EXPLOITATION OF LOW VALUE FOOD MATERIALS AS A NOVEL SOURCE OF FLAVOUR ENHANCERS

by

WEI XIA

BSc. MSc.

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

Division of Food Sciences

School of Biosciences

University of Nottingham

September 2017

ACKNOLEDGEMENTS

This PhD research would never have been completed without the contribution from many people that I wish to acknowledge.

I would like to thank Dr. Ian Fisk for his great supervision to improve my scientific research capability, for offering opportunities as many as possible to broaden my research horizon. I would especially thank Dr. Robert Linforth for his extraordinary technical support and critical comments throughout my whole PhD study. I also would like to thank Dr. Darren Greetham for proofreading my thesis with great patience and improving my thesis writing skills. Also thanks to Nick Dyson from Create Flavours Ltd for his kind accommodation during my visit to the company for their spray-drying technique.

I would like to thank my parents for their tremendous financial support during my PhD and constant encouragement. Without my parents' unrequited care, nothing would ever have been achieved. A special thank goes to Marlow Foods Ltd for their additional finical help during my second year of my PhD, I have gained valuable experience working with them.

Finally, I would like to thank my best friend Dominik Marko who truly broadens the horizon of my life experience during my study time in UK. Thanks to him and his family, for brining me to many other places in Europe and experiencing various cultures, making my whole time in Europe vivid.

ABSTRACT

There is demand from the food industry for novel savoury seasonings based on low-cost food ingredients, sourced from within the EU. A nucleotide and protein rich spray dried powder which was derived from a *Fusarium venenatum* fermenter waste stream and food-grade *Alphitobius diaperinus* with potential as a savoury flavour enhancer was evaluated.

Enzymatic digestion of these two raw materials as a source of flavour precursors was evaluated. Serial enzyme combinations, enzyme dosages, sequence of enzyme application, pH, temperature and length of digestion for the liberation of amino acids and nucleotides were optimised for the liberation of taste active compounds.

For amino acids, free glutamine (GLN) and glutamic acid (GLU) could be enhanced using a combination of peptidases on both raw materials. Digesting a spray dried powder derived from the fermenter waste stream of *Fusarium venenatum* with exopeptidase (1% Flavourzyme™), resulted in an improved yield of GLN (from 0.1 mg/g to 28.9 mg/g powder) and GLU (from 1.71 mg/g to 5.98 mg/g powder). For milled *Alphitobius diaperinus,* mixed use of exopeptidase (1% Flavourzyme™) and endopeptidase (2% Alcalase™) gains a 1.4-fold increased yield of GLU (17.5 mg/g powder) and 1.7-fold increased yield of GLN (1.2 mg/g) as best production was obtained.

For nucleotides, digestions of the waste stream with a yeast lytic enzyme (YL-TL™) followed by a nuclease (RP-1G™) resulted in the highest 5'-guanosine monophosphate (GMP) and 5'-adenosine monophosphate (AMP) production. Specifically, a 2% and 0.05% treatment by YL-TL™ and RP-1G™ respectively

was shown to be optimal, followed by a 0.05% Deamizyme^{TM} treatment for the conversion of AMP to 5'-Inosinic acid (IMP) of 38 mg/g. For the solid digestion of *Alphitobius diaperinus*, being treated with a nuclease tretment (2% RP-1G^{TM}) followed by a 0.2% Deamizyme^{TM} treatment for the conversion of AMP to IMP, resulted in the highest GMP yield, a 3.5-fold increased (2.6mg/g), and 7.8-fold increased IMP (4.7mg/g) production.

Table of Contents

ACKNOLEDGEMENTS 2
ABSTRACT 3
1. INTRODUCTION 8
1.1 BACKGROUND 8
1.1.1 Food waste in the UK 8
1.1.2 Trend of edible insect 9
1.2 UMAMI FLAVOUR 12
1.2.1 Introduction: food preferences and umami taste 12
1.2.2 How do we perceive umami? 17
1.2.3 Synergy effect of ribonucleotide on umami 19
1.3 UMAMI ENHANCEMENT 25
1.3.1 Yeast extract as one important umami enhancer 26
1.3.2 Protein hydrolysis 27
1.3.3 Nuclease digestion 32
1.4 EXPERIMENTAL AIM AND HYPOTHESES 37
2 MATERIALS AND METHODS 39
2.1 MATERIALS 39
2.2 METHODS FOR SPRAY DRIED POWDER OF FERMENTED WASTE WATER ANALYSIS 40
2.2.1 Quantification of total protein by bicinchoninic acid (BCA) assay 40
2.2.2 Acid hydrolysis of water soluble protein 40
2.2.3 RNA hydrolysis 41
2.2.4 pH adjustment 41
2.2.5 Digestion of solubilised protein by peptidase treatment 41
2.2.6 Digestion of RNA by nuclease treatment 42
2.3 METHODS FOR FOOD-GRADE INSECTS 43
2.3.1 Digestion of solubilised protein by peptidase treatment 43
2.3.2 Digestion of RNA by nuclease treatment 43
2.3.3 Response surface modelling for enzyme treatment optima 44
2.4 NUCLEOTIDE ANLYSIS BY HPLC 45
2.5 AMINO ACID ANLYSIS BY GC-MS 47
2.6 DATA ANALYSIS 48
3 ENZYMATIC LIBERATION OF UMAMI COMPOUNDS FROM SPRAY DRIED FERMENTER WASTE WATER 49

3.1 THE POTENTIAL VALUE OF FUSARIUM VENENATUM FERMENTED WASTE WATER 49
3.2 EXPERIMENTAL HYPOTHESES 50
3.3 RESULT AND DISCUSSION 51
3.3.1 Glutamic acid content present in post-fermentation spray-dried powder from wastewater 51
3.3.2 Quantification of nucleotides in post-fermentation spray-dried powder from wastewater 53
3.3.3 The effect of pH on the concentration of nucleotides 54
3.3.4 Yield of nucleotides after nuclease treatment following variable enzyme treatment 55
3.3.5 Nuclease activity time course 60
3.3.6 Peptidase treatment with following variable enzyme treatment 63
3.3.7 Effect of a two-step peptidase digestion on the yield of GLU and GLN 65
3.3.8 Time course of Flavourzyme activity 67
3.3.9 Glutaminase digestion of GLN into GLU 67
3.4 CONCLUSION 69
4. THE LIBERATION OF UMAMI COMPOUNDS FROM ALPHITOBIUS DIAPERINUS FOLLOWING ENZYMATIC DIGESTION 71
4.1 INTROCUTION 71
4.1.1 The value of Alphitobius diaperinus as umami flavour enhancer 71
4.1.2 Hydrolysis approach to yield umami property compound from food-grade Alphitobius diaperinus 72
4.2 EXPERIMENTAL HYPOTHESES 72
4.3 RESULT 72
4.3.1 Amino acid profiling of Alphitobius diaperinus treated with various peptidase 72
4.3.2 Nucleotide profile of Alphitobius diaperinus after enzyme treatment 89
4.4 CONCLUSION 108
5. FURTHER DISCUSSION 110
5.1 INTRODUCTION 110
5.2 WHY PEPTIDASE AND NUCLEASE CANNOT BE TREATED TOGETHER 111
5.3 MECHANISM OF ENZYMES TREATED IN THE STUDY 113
5.3.1 Nuclease P_1 (RP-1G) 113
5.3.2 Yeast-lytic enzyme (YL-TL) 115

5.3.3 Nucleodeaminase (Deamizyme) 118
5.4 CONCLUSION 119
6. FUTURE WORK 122
6.1 SENSORY STUDY 122
6.2 LOOKING FOR BETTER ENZYMES 122
6.3 KOKUMI, A NEW CONCEPT OF UMAMI SYNSERGY? 123
REFERENCE 125
Appendix I (Thermofisher.com, 2017) 134
Appendix II (Thermofisher.com, 2017) 141
Appendix III 143

1. INTRODUCTION

1.1 BACKGROUND

1.1.1 Food waste in the UK

Food waste has always been a serious issue in the UK. In 2013, 15 million tonnes of food and drink was wasted in the food chain, which is equivalent to around one third of the 41 million tonnes of food that is bought annually in the UK (Wrap.org.uk, 2016). The highest proportion of this waste in the food chain was wasted in households, with 7 million tonnes being thrown away in the UK in 2013. Of the 7 million tonnes of household food and drink waste, 60% were avoidable, 17% were possibly avoidable and 23% were unavoidable (Wrap.org.uk, 2016).

Among these three types of waste, avoidable waste is food and drink thrown away because it is no longer wanted or has been allowed to go past its best. Possibly avoidable waste is food that some but not all people would eat, and unavoidable waste is elements that are not suitable for consumption (Wrap.org.uk, 2016).

Since the vast majority of avoidable food is composed of material that was prior to disposal, edible, there may be a significant decrease in this avoidable waste if people's awareness of food waste is increased. In fact, the waste tonnage in 2014 has been decrease by 0.6% since 2010. However, there is still huge amount of other waste awaiting to be taken advantage of (Wrap.org.uk, 2016).

Apart from household waste, the second biggest food waste belongs to manufacturing, taking up near 26% of the total food waste in the food chain. In the UK, businesses generate approximately one quarter of all waste each year.

Levels of food and drink waste by commercial and industrial businesses in the food sector amount to around 2.8 million tonnes, with a value of over £4.3 billion (Wrap.org.uk, 2016).

Some recent research has begun the exploitation of the protein in the toufu wastewater with various proteases for savoury flavour enhancement (Sun, Zhao and Guo, 2016). The commercial and industrial manufacturers should also pay more attention to the treatment of certain waste such as fermentation waste from brewery and fungi processing, improving the productivity of the waste re-usage and generate new opportunities such as brand-new product or energy derived from waste.

1.1.2 Trend of edible insect

1.1.2.1 Background

Because of the growing demand for meat and the declining availability of agricultural land, there is an urgent need to find alternative protein sources. By 2050, the world's population is expected to exceed 9 billion people, adding more than 2 billion individuals to an already crowded planet. Coupled with expanding economic wealth and purchasing power, Food and Agriculture Organization (FAO) estimates indicate that global food production will need to expand by an estimated 60 percent from current levels to meet global food requirements in 2050 (FAO, 2017). Meeting this massive additional demand for food will require concerted action on a number of fronts. Part of this will focus on increasing the production and consumption of currently under-utilized and under-appreciated foods. Although the increased consumption of these foods is constrained by production, processing and trade, often it is simply a lack recognition and appreciation of their potential value to future food security. Edible insects

comprise one such category of under-utilized foods that offer significant potential to contribute to meeting future global food demands.

1.1.2.2 Edible insect farming

Insect farming has been suggested as a good alternative to conventional livestock farming for future food production. Livestock production accounts for 70% of all agricultural land use and is a major contributor to global warming (Jansson and Berggren, 2015) while edible insects can be produced with a smaller environmental footprint than livestock. Insects have a high feed conversion rate, which in a rearing system limits the demand for land for feed production. Edible insects can also alleviate waste disposal problems by growing them on organic by-products. Moreover, insects can potentially emit less greenhouse gases (GHG) than 12% anthropogenic GHG emissions of conventional livestock. It has also been suggested that the volume of water required to produce edible insects is low compared with that needed in conventional livestock production such as beef at 22,000 litre/kg water usage (van Huis et al., 2013).

1.2.2.3 Nutrition value of edible insect

Insects are a good source of nutrients and are comparable to conventional animal sources with a high protein content. Their nutritional values vary according to the species and how they are prepared for consumption. In general, insects can supply protein (20-70% of raw protein), amino acids (30-60%), fats (10-50%), minerals and vitamins important for human health. This is comparable to conventional meat products in terms of protein content (Yi et al., 2013). There are adequate protein quantity and quality with a high content of unsaturated fatty acids and minerals like iron and zinc in some certain insects, which can even replace scarce fishmeal. Thus, entomophagy (eating of insects) has been

proposed to tackle the deficiencies of these minerals in developing countries (Christensen et al., 2006), in particular in view of the fact that the percentage of the world population at risk for these deficiencies is more than 17 % for zinc (Gibson, 2015) and 25 % for iron (McLean et al., 2008).

1.2.2.4 Edible insect as foodstuffs

Although about 2000 insect species are eaten worldwide, but mostly in tropical countries such as Cambodia, Thailand, Mexico where insects are regarded as an important protein source (Jansson and Berggren, 2015), food safety issues have been addressed by a number of authors with immediate impact on legislative issues (van der Spiegel, Noordam and van der Fels-Klerx, 2013, Rumpold and Schlüter, 2013, Charlton et al., 2015, Amadi and Kiin Kabari, 2016).

Contaminants of insects such as mycotoxins, pathogens, heavy metal and pesticide residues are questioned as possible hazards. However, the information available shows that insect pathogens are specific for invertebrates and do not harm vertebrates (Eilenberg et al., 2015). In terms of legislation, in the EU, at the time the legislation was made, very few people considered insects as either food or feed, and when in the legislation the word “animal” is used, insects are often automatically part of that legislation. Regarding insects as food, it has not been decided yet in the EU whether a product made from insects is considered a novel food (definition: not consumed “in a significant degree” in the EU before May 15, 1997) or not (van Huis, 2015). If so, the producer can follow the conventional food regulations otherwise they have to provide a Novel Food Dossier to prove the product is safe for consumers.

There is increasing number of people consuming insect in the EU such as in Belgium (House, 2016) and the Netherlands (Caparros Megido et al., 2013).

