analysis. The differences in the mean optical density readings between all the control samples and the samples containing either thermolysin or crude enzyme fraction were significant ($P < 0.01$). This clearly indicated that the mean optical density measurements were not the same for the control groups (with no enzyme) and for the test group consisting of either commercial grade thermolysin, or crude enzyme fraction from *Bacillus subtilis* DB428. Since the change in optical density readings at 492 nm in the wells was related to the relative amount of dipeptides formed, the treatment effect was significant on the amounts of DNP-asn-leu formed in the microtiter wells. Hence, the experimental data, and subsequent statistical analyses confirmed that the proposed rapid screening method was sensitive enough to detect the formation of DNP-asn-leu dipeptides on a solid-phase even when crude enzyme fraction isolated from bacterial culture was used as a catalyst.

Although the proposed rapid screening method can be used to

Table 9. 2-Way analysis of variance on O.D._{492nm} in microtiter wells from five experiments of thermolysin or neutral protease T catalysed synthesis of Asn-Leu dipeptides

Fig.	Exp.	Source of Variation	DF	F ratio
14 ^d	6	Time	3	62.51**
		Treatment ^a	2	197.4**
		Time * Treatment	6	22.10**
		Error	36	
15a ^c	7a	Time	3	407.5**
		Treatment ^b	3	365.4**
		Time * Treatment	9	28.23**
		Error	32	
16 ^d	8	Time	12	35.14**
		Treatment ^a	2	1146**
		Time * Treatment	24	12.62**
		Error	117	
17 ^d	9	Time	12	29.94**
		Treatment ^a	2	829.8**
		Time * Treatment	24	6.660**
		Error	117	
18 ^d	10	Time	12	48.15**
		Treatment ^c	3	668.8**
		Time * Treatment	36	8.260**
		Error	156	

^aTreatment 1 = Control sample (DNP-L-asparagine + buffer)

Treatment 2 = Test sample 1 (DNP-L-asparagine + buffer + thermolysin)

Treatment 3 = Test sample 2 (DNP-L-asparagine + buffer + crude enzyme fraction)

^bTreatment 1 = Control sample (DNP-L-asparagine + buffer)

Treatment 2 = Test sample 2 (DNP-L-asparagine + buffer + 6.52 ug of crude enzyme fraction)

Treatment 3 = Test sample 2 (DNP-L-asparagine + buffer + 13.04 ug of crude enzyme fraction)

Treatment 4 = Test sample 2 (DNP-L-asparagine + buffer + 19.56 ug of crude enzyme fraction)

^cTreatment 1 = Control sample (DNP-L-asparagine + buffer)

Treatment 2 = Test sample 1 (DNP-L-asparagine + buffer + thermolysin)

Treatment 3 = Test sample 2 (DNP-L-asparagine + buffer + 5.27 ug crude enzyme fraction)

Treatment 4 = Test sample 4 (DNP-L-asparagine + buffer + 26.04 ug crude enzyme fraction)

^d 4 wells per time-treatment combination

^c 3 wells per time-treatment combination

** Significant at P < 0.01.

detect the formation of DNP-asn-leu dipeptides, the optical density readings measured at 492 nm for all of the experiments in this thesis were really quite low: even the highest readings were under 0.2. There are several possible explanations for the low readings. First, each well on the Nunc Immuno Module can only bind a limited number of L-leucine molecules. There are roughly 10^{14} secondary amino groups that are anchored to the surface of each well. This would mean that only a maximum of 21.8 ng or 0.17 nmol of leucine could bind to each well when all the amino groups are coupled with leucine molecules (Figure 20). Assuming that the dipeptide synthesis reaction was 100% successful, there would only be a maximum of 0.17 nmol of L-asparagine molecules that could form peptide bond with the immobilized L-leucine molecules. Second, there was the possibility that a portion of the added DNP-L-asparagine molecules were unable to form a bond with the immobilized L-leucine molecules either because of steric hindrance or of their inability to assume the conformation for proper binding. Third, some of the antibody peroxidase conjugate molecules might not have been able to complex with the dinitrophenol group on L-asparagine for the same reasons given above. Fourth, all of the synthesis reactions took place in an aqueous environment, which could have significantly lowered the yield of dipeptides as water limits yield because of hydrolysis of the enzyme-substrate intermediate, or the final product. It has been reported that product yield could be enhanced significantly if the peptide synthesis reaction is carried out in the presence of