They adapted insects as a protein source, strongly influenced by motivations towards sustainable food consumption. Eating insects is still however an enigma in most of the western world and people are reluctant to use insects as food. Almost a hundred years ago, Bequaert attributed the aversion of western people to include insects in their diet to 'prejudice', and cultural conditioning: "What we eat and what we do not eat is, after all, a matter of custom and fashion rather than anything else". Take consumers in Thailand for example, where insects are part of the local food culture, considered insects more in terms of their taste than Dutch consumers. Thai consumers are strongly repulsed by mealworms due to the association with larvae often seen in decaying matter. However, this association was absent among Dutch consumers who are familiar with mealworms as food. Therefore, the development of strategies to overcome the psychological and cultural barriers to entomophagy is the priority task.

1.2 UMAMI FLAVOUR

1.2.1 Introduction: food preferences and umami taste

One of the effective approaches to make individuals more easily accept under-appreciated food such as insects is to explore and develop more satisfying flavour. But before further discussion of flavour modification, we need to be clearly aware of the mechanism behind our taste sensation.

Our sense of taste arises from the contact of chemical compounds, including those in foods, with taste receptors on the surface of their cells that are located mostly within the taste buds on the tongue (Figure 1.1), which are embedded in papillae located mainly on the top of the tongue and distributed over the soft palate, pharynx, epiglottis, and the entrance to the esophagus. There are two principal types of taste receptors: receptor protein and ion channels. The first

type is called the G-protein-coupled receptor and are sensitive to sweet, bitter, and umami taste, which cross the membrane of the receptor cell. When such a receptor on the surface of the cell has interacted with a taste molecule for which it is adapted, a G-protein on the other side of the membrane adopt a new shape and charge. This charge sets in motion a cascade of biochemical processes which ultimately cause particular sodium channels in the cell membrane to open. Sodium ions flow through the channel, resulting in a drop in the electrical potential across the membrane. This generates an electrical signal that passes through efferent neurons system to the cell (Chaudhari and Roper, 2010). The other type of taste receptors is sensitive to sour and salty tastes, especially hydrogen (sour), sodium (salt) and potassium ions. These receptors are ion channels that cut across the cell membrane. A change in the concentration of these ions leads to a change in the electrical potential across the membrane, and if over a threshold difference, resulting an electrical impulse is sent to the brain (Chaudhari and Roper, 2010).

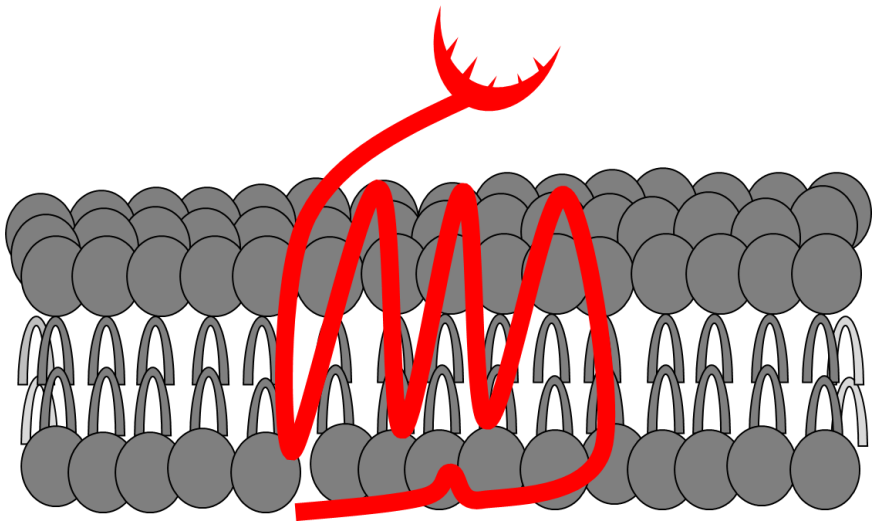
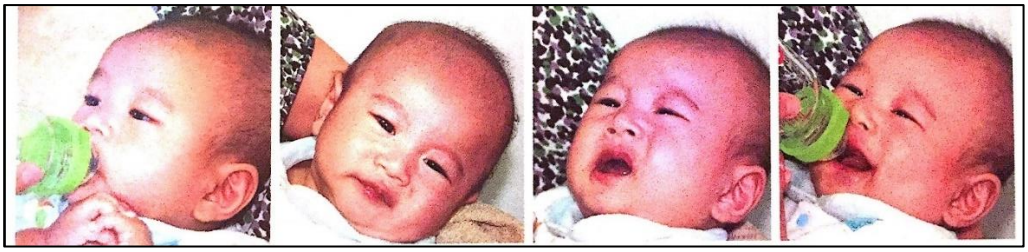


Figure 1.1 Schematic illustration of a receptor (hinge-like structure in red) embedded in the cell membrane (oval linkage in grey) of a sensory cell.

Sweetness, saltiness, sourness and bitterness have been traditionally recognized as fundamental taste in all foods and drinks. However, these qualities are insufficient to describe our experiences from eating foods. Compared to the rich inventory of thousands of different odourant that contribute to the flavours in our diet, the range of taste qualities seems remarkably limited (Keller et al., 2014).

Being responsive to a small set of basic tastes does suggest that each of the qualities must be significant in some way. In contrast to our preferences for odours or flavours, which are molded by exposure and learning from an early age, preferences for pure tastes are remarkably fixed. Distinct, and unambiguous, facial expressions to sweetness and bitterness are present at birth in humans, monkeys, and rats, and are essentially the same response that we have as adults (Figure 1.2). Both in terms of willingness to ingest, and also in the quality of these characteristic facial expressions, sweetness is highly liked and bitterness rejected in infants (Desor, Maller and Turner, 1973) (Steiner et al., 2001). Sourness is also disliked, especially at high levels. A preference for saltiness, on the other hand, develops in the first few post-natal months (Getchell, 1991). Liking for pure tastes also seems to be relatively independent of culture or diet. Comparisons between cultures whose diets are very different, such as Japan, Taiwan and Australia, have found remarkably similar patterns of likes and dislikes for basic tastes (Prescott, 1998).



Sweet Sour Bitter Umami

Figure 1.2 The reactions of a four-month-old boy after tasting sweet, sour, bitter and umami (Keller et al., 2014).

The explanation for these responses to taste qualities lies in their ability to signal nutrients and toxins. The palatability of sweetness is thought to signal the presence of energy in the form of calories provided by sugars and other plant carbohydrates. Consistent with this interpretation, animal such as cats that are solely carnivores are indifferent to sweetness. Saltiness signals the presence of the sodium ion (Na+), crucial for maintaining the body's fluid balance. The strong dislike that animal naturally have to bitterness is thought to be protective mechanism as many plants manufacture bitter toxins as a defense against predators, and many of these toxins are bitter. The significance of the dislike for high levels of sourness is not as clear-cut. It may be a signal for unripeness or spoilage in foods, or the fact that concentrated, and thus extremely sour, acids can cause tissue damage. One way of looking at our sense of taste to the flavour is that there is a balance between the external and internal environment of the body whose function is to ensure that ingested substances maximize our survival.

In addition to these four well-known qualities, we now recognize the existence of another basic taste. In the early twentieth century, Umami was defined as the fifth basic taste and was described as meaty and broth-like. This was first discovered by Kikunae Ikeda in 1908 through his investigations on konbu (Kurihara, 2009). The choice of konbu was based on its availability as a soup base in Japanese cuisine and Ikeda originally found that the taste was a significant factor in konbu broth (Ikeda, 1909). He is also the first person who synthesized glutamate from the seaweed kombu. He used the term umami to describe the quality that glutamate produced when added to savoury foods. Ikeda described umami as the "peculiar taste which we feel as 'umai' (meaning brothy,

meaty or savory), arising from fish, meat and so forth (Ikeda, 2002).” The Japanese word umami, translated approximately as “savory deliciousness”, refers to the quality of foods containing significant amounts of naturally occurring glutamate (a derivative of glutamic acid, a non-essential amino acid), its sodium salt, monosodium glutamate (MSG), or 5’-ribonucleotides such as guanosine monophosphate (GMP) and inosine monophosphate (IMP). If the glutamate is simply dissolved in water, is not particularly palatable, and it is hard to define its quality. Some people may even be confused it with saltiness. It is often described as “mouth-filling”, suggesting a texture. One of the common forms of glutamate, the flavour-enhancer mono-sodium glutamate (MSG), is somewhat salty (because of the sodium component), but it also has an additional quality.

Nevertheless, by adding MSG is one of the simplest ways to impart umami. Increase in food acceptability are clearly mediated by changes in the sensory properties of the foods produced by added MSG (Figure 1.3).

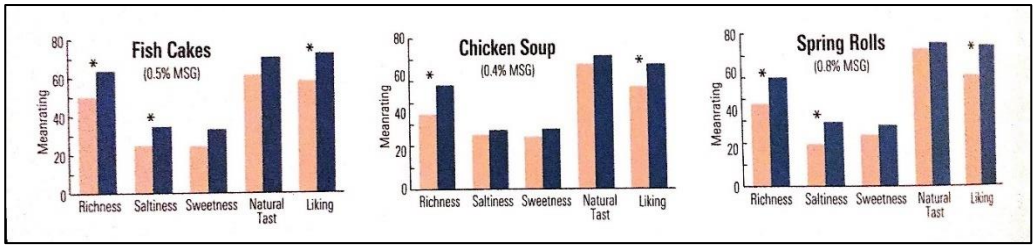


Figure 1.3 Effects of adding MSG (dark bars) to three savoury foods on ratings of liking, richness, sweetness, saltiness and natural taste. (* indicates significant increase in ratings by adding MSG) (Keller et al., 2014)

Although adding MSG to a food does influence its saltiness, it is clear that the effect on other sensory properties and on palatability are not just an effect of salt on flavour. For example, if one equates the amount of sodium in two savoury products of the same type, the one that contains the added glutamate will always

be preferred (OKIYAMA and BEAUCHAMP, 1998). Alternatively, other glutamate compounds, such a calcium glutamate, do not add saltiness but still increase the palatability of foods to which it is added (Bellisle, Dartois and Broyer, 1992).

Ikeda was convinced that umami represented a new, distinct taste. However, it is only during the past two decades that there has come to be general agreement with Ikeda's proposition from within the scientific taste community. A considerable amount of recent research has shown that human has glutamate receptor and hence that ended the argument.

1.2.2 How do we perceive umami?

1.2.2.1 Receptor mGluR4 and mGluR1

Although Ikeda has already determined umami flavour could be elicited by glutamate in 1908, a few researches in 2000 were finally in a position to investigate the molecular basis of umami that the specific receptor known as taste-mGluR4 for umami taste (Chaudhari, Landin and Roper, 2000). This taste-mGluR4 shows us that it is related to the already known mGluR4 in the brain, which is sensitive to glutamate when it acts as a neurotransmitter. The difference between taste-mGluR4 and mGluR4 is that the former is a truncated version of the latter; the part of the taste receptor that projects from the taste cell is only about half as big as that of brain-mGluR4. As the outermost part of the receptor molecule is exactly the site where glutamate is bound, the consequence is that the taste-mGluR4 is more than 100 times less sensitive than its larger counterpart in the brain brain-mGluR4. Having said that, it is not important for the taste receptors to be nearly as sensitive as those in the brain because the concentration

of glutamate in food is much greater than the concentration of glutamate in the neural cells.

Another mGlu glutamate receptor in human, related to taste-mGluR4, has been identified by a few researchers in 2009 (Raliou et al., 2009). The new receptor is a truncated variant of glutamate receptor in the brain: mGluR1.

1.2.2.2 Receptor T1R1/T1R3

A big breakthrough followed the discovery of mGluR4 in 2002, when two research groups in the United States found another glutamate receptor (Li et al., 2002). It is a combination of T1R1 and T1R3, which are two different receptors from the T1R family of receptors that are present only in sensory cells. Members of the T1R family of receptors assemble into heterodimer, forming two related complexes, T1R1/T1R3 and T1R2/T1R3, which are sensitive to umami and sweet taste, respectively. This can also indicate that the biochemical pathways for the recognition of sweetness and umami are very closely connected. In fact, they may be so intimately related that not only would it explain why rats experience umami as a sweet taste and stop eating sugar when they have ingested too much glutamate (San Gabriel et al., 2007), but also may explain why umami can enhance the perceived sweetness of some food (Fuke and Ueda, 1996). The T1R1/T1R3 pair in human is most sensitive to glutamate and aspartate and the binding site for glutamate in the T1R1/T1R3 receptor is found in the T1R1 part that has the same molecular structure as in the mGluR4 and taste-mGluR4 receptors. In contrast to taste-mGluR4, T1R1/T1R3 is not sensitive to L-aspartate that confers only a little umami taste. Moreover, the interference of substances binding to T1R3 will also supress both sweetness and umami. Lactisole, a commercial sweet inhibitor that binds itself to T1R3 and block the

taste of sweetness things, also suppresses the umami flavour. This important receptor reveals the nature of umami and how the perception of this taste is possibly related to that of sweetness.

1.2.3 Synergy effect of ribonucleotide on umami

Beside glutamate as umami taste active amino acid, the presence of 5'-nucleotides have been shown to have a synergistic relationship with GLU and presence of nucleotides and glutamic acid is associated with greatly elevated perception of umami flavours in food (Yamaguchi et al., 1968).

The discovery of ribonucleotides' synergistic impact on umami taste started within the discovery of another umami substance inosine monophosphate in dried bonito flakes by Shintaro Kodama in 1913 (Kodama, 1913). In 1960s, a few Japanese researchers demonstrated that ribonucleotides can act as potential umami flavour compounds in plant source, these also included guanosine monophosphate (GMP) and adenosine monophosphate (AMP) (Kuninaka, 1960).

Among these studies, one Japanese chemist, Kuninaka, systematically studied the relationship between umami taste and the chemical structure of nucleotide-related substances and concluded that the 6'-carbon of the purine ring and phosphate ester on the 5'-carbon of ribose moiety (i.e. 5 prime nucleotide) have umami taste while purine ribonucleotides phosphorylated on the 2'-C or 3'-C of the ribose moiety (2 or 3 prime nucleotides) are tasteless (Figure 1.4) (Kuninaka, 1960). Moreover, he further confirmed that only GMP, IMP and XMP (Xanthosine monophosphate, formed from IMP via the action of IMP dehydrogenase) have umami taste. In addition, another study reported that the phosphate ester linkage on the 5'-C of the ribose moiety is necessary for

imparting umami taste. Besides that, the 5'-C phosphate must have both primary and secondary dissociation of OH to exhibit umami taste; if the OH is esterified or acidified, there will be no more umami taste present.

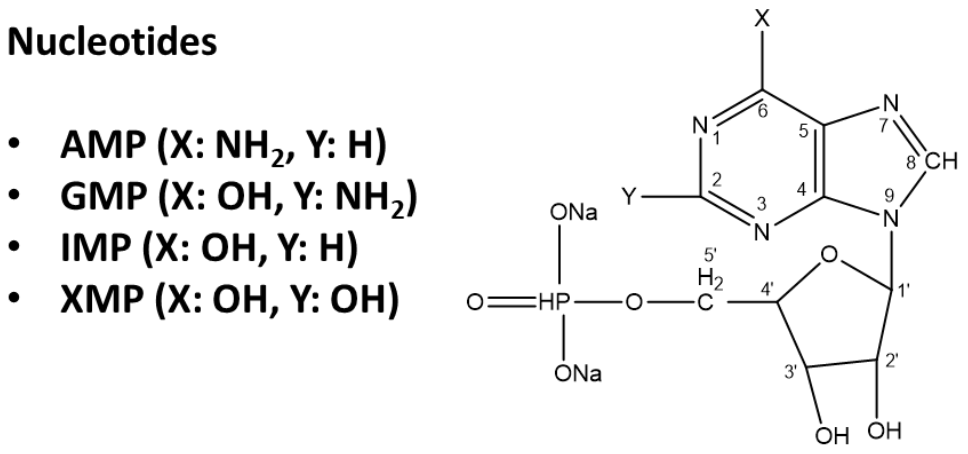


Figure 1.4 Chemical structure of nucleotides (AMP, GMP, IMP and XMP)

The threshold values of IMP (0.025 g/100mL), GMP (0.0125 g/100mL) and MSG (0.03g/100mL) are all quite low in plain water. Although MSG and IMP's threshold values are very similar, the relationship between concentration and intensity of taste is very different especially at higher concentrations (Figure 1.5). In general, the MSG exhibits a marked umami taste by itself while nucleotides do not, which indicates the existence of MSG is necessary in order for nucleotides to enhance the umami taste.

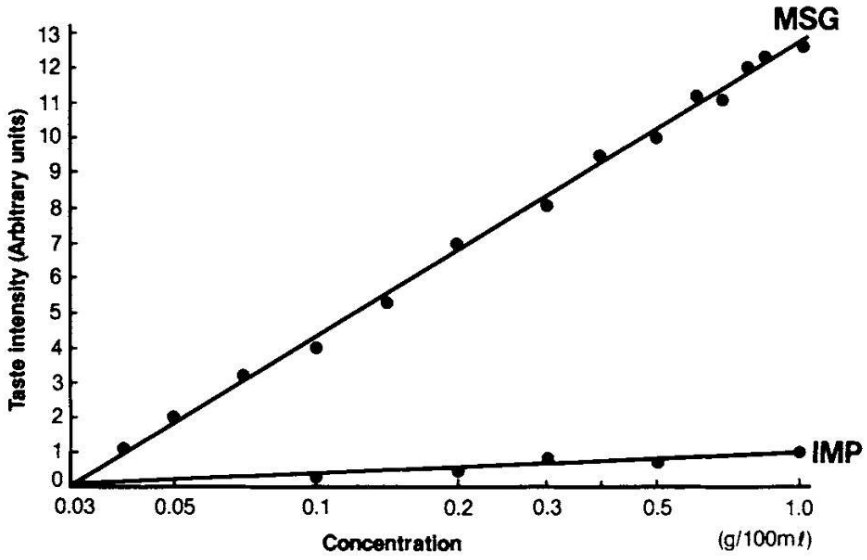


Figure 1.5 Relationship between taste intensity and concentration of MSG solutions and IMP solutions (Charalambous and INGLETT, 1982)

In 1967, Yamaguchi concluded the equations of umami taste synergism between nucleotides and MSG carefully measured by sensory test. As seen in Figure 1.6, the equivalent taste intensity between mixtures of IMP and MSG, and MSG alone are plotted. The taste intensity of mixtures increases exponentially against their concentration, with the degree of synergism depending on the ratio of IMP to MSG.

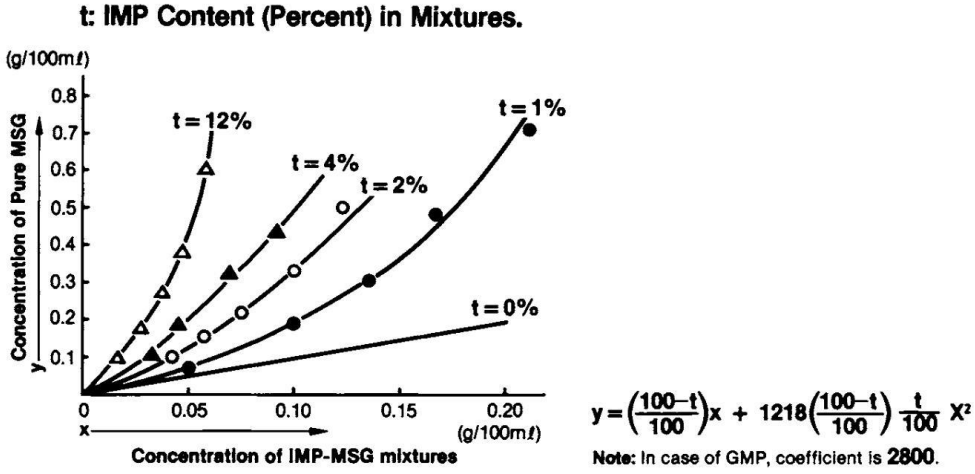


Figure 1.6 Taste equivalency of mixtures of IMP and MSG to MSG alone (Charalambous and INGLETT, 1982)

There are two equations obtained from the result to determine the adequate ratio of nucleotide to MSG. When the concentration of MSG in the mixture is u g/100 mL and that of GMP or IMP is v g/100 mL, and the concentration of MSG alone which shows an equivalent taste intensity is Y g/100mL, the relationship can be expressed as

- $Y = u + 1218\,uv$ in the case of mixture of IMP and MSG
- $Y = u + 2800\,uv$ in the case of mixture of GMP and MSG

Comparing those two equations, the only difference is the coefficients, which GMP's one is 2.3 times greater than IMP's. This indicates that the synergistic effect of GMP is 2.3 times stronger than that of IMP.

For a mixture of IMP, GMP and MSG, if the concentration of IMP plus GMP is v' g/100 mL and the ratio of IMP to GMP is p: q, the equation is shown as below:

$$y = u + \frac{(P+2.3q)}{P+q} \cdot 1218\,uv'$$

Yamaguchi also determined the variation of the taste intensity of mixtures of IMP with MSG at a fixed concentration of the mixture but with the components present in various ratios from 0:100 to 100:0 by using the method of paired comparison using an incomplete block design (Gulliksen, 1956). The result is illustrated in the Figure 1.7, the curve suggests that when small quantities of one are added to the other, the taste intensity rises dramatically and taste intensity is strongest when there are approximately equal amounts of the two components.

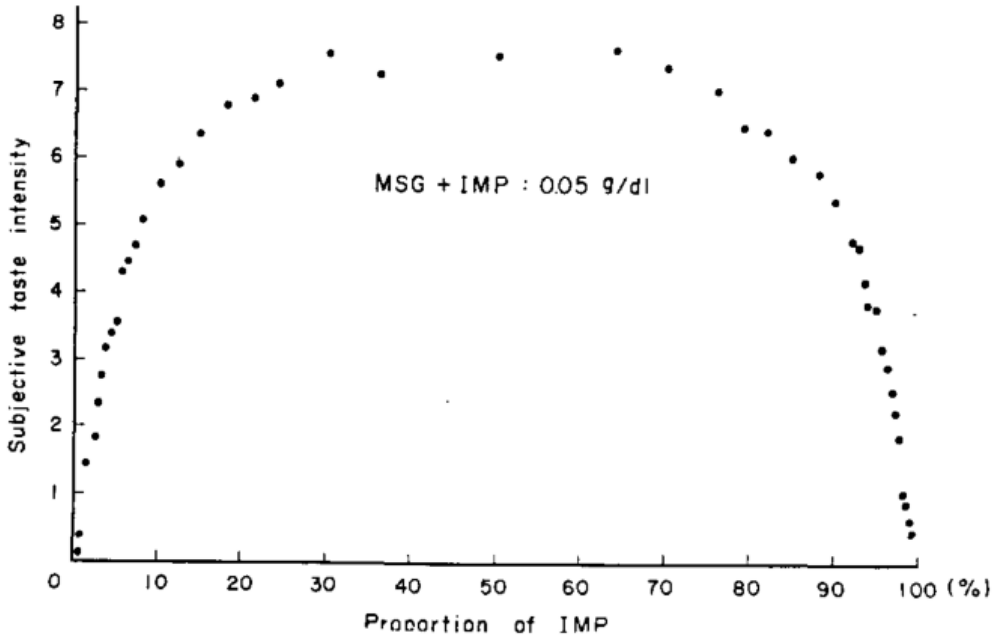


Figure 1.7 Taste intensity for varying mixtures of MSG and IMP (Yamaguchi, 1967)

The discovery of how the taste receptors T1R1 and T1R3 work in tandem in the combination T1R1/T1R3 can also explain how the umami is being synergistically induced by other substances like ribonucleotides (Figure 1.8). 5'-GMP and IMP do not activate T1R1/T1R3 by themselves, but can enhance the activity of glutamate. On the contrary, other ribonucleotides such 2 or 3'-GMP, IMP that do not possess umami property have no effect on the receptor.

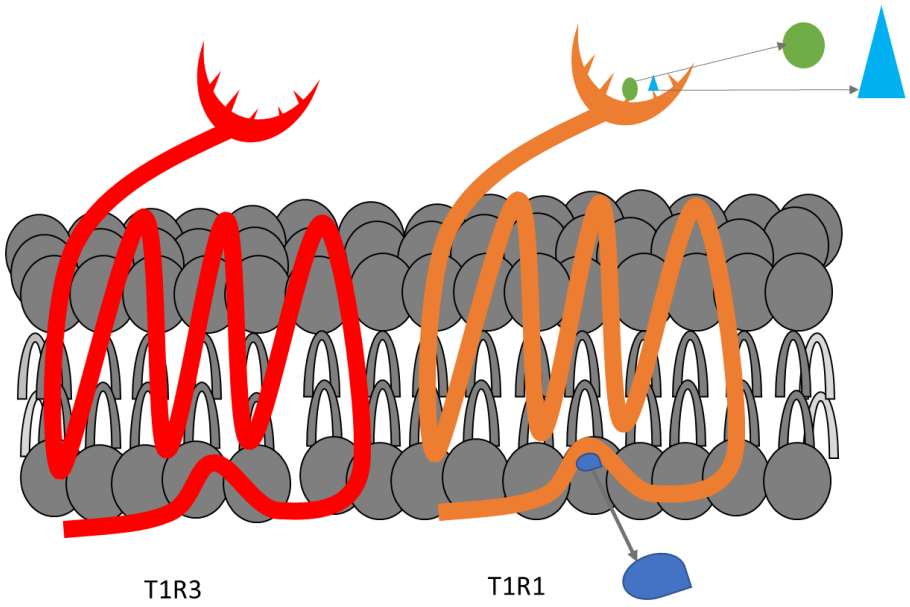


Figure 1.8 Schematic illustration of the umami receptor T1R1/T1R3 embedded in the cell membrane of a sensory cell. The T1R1 part binds a glutamate ion (the green circle) and a nucleotide (the blue triangle), which triggers a signal through the receptor that a G-protein (the blue oval) is to be bound on the other side of the membrane.

The synergic activity takes place in the T1R1 part of the receptor, which has a hinge-like structure. L-glutamate binds near the place where the hinge will bend. Like the mechanism in the Venus flytrap, this causes the hinge to snap shut, trapping the glutamate molecule securely. GMP or IMP binds to a place at the edge of the hinge, where there is a spot that resembles a cleft (Zhang et al., 2008). This further stabilize the closed conformation of Venus flytrap and results in a stronger binding of the glutamate molecules. Stabilizing the glutamate in this way is equivalent to making the receptor more sensitive against glutamate. However, neither of the two ribonucleotides would be able to stimulate the receptor on its own in the absence of glutamate. This effect is referred as allostery, which suggest that the effect of the protein's function is dependent on something that happens in a place on the protein other than its active binding site. The allosteric action was also proved in the dynamic mechanism of the Venus flytrap: the flytrap is very dynamic without bound glutamate while the dynamics

become extremely slow once a nucleotide binds (Zhang et al., 2008) (Figure 1.9). Moreover, glutamate is also involved in stabilizing the formation of the dimer of T1R1/T1R3 (Morikawa et al., 2000).