Approximate surface density of secondary amino groups on the surface of Covalink module = 10^{14} /cm²

Approximate surface area of each microtiter well = 1 cm²

Avogadro number = 6×10^{23} molecules per mole

If every secondary amino group in each well is coupled with a leucine molecule, then there should be 10^{14} molecules leucine per well.

$$10^{14} \text{ molecules leucine} \times \frac{1 \text{ mole}}{6 \times 10^{23} \text{ molecules}} \times 131 \frac{\text{g}}{\text{mole}} = 0.17 \text{ nmol leucine or } 21.8 \text{ ng leucine per well}$$

Figure 20. Calculation of the Maximum Concentration of Leucine Immobilized on Nunc Immuno Module

mixed or pure organic solvents instead of water (Chen et al., 1991). Organic solvents were not used in all of the experiments in this thesis because of concerns over the possibility that organic solvent might damage the polystyrene surface, which would result in problems in measuring the optical density readings at 492 nm. Moreover, most enzymes are usually not very stable in organic solvents. Lastly, the reaction conditions for synthesis of dipeptides on microtiter plates had not been completely optimized. Miranda and Tominaga (1991) reported that product yield for the synthesis of Z-Asn-Leu-OEt was highest when the following experimental conditions were used: 1.0 mmol Z-Asn-OH, 1.0 mmole H-Leu-OEt.HCl, 21 nmol thermolysin, 2 g ammonium sulfate, 0.2 M sodium acetate pH 6, incubation temperature at 48°C. The recommended concentration of the various reactants could not be used in the solid-phase synthesis of DNP-asn-leu because there were restrictions on how much one can add to a microtiter well. The use of ammonium sulfate to improve product yield by precipitating the formed peptides was clearly not applicable on a microtiter plate. More work can still be done to determine the pH and temperature conditions that are best suited for peptide synthesis reactions on microtiter plates. The concentration of enzyme can be lowered even more in the case of thermolysin catalysed reactions while the opposite is true for the crude enzyme fraction.

Future investigation should be focused on increasing the sensitivity of this rapid screening assay. One possible solution involves the use of fluorescent substrates for peroxidase enzymes.

The use of other enzymes with higher substrate turnover rates is another possibility. An example of such an enzyme would be catalase. Catalase can react with 30,000 μmol of H_2O_2 /min per mg of enzyme, which is 30-fold greater than that of other enzymes used in enzyme immunoassays (Yolken, 1982).

CONCLUSIONS

A rapid screening assay was developed to detect the formation of DNP-asparagine-leucine dipeptides on microtiter plates, which had secondary amino groups grafted on the surface. Conventional detection methods including reverse-phase high performance liquid chromatography and thin layer chromatography are not suitable methods for rapid screening purpose as they are much more time consuming and labour intensive. Commercial grade thermolysin was first used as the catalyst for the synthesis reactions. The best reaction conditions for thermolysin catalysed synthesis were as follows: 76.3 nmol L-leucine, 10 μ g thermolysin with 7.8 units of protease activity (using Hammerstein casein as a substrate), 7.56 nmol DNP-L-asparagine in 0.2 M sodium acetate buffer, pH 6, with 50 mM calcium acetate, at 48°C for 2 hours. The presence of the final products was detected by the addition of anti-DNP antibody horseradish peroxidase conjugate, which reacted with the dinitrophenyl group on the asparagine molecule to produce a colour that could be measured at 492 nm. The effect of the enzyme treatment on the mean optical density measurements was found to be significant ($P < 0.01$).

Crude enzymes secreted by transformed *Bacillus subtilis* DB428 and WB600 was then used to catalyse DNP-asparagine-leucine synthesis. The reaction conditions employed were similar to those described for thermolysin catalysed reactions except that 5 to 26 μ g of crude enzyme with protease activity in the range of 32 to 715 units (using azocasein as a substrate) was used. The effect of the

enzyme treatment on the mean optical density measurements at 492 nm was found to be significant ($P < 0.01$).

For future research studies, more work should be done on improving the sensitivity of the rapid screening assay. At the same time, random mutagenesis experiments should be carried out on targeted regions of the gene (nprT) which encodes for neutral protease T from *Bacillus stearothermophilus* in an attempt to produce mutant enzymes with enhanced reverse proteolysis activity. The developed rapid screening assay could then be used to identify those positive mutants. Once these mutants are identified, mutant enzymes can be produced in fermentation vessels. Harvested mutant enzymes should then be purified and used to synthesize dipeptide on a larger scale (i.e. test tubes versus microtiter wells). The peptide synthesis activities of these mutant enzymes could then be accurately quantified by reverse-phase high performance liquid chromatography.