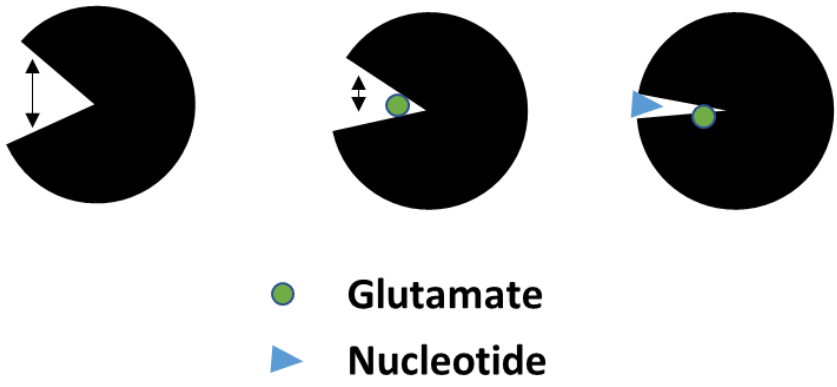


Figure 1.9 Schematic illustration of the synergistic action by simultaneous binding of glutamate and a 5'-nucleotide.

Therefore, the whole process of umami taste generation is a cooperative effort with separate entities including amino acid and umami property nucleotides.

1.3 UMAMI ENHANCEMENT

Umami flavour appears quite frequently in our daily lives from the point of view of our own food experience. Savoury foods can be more or less rich-tasting, of which the quality is mainly determined by the presence of umami compound. For instance, the preparation of a pasta sauce from basic ingredients such as onions, carrots, celery, tomatoes, mushrooms, chicken stock and Parmesan cheese is fundamentally an exercise in getting umami to our taste buds. By cooking, glutamate or ribonucleotides in relatively high level in these ingredients are released and perfectly developed into one cuisine after another. However, there are many other approaches to make the most of umami.

1.3.1 Yeast extract as one important umami enhancer

Besides MSG, yeast extract is another umami enhancer that is widely used in savoury-type food applications. Marmite supplied by Unilever is a good example of a yeast extract product. The reason for the umami flavour of yeast extract possessing is its high-level of free protein, peptides, amino acids. In addition to a high-level 5'-nucleotides (14-20% 5'GMP + 5'IMP) (Taylor and Hort, 2008).

The yeast extract can be obtained by hydrolysing yeast with three approaches: autolysis, plasmolysis or acid (Reineccius, 2005). For the autolysis, the cell is killed without denaturing its proteolytic enzymes. Under the suitable temperature and pH, the cell protein and nucleases will be solubilized by the proteolytic enzymes upon on the RNA to enhance the flavour of the product. If the yeast is produced by plasmolysis, the high salt levels will initiate the cellular degradation, resulting in death and liberation of cellular enzymes. The acid hydrolysis yields a product very similar to a protein called hydrolysed vegetable protein (HVP), which is slurried at 65 to 80% solids, concentrated HCl added, and the mixture is held at 100°C for 6 to 12 h. However, this acidic hydrolysis leads to a much higher capital cost (acid and pressure) compared to autolysis and are also high in salt, which limit the market for acid hydrolysed yeast extracts (Reineccius, 2005).

As a result, the high-level of nucleotides combined with the amino acid profile derived from autolysed yeast at significant level of protein hydrolysis can confer umami flavour.

These high-nucleotides yeast extracts possessing less basic taste can be used at lower dosage in food products without introducing strong bouillon-like taste. Yeast extracts show a powerful interaction with other non-savoury, flavour

modalities and offer improvement through increased fullness while a decrease of bitter aftertaste. Therefore, these yeast extracts offer the possibility of improving flavours in a wide range of savoury and non-savoury applications (Taylor and Hort, 2008).

1.3.2 Protein hydrolysis

1.3.2.1 Taste of amino acid

As discussed in previous sections, glutamate has an umami taste, therefore the umami intensity should increase with more umami-property amino acids. Since amino acid is the sub-unit of peptide and protein, protein hydrolysis is consequently an effective way to liberate more amino acids in order to generate more umami flavour. However, the practice is more complicated than the theory.

In general, there are 20 amino acids present in the food. Each amino acid contributes a different taste (Figure 1.10), for instance, glycine and alanine have a sweet taste, valine and leucine have a bitter taste. Some amino acid has more than one taste, aspartic acid has sour and slight umami taste; though valine is called a bitter amino acid, it still has a slightly sweet taste (Solms, 1969). The combination of amino acids with their respective taste is a key determinant for the taste of food. For instance, crab, shrimp and sea urchin have a similar amino acid profile apart from two amino acids methionine and valine that presents in sea urchins (Table 1.1). If without methionine, a key ingredient and very bitter taste amino acid in the sea urchins, it will just taste like shrimps or crabs (Sen, 2005).

Table 1.1 Taste of different amino acid present in food

Umami and sour tastes	Sweet taste	Bitter taste
Glutamate	Glycine	Phenylalanine
Aspartic acid	Alanine	Tyrosine
	Threonine	Arginine
	Proline	Leucine
	Serine	Isoleucine
	Glutamine	Valine
		Methionine
		Histidine

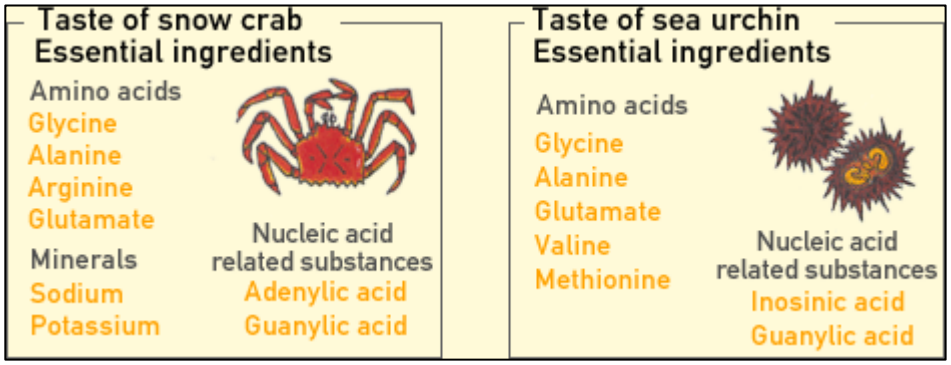


Figure 1.10 Taste compound profile of snow crab and sea urchin (Ajinomoto.com, 2017)

While some free amino acids are present in various natural foods bound amino acid can be further released in order to modify or enhance the flavour. One approach widely used by different nations around the world is fermentation. Although people developed various dietary culture to enhance the flavour without the specific intention of liberating amino acids, fermented food is a rich source of various amino acids. One good example of western and Asian fermentation culture is cheese and fermented soya product. Although proteins have no major tastes of their own such as milk and soya bean, by degrading the protein into amino acids through fermentation, the fermented products (cheese, soy sauce) offer a wide variety of taste profiles.

1.3.2.2 Protein hydrolysis approach

There are many ways to degrade protein into amino acid for different purposes from the most violent approach such as acidic hydrolysis to more specific enzymatic protein digestion. Through both routes, the water molecules break apart the peptides links of the protein molecules, leaving short-chain peptides and separate amino acid molecules depending on how strong the hydrolysis is (Figure 1.11).

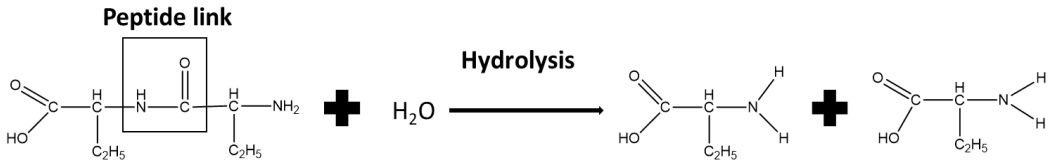


Figure 1.11 Schematic illustration of protein breakdown

By hydrolysing proteins with hydrochloric acid into amino acid, the assay normally heats the protein with 6M hydrochloric acid for 24 h at 110°C. However, through this strong acidic hydrolysis, some amino such tryptophan, glutamine, asparagine, serine and threonine degrades or partially destructed during acid hydrolysis.

Another effective way to hydrolyse protein is to use proteolytic enzymes. The proteolytic enzymes can be categorized into several groups with different terms. In 1986, one German literature clarified some confusion regarding the use of term among protease, proteinase and peptidases (Barrett and McDonald, 1986). Historically, these terms had slightly different meanings (Beynon and Bond, 2001), but largely the differences are being forgotten in the present day. However, the term peptidase is recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) as the general term for all the enzymes that hydrolyse the protein since the word

can properly lead to a set a term for the subtypes of proteolytic enzymes that are indispensable to communications (Rawlings and Salvesen, 2013).

In general, the peptidase can be divided into two types: exopeptidases cleaving one or a few amino acids from the N- or C-terminus of the polypeptide chain while endopeptidases that cleave internally (Figure 1.12).

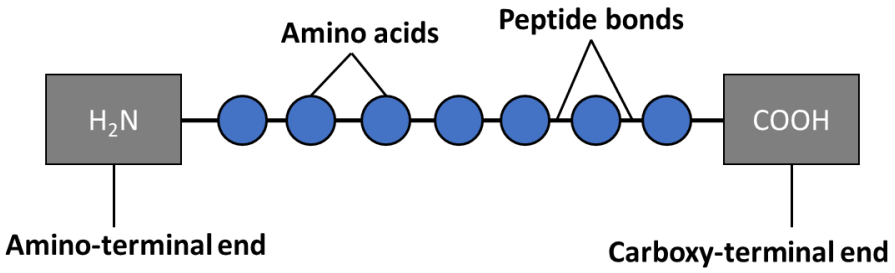


Figure 1.12 The structure of general protein

The exopeptidases that act at a free N-terminus liberate a single amino acid residue (aminopeptidases) or a dipeptide or a tripeptide (dipeptidyl-peptidases and tripeptidyl-peptidases). Those acting at a free C-terminus liberate a single residue (carboxypeptidases) or a dipeptide (peptidyl-dipeptidases) (Figure 1.13). Other exopeptidases are specific for dipeptides (dipeptidases) or remove terminal residues that are substituted, cyclized or linked by isopeptide bonds (peptide linkages other than those of α-carboxyl to α-amino groups) (isopeptidases and omega peptidases). The endopeptidases are divided on the basis of catalytic mechanism into aspartic endopeptidases, glutamic endopeptidases, cysteine endopeptidases, metalloendopeptidases, serine endopeptidases and threonine endopeptidases (Rawlings and Salvesen, 2013).

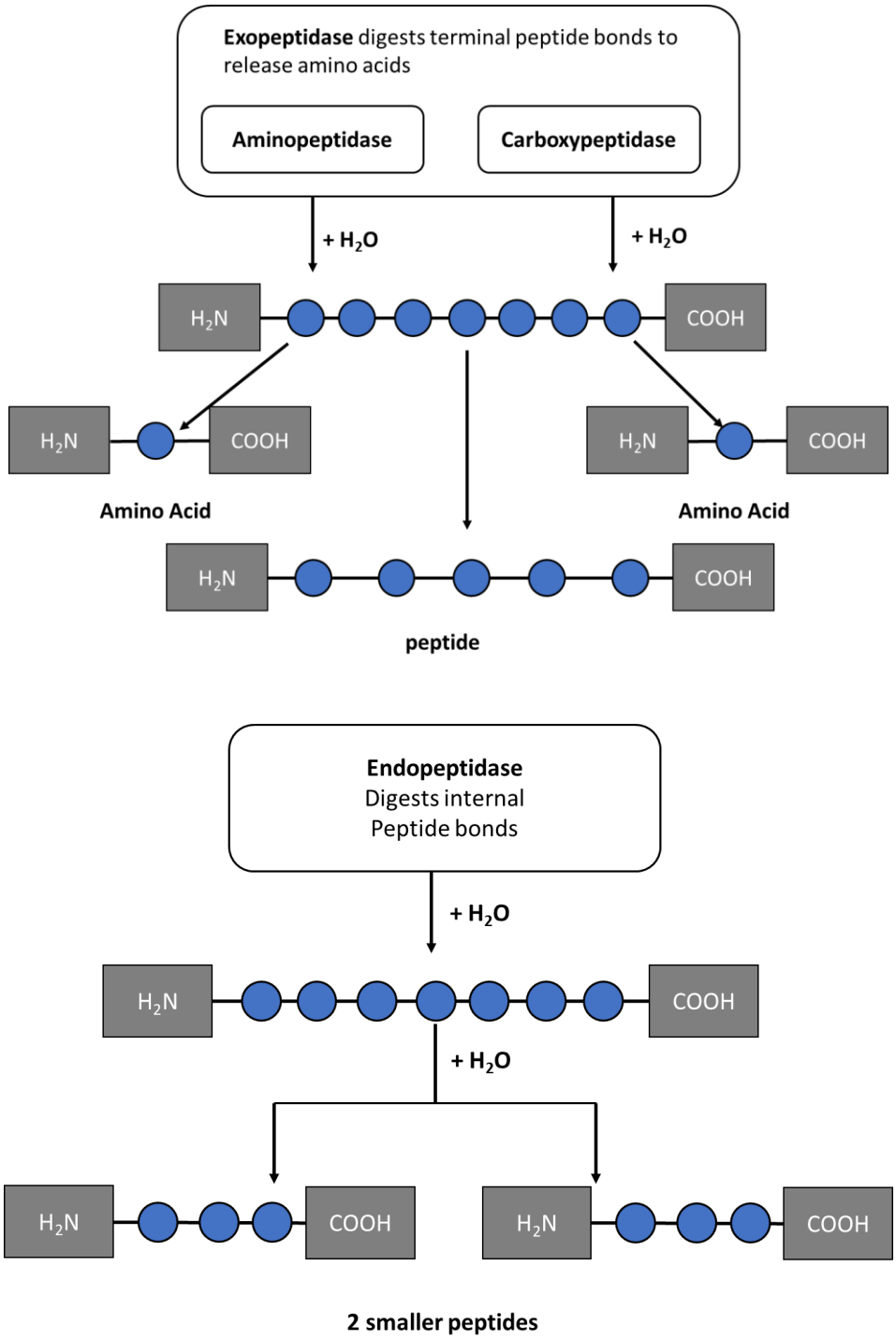


Figure 1.13 Schematic illustration of exopeptidase and endopeptidase digestion of peptide

Since the nature of endopeptidase and exopeptidase, it is impossible that the enzymatic protein hydrolysis liberates only glutamate without realising other amino acid so as to enhance the umami flavour specifically. How to take

advantage of various peptidases in order to enhance the savoury flavour without developing other bad-tasting amino acid profile is still a challenge.

1.3.3 Nuclease digestion

Although the peptidase cannot specifically release umami-property amino acid from the protein to enhance savoury flavour, nuclease has its advantage of enzymatic specificity in terms of savoury flavour enhancement. Since nucleotide are the unit composing deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), acid and alkaline hydrolysis can also effectively break down DNA/RNA into nucleotide but result in non-savoury nucleotides such as 2 or 3'-nucleotides. Therefore, those hydrolysis approach cannot be applied for savoury flavour enhancement.

Umami-property nucleotide such as 5'-GMP and 5'-IMP both occur naturally in foods. Animal muscles are generally rich in 5'-IMP while fresh seafood is rich in 5'-AMP, which may be converted to 5'-IMP due to the action of endogenous enzymes released during processing. Raw vegetables have neither 5'-IMP nor 5'-GMP but they are detected after cooking (Nguyen et al., 1988). Therefore, the natural presence of 5'-nucleotide in cooked food indicates the possible enzymatic degradation of intracellular RNA.

1.3.3.1 Source of nuclease substrate

Before applying any type of enzyme to degrade any nuclear material, it is import to understand the structure of these macromolecules and the specific bonds that enzymes break down during the process of hydrolysis. Similar to protein, nucleic acids are informational macromolecules and therefore contain non-identical monomeric units in a specific sequence. The two major types of nucleic acids are DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). Both RNA and

DNA are long chain polymers made up of mononucleotides that contain three characteristic components: a nitrogenous purine or pyrimidine base, a five-carbon sugar and a phosphate group (Figure 1.14).

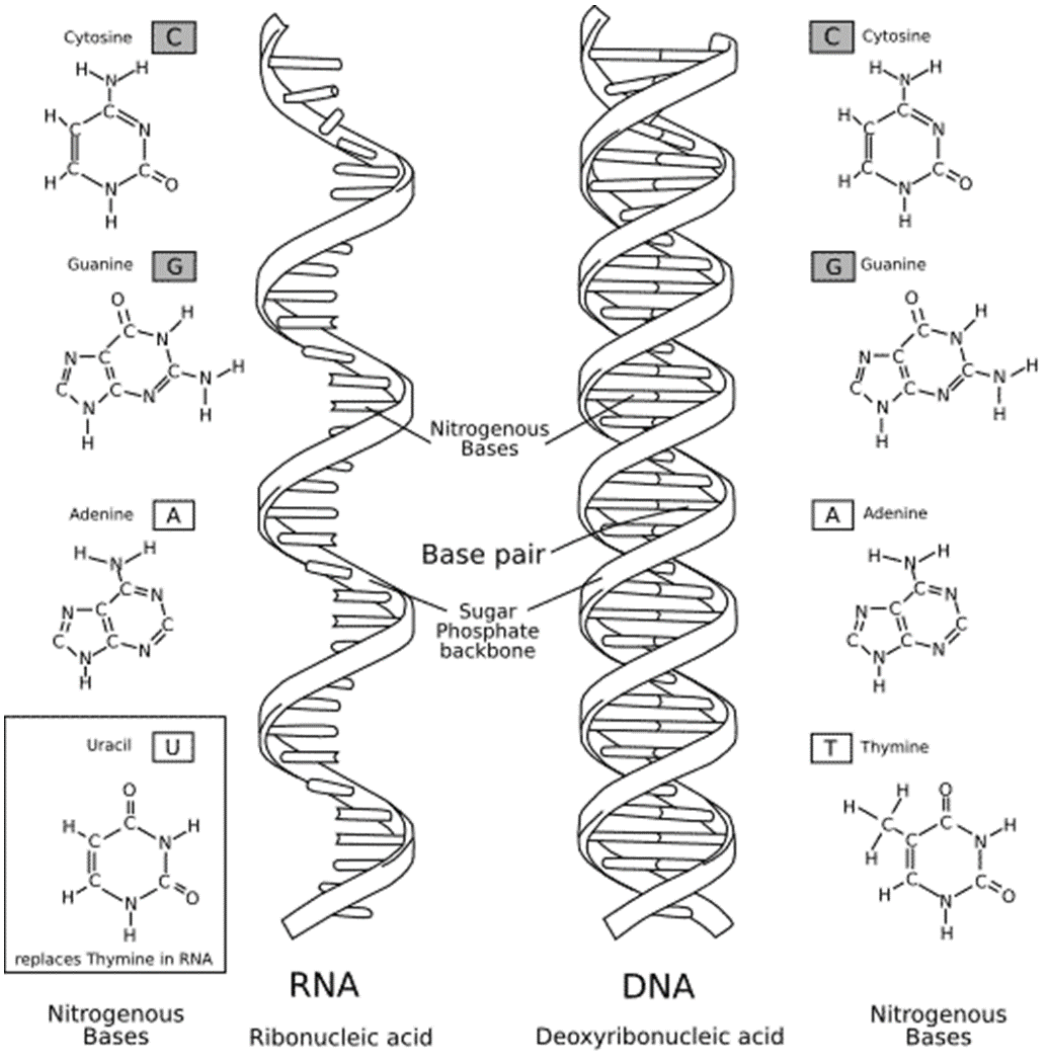


Figure 1.14 The structure of DNA and RNA (Saenger, 1995.)

The composition of RNA is very similar to DNA's apart from one nitrogenous base group. Thymine is replaced by uracil in the polymer chain of RNA. In addition, RNA contains D-ribose as the 5-carbon sugar component while DNA contains 2-deoxy-D-ribose sugar. In terms of functionality, DNA serves primarily as a repository of genetic information whilst RNA plays different roles in the expression of DNA's information.

Take yeast for instance since it is the source of famous umami flavour application – yeast extract, yeast contains both DNA and RNA, the latter typically makes up around 8-12% with less than 1% DNA on a dry solid basis. There are three basic classes of RNA distinguished by their molecular weight and the way they function during protein synthesis.

- Messenger RNA (mRNA) provides the information that dictates amino acid sequence during polypeptide synthesis, due its association with DNA during transcription process and may therefore be partially inaccessible to nuclease digestion such as 5'-phosphodiesterase.
- Transfer RNA (tRNA) directs the correct amino acids to the correct site in an elongating polypeptide chain and may contain up to 10% unusual base such as Dihydrouridine (D) and Pseudouridine (ψ) in addition to the normal bases A, G, C and U, which are generated by the saturation of a double bond, changing its ring structure and interchange of the positions of N and C atoms, respectively (Choudhary, 2012).
- Ribosomal RNA (rRNA) provides the site for protein synthesis that constitutes up to 65% of the weight of ribosomes in the yeast cell.

In order to meet steric requirements, one member of a nucleotide pair must be a purine and the other a pyrimidine. RNA pairing often occurs between guanine and cytosine, and adenine and uracil. Although the phosphate and sugar skeleton of the model is regular, but any sequence of base pairs will fit the overall structure. These four nucleotides can give rise to a variety of permutations, which molecular masses of different RNA range from 25000 daltons from tRNA to over 500,000 daltons for rRNA (Nagodawithana and Reed, 2013). Since these high values obtained for freshly isolated RNA, hence it would be possible only

if RNA escaped the action of nucleolytic enzymes during the purification process. Therefore, rRNA is by far the most abundant and most stable form of RNA in most cells.

In short, the indication underlying all those facts mentioned above is that the target substrate for nuclease digestion for savoury flavour enhancement, should primarily focus on the RNA, to be more specific, rRNA.

1.3.3.2 Nucleolytic enzyme approach

There are four major classes of nucleolytic enzymes: nuclease, phosphatases, nucleosidases, and nucleodeaminases as found in all biological system (Figure 1.15).

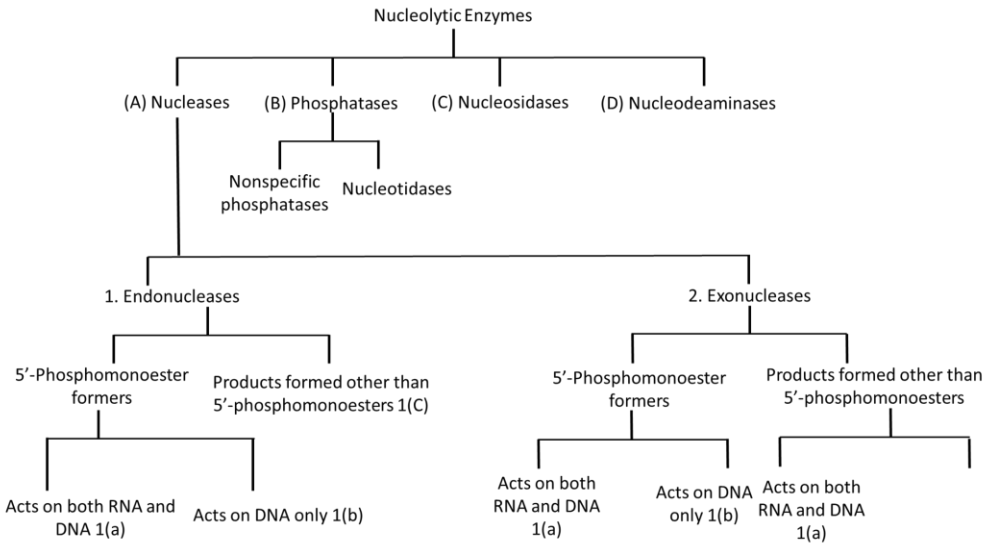


Figure 1.15 The classification of nucleolytic enzymes

Most of these enzymes are present in biological materials at varying concentrations. It is important for living cells to carry such an array of enzymes, both to break down the nuclear material that the cell may absorb from the environment during the growth phase and to break down the specific substrates inside that the cell no longer requires for sustenance. Under normal conditions, these hydrolytic enzymes must be sequestered carefully until actually needed by

the cell in case they digest vital cellular components that are not designed for destruction. Thus, it becomes critical for the enzyme preparation used in the production of 5'-nucleotide rich extracts to have the maximum activity for the formation of the expected product, with minimal activity of the other competing enzymes.

Similar to endopeptidase and exopeptidase for digesting proteins, there are three main features of nuclease activity can be used as the basis for classification. Firstly, polynucleotides can be attacked at points within the polymer chain endolytically or exolytically. The second feature is based on the substrate specificity to make action on RNA, DNA or both. The third feature is to cleave the bond of phosphoidester. The cleavage position depends on the hydrolysis of the bond occurs either between 3'-OH and the phosphate group of the phosphodiester bond so as to give 5'-phosphoryl end groups, or between 5'-OH and the phosphate group result in 3'-phosphoryl end groups (Figure 1.16).

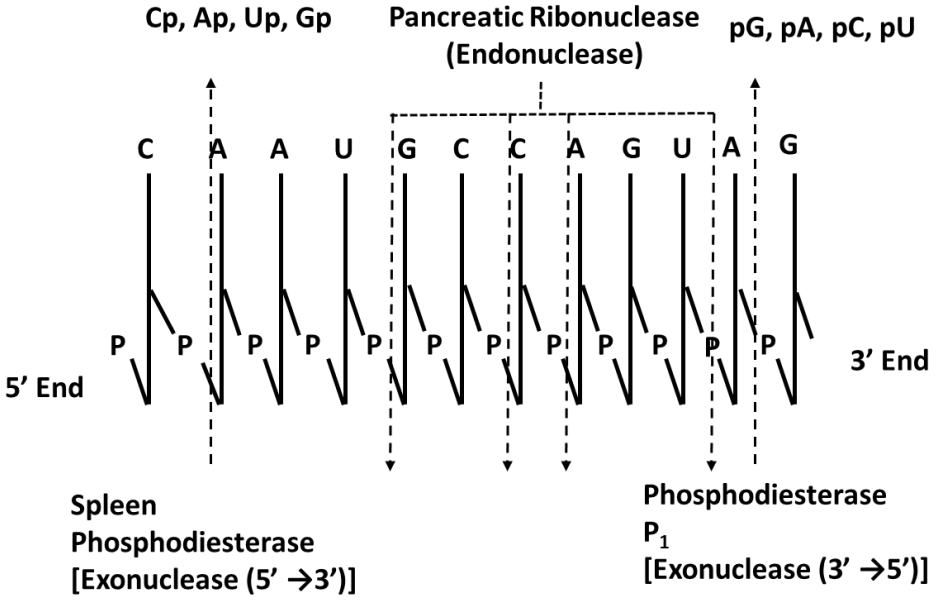


Figure 1.16 Schematic illustration of exonuclease and endonuclease on the nucleotide

Several important enzymes that have applications in flavour-nucleotide production have been identified in naturally occurring products of plants, animal, or microbial origin. Best known with this group are the two enzymes 5'-phosphodiesterase and adenylic deaminase, derived from *Penicillium citrinum* and *Asperqillus strains*, respectively and these enzymes has been used in some research for the uitilzation of brewer's yeast cells for the production of food-grade yeast extract (Chae, Joo and In, 2001).