REFERENCES

- Berkowitz, D.B. and Webert, D.W. 1981. The inactivation of horseradish peroxidase by a polystyrene surface. *J. Immunol. Methods.* 47:121.
- Bigbee, W. and Dahlquist, F. 1974. Magnetic Resonance studies of active-site region of thermolysin. *Biochem.* 13:3542.
- Blake, C. and Gould, B.J. 1984. Use of enzymes in immunoassay techniques. A review. *Analyst.* 109:533.
- Bodanszky, M. 1988. Formation of the peptide bond. In Peptide Synthesis, pp. 54 - 73, Springer-Verlag, NY.
- Breitling, R., Sorokin, A.V., Ellinger, T. and Behnke, D. 1990. Controlled gene expression in *Bacillus subtilis* based on temperature-sensitive λ cI repressor. In Genetics and Biotechnology of Bacilli, pp.3-12. M.M. Zukowski, A.T. Ganesan and J.A. Hoch (Eds.). Academic Press, San Diego, CA.
- Cantarero, L.A., Butler, J.E. and Osborne, J.W. 1980. The adsorptive characteristics of proteins for polystyrene and their significance in solid-phase immunoassays. *Anal. Biochem.* 105:375.
- Chen, K. and Arnold, F. H. 1991. Enzyme engineering for non-aqueous solvents: random mutagenesis to enhance activity of subtilisin E in polar organic media. *Biotechnol.* 9:1073.
- Chen, K., Robinson, A., Mariana, E., Martinez, P., Economou, C. and Arnold, F. 1991. Enzyme engineering for nonaqueous solvents. II. Additive effects of mutations on the stability and activity of Subtilisin E in polar organic media. *Biotechnol. Prog.* 7:125.
- Cheng, E., Miranda, M.T.M. and Tominaga, M. 1988. Thermolysin and α -chymotrypsin mediated synthesis of tripeptides containing proline. *Int. J. Peptide Protein Res.* 31:116.
- Cunningham, B., and Wells, J. 1987. Improvement in the alkaline stability of subtilisin using an efficient random mutagenesis and screening procedure. *Protein Engineering.* 1:319.
- De Savigny, D. and Voller, A. 1980. The communication of ELISA data from laboratory to clinician. *J. Immunoassay.* 1:105.
- Egmond, M., Antheunisse, W., van Bommel, C., Ravesteyn, P., de Vlieg, J., Peters and Branner, S. 1994. Engineering surface charges in a subtilisin: the effects on electrophoretic and ion-exchange behavior. *Protein Engineering.* 7:793.

- Estell, D., Graycar, T. and Wells, J. 1985. Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. *J. Biol. Chem.* 260:6518.
- Ewings, K. N., O'Connor, R.E. and Mitchell, G.E. 1984. Proteolytic microflora of refrigerated raw milk in south east Queensland. *Aust. J. Dairy Technol.* 39:65.
- Fontana, A. 1988. Structure and stability of thermophilic enzymes. *Studies on thermolysin. Biophys. Chem.* 29:181.
- Fuji, M., Tagagi, M., Imanaka, T. and Aiba, S. 1983. Molecular cloning of a thermostable neutral protease gene from *Bacillus stearothermophilus* in a vector plasmid and its expression in *Bacillus stearothermophilus* and *Bacillus subtilis*. *J. Bacteriol.* 154:831.
- Goheen, S.C. and Stevens, A. 1985. New techniques for separating proteins in hydrophobic interaction HPLC. *Biotechniques.* 3:48.
- He, X.S., Shyu, Y.T., Wong, S.L. and Doi, R.H. 1992. Construction and use of a *Bacillus subtilis* mutant in multiple protease genes for the expression of eucaryotic genes. *Ann. N.Y. Acad. Sci.* 646:69.
- Heringa, J., Argos, P., Egmond, M. and de Vlieg, J. 1995. Increasing thermal stability of subtilisin from mutations suggested by strongly interacting side-chain clusters. *Protein Engineering.* 8:21.
- Holden, H.M. and Matthews, B.W. 1988. The binding of L-valyl-L-tryptophan to crystalline thermolysin illustrates the mode of interaction of a product of peptide hydrolysis. *J. Biol. Chem.* 263:3526.
- Isowa, Y., Ohmori, M., Ichikawa, T., Mori, K., Nonaka, Y., Kihara, K. and Oyama, K. 1979. The thermolysin catalysed condensation reactions of N-substituted aspartic acid and glutamic acid with phenylalanine alkyl esters. *Tetrahedron Lett.* 28:2611.
- Kallio, P., Palva, A. and Palva, I. 1987. Enhancement of α -amylase production by integrating and amplifying the α -amylase gene of *Bacillus amyloliquefaciens* in the genome of *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* 27:64.
- Kawamura, F. and Doi, R.H. 1984. Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. *J. Bacteriol.* 160:442.