1.4 EXPERIMENTAL AIM AND HYPOTHESES

The overall aim of this research is to evaluate two low values foods products (fermentation food waste water and insects) for their ability to confer umami taste, and the ability of complex enzyme system to liberate and enhance the availability flavour precursors and umami taste active compounds for the two food products (fermentation food waste water and insects).

The liberation of umami compounds from spray dried fermenter waste water following enzymatic digestion (Chapter 3)

- The umami compound (umami active amino acid and nucleotide) will be enhanced by using various types of enzymes treating the *Fusarium venenatum* fermented waste water.

The liberation of umami compounds from freeze dried *Alphitobius diaperinus* following enzymatic digestion (Chapter 4)

- The umami compound will be enhanced (umami active amino acid and nucleotide) by using different types of enzymes treating the *Alphitobius diaperinus*. With response surface modelling design for the enzyme treatment (pH, time, dosage, temperature), umami compound will be

effectively released from *Alphitobius diaperinus* at optimum treatment condition.

In the following chapter of the thesis, Chapter 3, examines the effect of different enzymes towards umami compound release. Chapter 4 investigates the effect of enzyme treating condition such as temperature, pH, time, dosage and buffer system on umami compound release. Chapter 5 discusses the mechanism of some enzymes based on the result and Chapter 6 presents the possible opportunities for the future work.

2. MATERIALS AND METHODS

2.1 MATERIALS

Spray dried powder from the filamentous fungus fermenter waste water were provided from Marlow Foods Ltd (North Yorkshire, UK). Freeze dried buffalo worm (*Alphitobius diaperinus*) were purchased from Snow Pony Ltd (Derby, UK). Freeze dried buffalo worm was grinded into powder using Kenwood kMix blender (Havant, UK). Four types of peptidases: Flavourzyme™ (1000 LAPU/g), Alcalase™ 2.4L FG (2.4 AU-A/g) produced by Novozyme (Copenhagen, Denmark) and ProteAX™, Protease A 2SD™ and produced by Amano Enzyme Europe (Chipping Norton, UK) were used. A yeast-lytic enzyme YL-TL™ (Amano Enzyme) was used for the release of intercellular contents 5'-RNAse, RP-1G™ (Amano Enzyme) and Deamizyme™ (Amano Enzyme) were used for DNA/RNA digestion. The brief activity of each enzyme is listed in the table below.

Table 2.1 Activity detail of applied commercial enzyme

Enzyme name	**Claimed main activity**	**Function**
Flavourzyme™	exopeptidase	break the terminal peptide bond in a peptide chain
ProteAX™	exopeptidase	
Alcalase	endopeptidase	break the non-terminal amino acids
Protease A 2SD™	endopeptidase	
Glutaminase SD-CS100S™	glutaminase	catalyse the hydrolysis of L-glutamine to L-glutamic acid
YL-TL™	yeast lytic enzmye	lyse the yeast cell walls and release the intercellular contents
RP-1G	5'-phosphodiesterase	hydrolyse RNA to 5'-nucleotides
Deamizyme	adenine deaminase	Convert 5'-AMP to flavour enhancer 5'-IMP

Reverse osmosis (RO) water was used for amino acid analysis and ultrapure water (Millipore, Watford, UK) was used for nucleotide analysis. Nitrogen gas (99.998% minimum purity, oxygen free) used was obtained from BOC (BOC Gases, Nottingham, UK).

2.2 METHODS FOR SPRAY DRIED POWDER OF FERMENTED WASTE WATER ANALYSIS

2.2.1 Quantification of total protein by bicinchoninic acid (BCA) assay

A Pierce BCA protein Assay Kit (Thermo Fisher Scientific, UK) was used for the calculation of the protein concentration measured at 562 nm using a Varian Cary 50 Probe UV Visible spectrophotometer (Scientific Laboratory Supplies, UK). Fermentation waste water samples of 2mg/mL, 5 mg/mL and 10 mg/mL and Bovine Serum Albumin (BSA) standards were reconstituted and assayed using the test-tube procedure (Appendix I). A calibration curve was then plotted and protein concentration of samples were determined.

2.2.2 Acid hydrolysis of water soluble protein

A liquid phase HCl hydrolysis was applied for protein hydrolysis in which the protein sample (2mL) was heated in 6 M boiling hydrochloric acid 100 mL (Thermo Fisher Scientific, UK) under vacuum tube (Thermo Fisher Scientific, UK) at 110ºC in an oven (Sanyo Scientific Oven, Loughborough, UK) for 24 h (Appendix II). For fermented waste water, sample concentration used was 0.007 g/mL.

2.2.3 RNA hydrolysis

RNA was hydrolysed with 0.3 M KOH (Fischer, Loughborough UK) at 38ºC for 24 h followed by addition of 2M $HClO_4$ (Sigma-Aldrich, Dorset UK) which removes perchlorate ion by precipitating $KClO_4$ (Guschlbauer, 1976).

2.2.4 pH adjustment

1 g of sample was dissolved in 50 mL of ultrapure water and modified to pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 (2 mL each) using either 0.01 M HAc (Fischer) or 0.01 M Na_2CO_3 (Acros Organics, Loughborough UK), respectively. All pH modifications or adjustments were conducted using an inoLab pH meter (WTW, Munich, Germany).

2.2.5 Digestion of solubilised protein by peptidase treatment

Spray dried powder from the filamentous fungus fermenter waste water was re-dissolved in water and treated with Alcalase (pH 9, 68ºC) or Flavourzyme (pH 6, 50ºC) provided by Novozyme UK ltd (Novozyme, 2012). Further conversion from Glutamine to glutamic acid treatment was carried out with Amano Glutaminase SD-CS100S (pH 6~7, 60ºC) (Figure 2.1). Alcalase and Flavourzyme enzyme dosage (0.05%~0.2%) was based on calculated protein concentration while Glutaminase SD-C100S (0.05%~0.6%) was based on total solid. Treatment was carried out over 24 h. After enzymatic treatment, samples were put into the oven at 90 ºC for 20 min for enzyme deactivation. All samples were treated in scientific ovens (Sanyo Scientific Oven, Loughborough, UK).



Figure 2.1 The flow diagram of peptidase treatment of filamentous fungus fermenter waste water

2.2.6 Digestion of RNA by nuclease treatment

Samples of spray dried powder dissolved in Milli-Q ultrapure water was mixed with three different enzymes (Amano Enzyme Europe Limited) solutions just before the treatment: Nuclease RP-1G (0.05%~1%, pH 5, 70ºC) and Deamizyme 50000G (0.05%~0.2%, pH 5.6, 55ºC) with addition of peptidase YL-TL (0.5%~2%, pH 8, 60ºC), for various time up to 24 h (Figure 2.2). Enzyme dosage was based on total solid content. After enzymatic treatment, samples were put into the oven at 90 ºC for 20 min for enzyme deactivation. All samples were treated at controlled temperature in scientific oven (Sanyo Scientific Oven, Loughborough, UK).

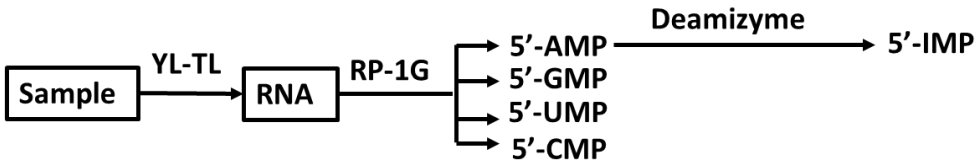


Figure 2.2 The flow diagram of yeast lytic enzyme and nuclease treatment of filamentous fungus fermenter waste water

2.3 METHODS FOR FOOD-GRADE INSECTS

2.3.1 Digestion of solubilised protein by peptidase treatment

0.25 g Grinded freeze dry buffalo worm prepared in 10 mL reverse osmosis distilled water were treated with four different peptidases: Alcalase, Flavourzyme, Prote AX, Protease A 2SD and Glutaminase at 50˚C at pH 7 for up to 24 h (Figure 2.3). These peptidases were either used separately or in a combined fashion. Samples were mixed on the magnetic stirrer (Fisher Scientific, Loughborough, UK) at 150 rpm in scientific ovens (Sanyo Scientific Oven, Loughborough, UK). After enzymatic treatment, samples were put into the oven at 90 ºC for 20 min for enzyme deactivation.

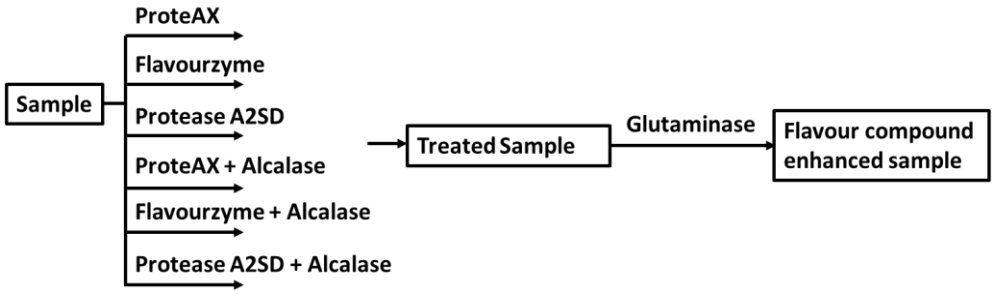


Figure 2.3 The flow diagram of peptidase treatment of *Alphitobius diaperinus*

2.3.2 Digestion of RNA by nuclease treatment

Grinded freeze dry buffalo worm prepared in buffer solution at certain pH was mixed with two different RNAase enzymes (Amano Enzyme Europe Limited): RP-1G (0.1-2%, pH 4-6.5, 45-75 ˚C), Deamizyme 50000G (0.01-0.2%, pH4&5.6, 30-60˚C) for up to 24 h (Figure 2.4). Different buffer solution was chosen for various pH settings. Sodium citrate buffer was used for pH 4, 4.5, 5.5 while sodium phosphate buffer was used for pH5, 6, 6.5. Sodium acetate solution was also prepared for pH 4-5.6 buffer. All buffer solution chemicals were of

analytical grade (>95% purity; Fisher Scientific, Loughborough, UK) and prepared in Milli-Q ultrapure water All samples were treated on the magnetic stirrer (Fisher Scientific, Loughborough, UK) at 150 rpm to reach an even mix in scientific ovens (Sanyo Scientific Oven, Loughborough, UK). After enzymatic treatment, samples were put into the oven at 90 ºC for 20 min for enzyme deactivation.

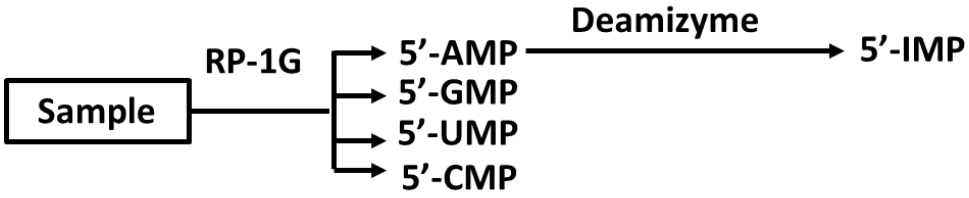


Figure 2.4 The flow diagram of nuclease treatment of *Alphitobius diaperinus*

2.3.3 Response surface modelling for enzyme treatment optima

To study the effect of pH, temperature, enzyme dosage and time on enzymes' performance to yield different types of nucleotides, several D-optimal designed experiments were created for creating a response surface using Design-Expert Version 10 software (Stat-Ease inc, Minneapolis, MN, USA). For enzyme RP-1G, treatment incubation temperature (45-75˚C), pH 4-6.5, treatment time (0.5 to 20 h) and enzyme dosage (0.1%-2%) were varied to produce two sets of 20 experimental design points with duplicates at the extremities and the edge of the design space. Likewise, for enzyme Deamizyme 50000G, treatment incubation temperature (30-60˚C), treatment time (0.5 -24 h) at pH 4 were varied to produce 30 experimental design points with duplicates at the extremities and centre of the design space (Figure 2.5).

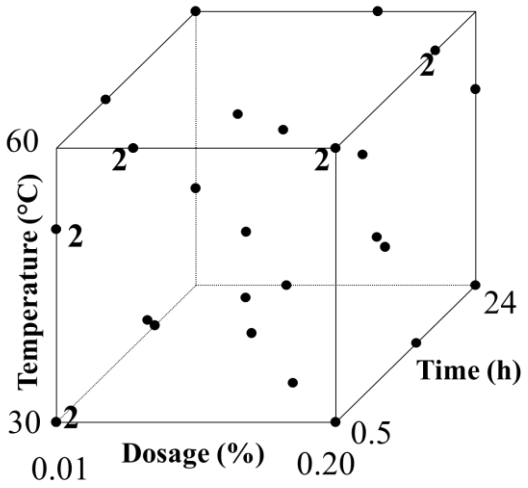


Figure 2.5 Response surface model of Deamizyme treatment generated by Design-Expert Version 10 (Stat-Ease inc, Minneapolis, MN, USA)

2.4 NUCLEOTIDE ANLYSIS BY HPLC

Nucleotide(s) were analysed by an Agilent 1100 series HPLC-UV (Agilent Technologies, Germany), using a Kinetex Biphenyl column (5 µl 100A 250×4.6mm, Phenomenex, UK) with isocratic mobile phase (10 mM NH_4Ac: Methanol 99:1) at 1 mL/min flow rate. 10 µL of sample was injected into the HPLC-UV system. UV detection was at 260 nm wavelength.