- Kemeny, D.M. and Challacombe, S.J. 1988. Microtitre plates and other solid phase supports. in ELISA and Other Solid Phase Immunoassays, pp. 32-55. D.M. Kemeny and S.J. Challacombe (Eds.). John Wiley and Sons Ltd., NY.
- Kemeny, D.M. and Chantler, S. 1988. An introduction to ELISA. In ELISA and Other Solid Phase Immunoassays. Theoretical and Practical Aspects, pp.1-28. D.M. Kemeny and S.J. Challacombe (Eds.). John Wiley and Sons Ltd., NY.
- Lars, A., Jeffrey, T., Burnier, J., Butcher, K., Kossiakoff, A. and Wells, J. 1991. Engineering subtilisin and its substrates for efficient ligation of peptide bond in aqueous solution. *Biochem.* 30:4151.
- Le Grice, S.F.J. 1990. Regulated promotor for high level expression of heterologous genes in *Bacillus subtilis*. *Methods in Enzymol.* 185:201.
- Lovett, P.S. and Ambulos, N.P. Jr. 1989. Genetic manipulation of *Bacillus subtilis*. In *Bacillus*, pp.115-155. C.R. Harwood (Ed.). Plenum Press, London.
- Martinez P., Mariana, E., Robinson, A., Chen, K. and Arnold, F. 1992. Stabilization of subtilisin E in organic solvents by site-directed mutagenesis. *Biotechnol. and Bioengineering.* 39:141.
- Matthews, B.W., Weaver, L.H. and Kester, W.R. 1974. The conformation of thermolysin. *J. Biol. Chem.* 249:8030.
- Matthews, B.W., Jansonius, J., Colman, P., Schoenborn, B. and Dupourque, D. 1972. Three-dimensional structure of thermolysin. *Nature*: 238:37.
- Miranda, M.T. and Tominaga, M. 1991. Thermolysin as a catalyst in enzymatic synthesis of aspartame-containing peptides II. *Int. J. Peptide Protein Res.* 37:128.
- Monzingo, A.F. and Mathews, B.W. 1982. Structure of a mercaptan-thermolysin complex illustrates mode of inhibition of zinc proteases by substrate-analogue mercaptans. *Biochem.* 21:3390.
- Morihara, K. and Oka, T. 1980. Peptide bond synthesis catalysed by subtilisin, papain and pepsin. *J. Biochem.* 89:385.
- Morihara, K., Ueno, Y. and Sakina, K. 1986. Influence of temperature on the enzymatic semi-synthesis of human insulin. *Biochem. J.* 240:803.
- Morihara, K. 1987. Using proteases in peptide synthesis. *TIBTECH.* 5:154.