Identification of peaks were conducted based on retention time when compared with chromatographs of standards. Calibration curves for each 5'-nucleotide was generated based on its absorbance values at different concentrations and these curves were used to determine the concentration of 5'-nucleotide in sample.

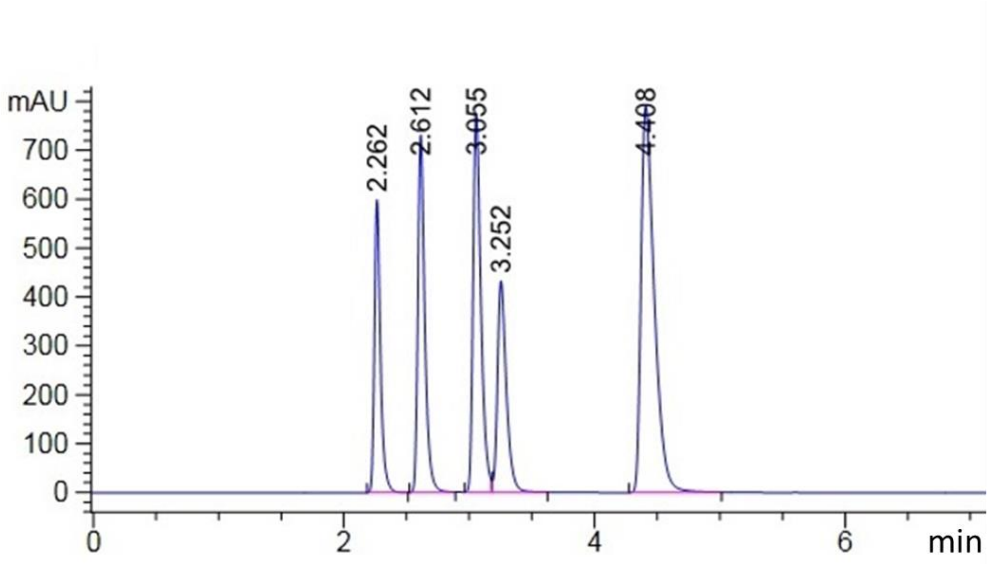


Figure 2.6 Chromatograph of 5'-nucleotide standards detected by HPLC-UV at 260 nm wavelength of nucleotides. The X-axis shows the retention time of the peak of each individual compound while Y-axis shows the height of the peak in milli-absorbance unit. Peak: 2.626, 5'-UMP; 2.612, 5'-CMP; 3.055, 5'-GMP; 3.252, 5'-IMP; 4.408, 5'-AMP.

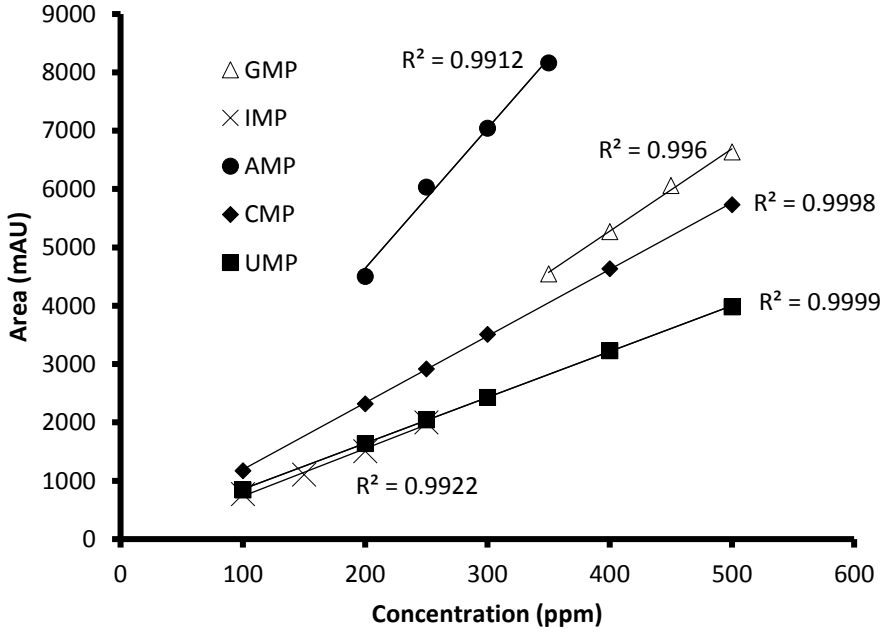


Figure 2.7 Standard calibration curves of 5'-nucleotides were obtained for dilution of 1000 ppm nucleotide standard solution. R^2 values are all above 0.99%.

2.5 AMINO ACID ANLYSIS BY GC-MS

Amino acid was analysed by EZ: faast kit method developed by Phenomenex (Badawy, Morgan and Turner, 2007) via Thermal ISQ GC-MS. Amino acids were isolated from samples and derivatized using the EZ:faast amino acid kit (Phenomenex, Macclesfield UK). 25 µL of each sample was combined with 10 nmol of norvaline which acted as an internal standard. This solution was passed through the EZ:faast solid phase extraction absorbent (contained within a pipette tip) which was subsequently washed with 200 µL propanol. A solution of propanol and sodium hydroxide (200 µL) was then used to remove the absorbent (and the amino acids were retained) from the pipette tip. 50 µL chloroform and 100 µL isooctane were then sequentially added to the solution to derivatize the amino acids. The amino acids were recovered in the upper organic layer, this was dried down by miVac duo centrifugal vacuum sample concentrator (Genevac Ltd, Ipswich, UK) and the sample re-dissolved in 100 µL Iso-octane: chloroform (80:20 v/v) prior to analysis using Gas Chromatography – Mass Spectrometry (GC-MS). Where necessary, samples were stored at 4°C for a maximum of 24h.

For GC-MS, 1 µL of the sample was injected in splitless mode (split closed for 10s) using an AS3000 autosampler (Thermo Fisher Scientific, UK). The injector of the Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, UK) was maintained at 250°C, with an initial oven temperature of 90°C which increased to 320°C at 20°C/min (transfer-line from the oven to mass spectrometer, 300°C). Helium (8 psi) was used as the carrier gas to elute the amino acids from the ZB-AAA column (10 m x 0.25 mm ID).

The ISQ mass spectrometer (Thermo Fisher Scientific, UK) was operated in selected ion mode recording ions 84, 101, 114, 116, 130, 144, 146, 155, 156, 158, 172, 180, 184, 243 and 244 with a dwell time of 0.03s. Calibration was achieved by comparison of peak areas for the amino acids in standard and sample runs after adjustment for variation in the peak area of the internal standard (Appendix III).

2.6 DATA ANALYSIS

Microsoft Excel software (Microsoft Corp., Redmond, WA, USA) was used to fit linear regressions to the calibration of standard curve for determining the concentration of samples. The Data Analysis ToolPak add-in (Microsoft Corp., Redmond, WA, USA) and the SPSS 23.00 (SPSS Inc., Chicago, USA) were used to perform paired comparison test, two-way analysis of variance (ANOVA) and Tukey's range statistical test, respectively. Design-Expert Version 10 (Stat-Ease inc, Minneapolis, MN, USA) was used for creating D-optimal responding surface experiments and significance evaluation.

3. ENZYMATIC LIBERATION OF UMAMI COMPOUNDS FROM SPRAY DRIED FERMENTER WASTE WATER

3.1 THE POTENTIAL VALUE OF *FUSARIUM VENENATUM* FERMENTED WASTE WATER

Fusarium venenatum is also known as the filamentous fungus, a microfungus of the genus Fusarium that has a high protein content, which was first discovered in a soil sample in 1967 (Nadathur, Wanasundara and Scanlin, 2016). During the 1960s, it was predicted that there would be a shortage of protein-rich foods by the 1980s. Then a research centre at Rank Hovis McDougall (RHM) began to investigate converting the *Fusarium venenatum* into a protein-rich food for human consumption (ICI). After a ten-year evaluation programme, RHM was given permission to sell mycoprotein for human consumption in 1985. In the same year, Quorn was founded as a joint venture named Marlow Foods Ltd, between RHM and Imperial Chemical Industries (ICI). It is now owned by the Monde Nissin Corporation.

All Quorn foods contain mycoprotein as an ingredient, derived from the *Fusarium venenatum* fungus (Phillips and Williams, 2011). The waste water from this fermentation process can contain leftover protein, amino acid, nucleotides due to the breakdown of protein and cells of *Fusarium venenatum* fungus and can be dried into powder.

The amino acid profile and the amount of nucleotide existing in the waste water may have an important impact in terms of the flavour enhancement. If by using a range of hydrolytic approach to fully release the peptides, amino acid and nucleotides in a good proportion from the leftover protein and fungus, further breakdown of peptide and nucleotides through enzymic treatment may result in the generation of a flavour enhancer from the waste water.

There are three hydrolysis approaches used in order to liberate and/or generate amino acid and nucleotides in this study.

1. Acid hydrolysis of water soluble protein
2. Alkaline hydrolysis of DNA and RNA
3. Enzymatic hydrolysis of protein, DNA and RNA

The acid and alkaline hydrolysis was used to determine the potential increase in total free amino acid and nucleotides in the waste water, while the enzymatic hydrolysis was used to generate specific amino acid and nucleotide possessing umami property.

3.2 EXPERIMENTAL HYPOTHESES

The umami compound (umami active amino acid and nucleotide) will be enhanced by using various types of enzymes treating the *Fusarium venenatum* fermented waste water.

3.3 RESULT AND DISCUSSION

3.3.1 Glutamic acid content present in post-fermentation spray-dried powder from wastewater

Protein, peptide and free amino acid is the source for certain amino acids such as glutamate (GLU) and glutamine (GLN), thus protein content of spray-dried powder sourced from post-fermentation wastewater was determined. Assays using a BCA assay revealed that there was 375 mg protein/g sample. Protein acid hydrolysis used HCl and was compared with digests using RO water (pre-hydrolysis). There was 1.19 ± 0.03 mg/g of GLU pre-hydrolysis which increased to 1.90 ± 0.04 mg/g post-hydrolysis (Figure 3.1). Suggesting that liberation of free amino acid from protein or peptide complexes may increase the abundance of the umami taste active compound – glutamic acid by a maximum of 58.5% ($P<0.05$). There was 0.14 mg/g GLN in pre-hydrolysis samples and no GLN was detected post-hydrolysis.

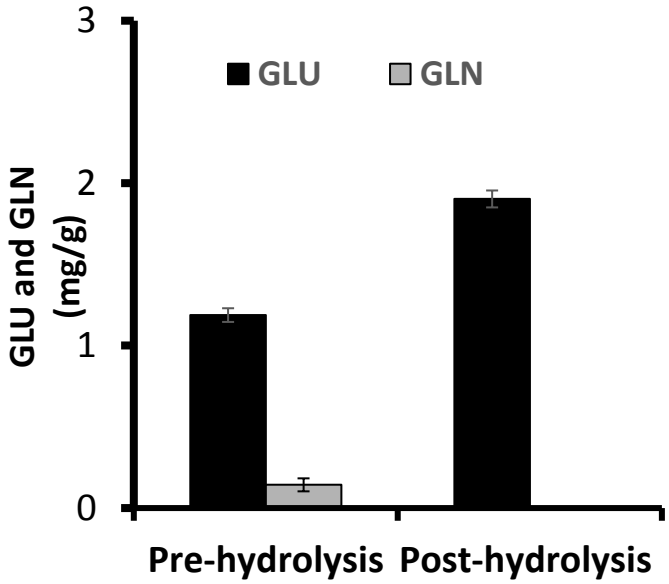


Figure 3.1 Average concentrations of GLU and GLN (mg/g solid content) before and after acid hydrolysis. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

Data obtained from the protein assay (375 mg protein per 1 g sample) and protein hydrolysis (1.88 mg GLU, no GLN) did not correlate. This is because the copper-based assay used (Pierce™ Protein Assay Kit) chelates with proteins ranging from tripeptide chains to large tertiary structures (Thermo, 2017) while protein hydrolysis only hydrolyses large protein structures. Moreover, presence of short-chain peptides may lead to protein content being lower than results suggest as these peptides could be regarded as a protein during the BCA assay. Thus, there was an over-estimation of protein detected from the protein assay.

No GLN was detected from protein hydrolysis assays and the degradation of GLN could be a possible explanation for this finding. GLN is a very fragile amino acid that constantly degrades to equimolar formation of ammonia and

does not form glutamate at room temperature (Khan and Elia, 1991) and is only stable at pH 5.0-7.5 (Arii et al., 1999), this explains the loss of GLN during hydrolysis. Hydrolysis conditions are outside GLN's stability in terms of pH and therefore GLN was probably degraded immediately after it was released.

3.3.2 Quantification of nucleotides in post-fermentation spray-dried powder from wastewater

Prior to RNA alkaline hydrolysis treatment, there was 20 mg/g 5'-GMP, 9.6 mg/g 5'-IMP and 10 mg/g 5'AMP present in the samples. Analysis of nucleotides after alkaline hydrolysis revealed the appearance of 2, 3'-AMP and 2,3'-GMP in addition to 5'-AMP or 5'-GMP, suggesting that nucleotides can be liberated from RNA.