- Mountain, A. 1989. Gene expression systems for *Bacillus subtilis*. In *Bacillus*, pp.73-114. C.R. Harwood (Ed.). Plenum Press, London.
- Nunc InterMed publication. Covalent binding. Roskilde, Denmark.
- Oka, T., and Morihara, K. 1978. Peptide bond synthesis catalysed by α -chymotrypsin. *J. Biochem.* 84:1277.
- Osburne, M.S., Craig, R.J. and Rothstein, D.M. 1984. Thermoinducible transcription system for *Bacillus subtilis* that utilizes control elements from temperate phage ϕ 105. *J. Bacteriol.* 163:1101.
- Parsons, G.H. 1981. Antibody-coated plastic tubes in radioimmunoassay. *Methods in Enzymol.* 73:224.
- Paul, F., Auriol, D. and Monsan, P. 1988. Direct enzymatic synthesis of aspartame. *Annals New York Acad. Sci.* 542:351.
- Pearse, B.F. 1980. Chromatography. In Basic Instrumental Analysis, pp. 358-401. D. Van Nostrand Company, NY.
- Personal Communication with T. Imanaka.
- Pharmacia. PhastSystem Development Technique File NO.210. Uppsala, Sweden.
- Pitcher, W.H. 1986. Genetic modification of enzymes used in food processing. *Food Technol.* 40(10):62.
- Rotman, J.P. and Delwel, H.R. 1983. Cross-linking of *Schistosoma mansoni* antigens and their covalent binding on the surface of polystyrene microtiter plates for use in the ELISA. *J. Immun. Methods.* 33:87.
- Sakina, K., Ueno, Y. and Morihara, K. 1987. In Peptide Chemistry 1986, pp.305-310. T. Miyazawa (Ed.) Protein Research Foundation, Osaka.
- Sakina, K., Kawazura, K., Morihara, K. and Yajima, H. 1988. Enzymatic synthesis of cholecystokinin-octapeptide. *Chem. Pharm. Bull.* 36:3915.
- Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Plasmid vectors. In Molecular Cloning: A Laboratory Manual, pp. 1-105. Cold Spring Harbor Laboratory, Cold Spring Harbor Press, Cold Spring Harbour, N.Y.
- Self, C.H. 1985. Enzyme amplification - a general method applied to provide an immune assisted assay for placental alkaline phosphatase. *J. Immun. Methods.* 83:89.

- Sondergard-Andersen, J., Lauritzen, E., Lind, K. and Holm A. 1990. Covalently linked peptides for enzyme-linked immunosorbent assay. *J. Immun. Methods*. 131:99.
- Steinke, D., Schwarz, A., Wandrey, C. and Kula, M.R. 1991. Formation of peptide bonds by carboxypeptidase c from orange leaves. *Enzyme Microb. Technol.* 13:262.
- Takagi, H. 1993. Minireview: protein engineering on subtilisin. *Int. J. Biochem.* 25:307.
- Takagi, M., Imanaka, T. and Aiba, S. 1985. Nucleotide sequence and promoter region for the neutral protease gene from *Bacillus stearothermophilus*. *J. Bacteriol.* 163:324.
- Takagi, M. and Imanaka, T. 1989. Addition of a methyl group changes both the catalytic velocity and thermostability of the neutral protease from *Bacillus stearothermophilus*. *Febs Lett.* 254:43.
- Takagi, H., Takahashi, T., Momose, H., Inouye, M., Maeda, Y., Matsuzama, H. and Ohta, T. 1990. Enhancement of the thermostability of subtilisin E by introduction of a disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease. *J. Biol. Chem.* 265:6874.
- Titani, K., Hermodson, M., Ericsson, L., Walsh, K. and Neurath, H. 1972. Amino-acid sequence of thermolysin. *Nature*: 238:35.
- Tran, L., Wu, X.C. and Wong, S.L. 1991. Cloning and expression of a novel protease gene encoding an extracellular neutral protease from *Bacillus subtilis*. *J. Bacteriol.* 173:6364.
- Urbanek, R., Kemeny, D.M. and Samuel, D. 1985. Use of enzyme-linked immunosorbent assay for measurement of allergen-specific antibodies. *J. Immun. Methods*. 79:123.
- Wilson, M.B. and Nakane, P.K. 1978. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In Immunofluorescence and Related Staining Techniques, pp. 215-225. W. Knapp, K. Holubar and G. Wick (Eds). Elsevier, North Holland.
- Wong, C. 1992. Engineering enzymes for chemoenzymatic synthesis. Part 2: modifying proteases for peptide synthesis. *TIBTECH*. 10:378.
- Wong, C., Chen, S., Hennen, W., Bibbs, J., Wang, Y., Liu, J., Pantoliano, M., Whitlow, M. and Bryan, P. 1990. Enzymes in organic synthesis: use of subtilisin and a highly stable mutant derived from multiple site-specific mutations. *J. Am. Chem. Soc.* 112:945.

Xu, C.W., Lee, W., Tran, L. and Wong, S.L. 1991. Engineering a *Bacillus subtilis*. Expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. 173:4952.

Volken, R.H. 1982. Enzyme immunoassays for the detection of infectious antigens in body fluids: current limitations and future prospects. Rev. Infect. Dis. 4:35.