As previously discussed in the chapter one, there are three main types of RNA present in the cell; ribosomal RNA (rRNA) is the most abundant type of RNA in the cell (80%) while transfer RNA (tRNA) and messenger RNA make up 15% and 5%, respectively (Ogle et al, 2005.) During the fermentation process, the fungal cell wall can become disrupted resulting in a release of free RNA, this free RNA is present in the cytoplasm but is instantly degraded into free nucleotides by the action of RNases. The action of RNases is the reason of the presence of 5'-nucleotides in the sample as the peak of UMP, CMP, GMP, IMP and AMP appears at retention time at 2.2, 2.6, 3.1, 3.5 and 4.5 min, respectively (Figure 3.2). However, some polymers of RNA which are not fully degraded into polymers can still be manipulated further. Hence, by using alkaline treatment to degrade polymers of RNA, the presence of nucleotides derived from RNA could increase. Following alkaline hydrolysis of the samples, 2'-3'-nuclotied circled

in red are present compared to none in samples before alkaline hydrolysis (Figure 3.2). 2'-3'-CMP 2'-3'-GMP, 2'-3'-AMP appeared at retention time 3.3, 3.9 and 8.8 minute, respectively (Figure 3.2). Although the presence of 2'-3'-nucleotides cannot quantify the concentrations of RNA present in the sample, it suggests that there were still potential nucleotides could be liberated.

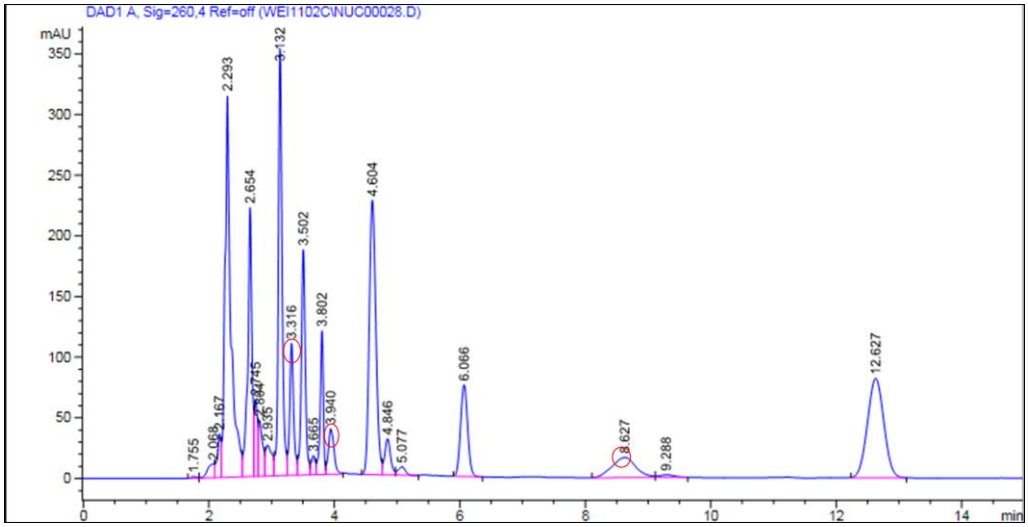


Figure 3.2 Chromatography detected by HPLC-UV at 260 nm wavelength of nucleotides with alkaline hydrolysis. The X-axis shows the retention time of the peak of each individual compound while Y-axis shows the height of the peak in arbitrary unit.

3.3.3 The effect of pH on the concentration of nucleotides

Perception of umami is associated with presence of 5'-GMP, 5'-IMP and 5'-AMP so only these nucleotides were included in this study. Different enzyme treatments have different optimal pH's, therefore an assessment of the influence of pH on the stability of nucleotides at different pH's was required. In general, 5'-prime nucleotides GMP, AMP and IMP were sensitive to alkaline conditions (Figure 3.3). By increasing the samples pH to pH 9, a significant decrease was observed in GMP (67%), AMP (77%) and IMP (70%) ($P<0.05$), indicating alkaline pH denatured free nucleotides (McLennan et al., 2012). By comparison,

acidic conditions (pH 4-6) did not alter the concentration of these nucleotides. At pH 4 and 6, there was no significant difference in concentration of nucleotides 5'-GMP, 5'-IMP and 5'-AMP when compared to the original pH of samples at 5 ($P>0.05$) (Figure 3.3). To minimise nucleotide degradation during enzymic hydrolysis of RNA, this should be carried out at a pH range of 4-6.

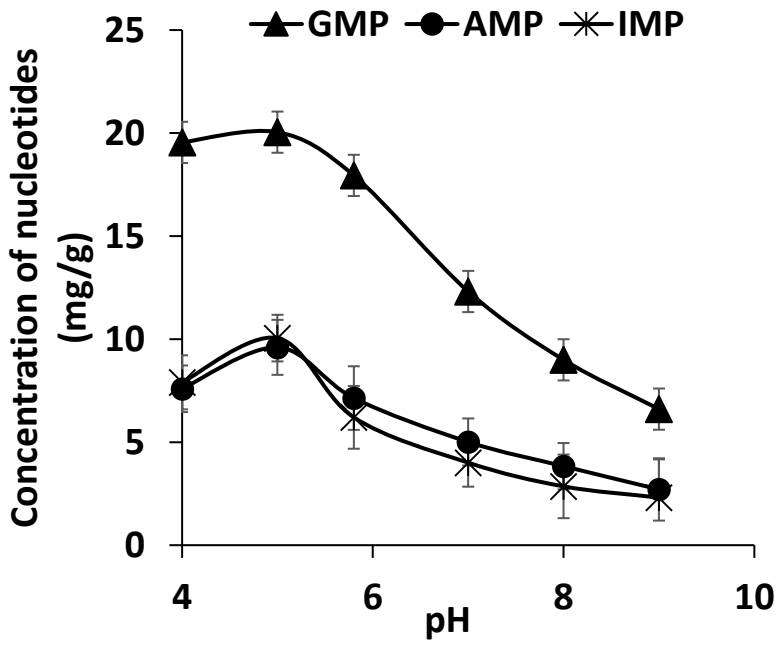


Figure 3.3 : Average concentration of 5'-GMP, IMP, AMP at different pH at room temperature. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

3.3.4 Yield of nucleotides after nuclease treatment following variable enzyme treatment

RP-1G is a 5'-Phosphodiesterase produced by *Penicillium citrinum* fermentation which digests RNA/DNA at the 5' position liberating 5'-nucleotides. This was used to treat the extracts to increase nucleotide concentration. There was a 2-fold increase in 5'-CMP, a decrease in 5'-IMP, no significant differences in 5'-UMP

and 5’-GMP (P>0.05) and a 70-85% significant increase in 5’-AMP concentrations (P<0.05) (Figure 3.4).

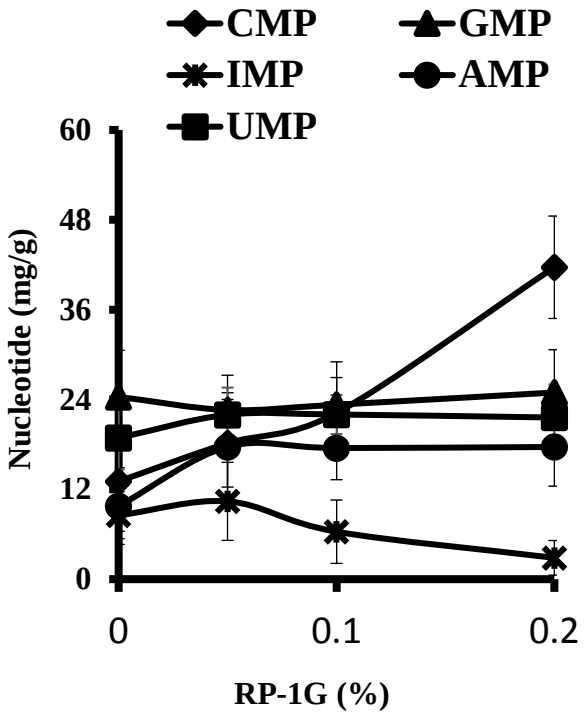


Figure 3.4 Average of concentration of 5’-nucleotides after treatment of RP-1G at pH 5, 70°C for 24 h Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

Incubation with 0.05% RP-1G elicited the greater yield of 5’-AMP, 5’-GMP and 5’-IMP compared to the ones treated with 0.1% and 0.2% RP-1G. A similar study using RP-1G for the production of yeast extracts reported that 0.045% was the best concentration (Guan, 2013) and this was similar to that observed with our study.

YL-TL is a yeast-lytic enzyme preparation derived from *Geobacillus stearothermophilus* which can lyse the cell wall and releases intercellular contents and this may enhance the availability of the precursors.

Digestions with 2 % YL-TL resulted in an increase in 5'-GMP (112%), 5'-AMP (16.7%), 5'-CMP (84%) and a decrease in 5'-UMP (36%) and 5'-IMP (45%) respectively (Figure 3.5).

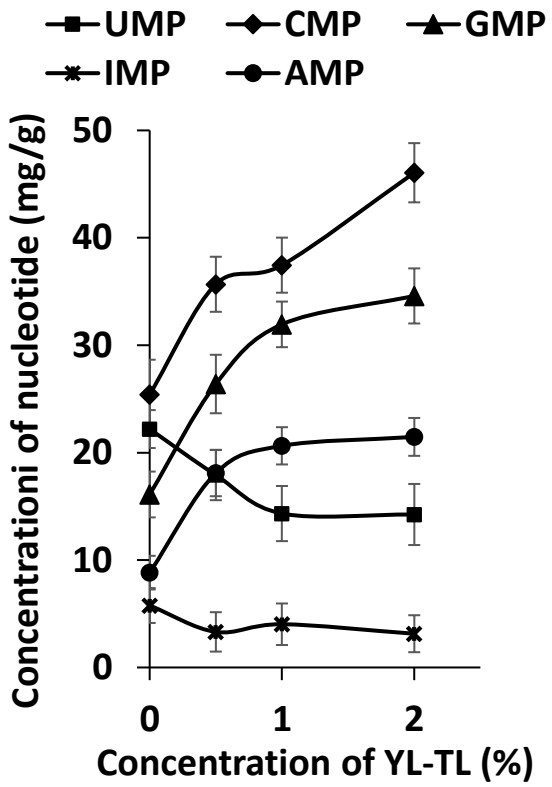


Figure 3.5 Average concentration of 5'-nucleotides with treatment of YL-TL at 60°C, pH 8 for 24 h before 0.05% RP-1G digestion at pH 5, 70°C for 24 h. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

Treatment with 2% YL-TL revealed that the greatest increase in 5'-GMP (112%) and 5'-AMP (16.7%) concentrations. 2% YL-TL was used since it yielded the greatest concentration of 5'-GMP and 5'-AMP. 2% was not exceeded due to the cost of the enzyme preparation.

To determine, the smallest concentration of Deamizyme needed for maximum conversion of 5'-AMP to 5'-IMP, assays with Deamizyme at different dosages

for 24 h were performed. Full conversion of 5'-AMP to 5'-IMP was observed based on molar basis when using 0.05% Deamizyme.

Deamizyme was added after 0.05% RP-1G but without YL-TL treatment for 24 h to observe the conditions for converting 5'-AMP to 5'-IMP. Concentrations of 5'-UMP, 5'-CMP and 5'-GMP were unaffected when treated with Deamizyme for 24 h, and there was a 100% conversion from 5'-AMP to 5'-IMP in assays using 0.05% Deamizyme (Figure 3.6). Therefore, the concentration selected is 0.05%.

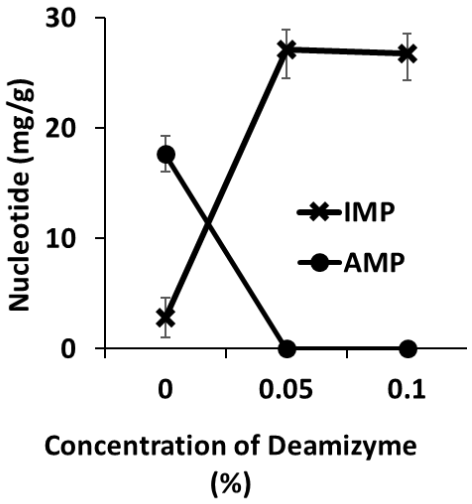


Figure 3.6 Average concentration of 5'-IMP and 5'-AMP with treatment of 0.05% RP-1G at pH 5, 70ºC for 24 h followed by Deamizyme pH 5.6, 55ºC for 24 h. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

Three enzymes were evaluated to maximise the yield of 5'-nucleotides that could be achieved when treated with enzymes. The sequence for enzyme addition was important since YL-TL itself does not yield any nucleotides. YL-TL was added first to lyse any intact cells and release its intracellular contents, subsequently RP-1G was added to hydrolyse the RNA to 5'-nucleotides and then Deamizyme to convert 5'-AMP to 5'-IMP.

Treatment without enzyme had 98 ± 11 mg/g 5'-nucleotides, while assays with enzyme treatment had 119 ± 10 mg/g 5'-nucleotides (Figure 3.7), achieving a 21 mg/g increase ($P<0.05$). In terms of umami-property nucleotide, there was a slight decrease of 5'-GMP (12.5%) and a significant increase of 5'-IMP by 3-fold. The final maximum nucleotides produced with the exception of 5'-GMP correlated with the increase in other nucleotides. Overall, a 21 mg/g sample increase in 5'-nucleotides was obtained.

5'-GMP was found to decrease upon digestion. This decrease could be attributed to a 24h digestion with RP-1G which was characterised by a slight decline in 5'-GMP suggesting that long-term thermal incubation may degrade 5'-GMP. Therefore, positive effects in 5'-GMP concentration may be achieved if the assay was not allowed to run for 24 h but for a shorter duration of time instead.

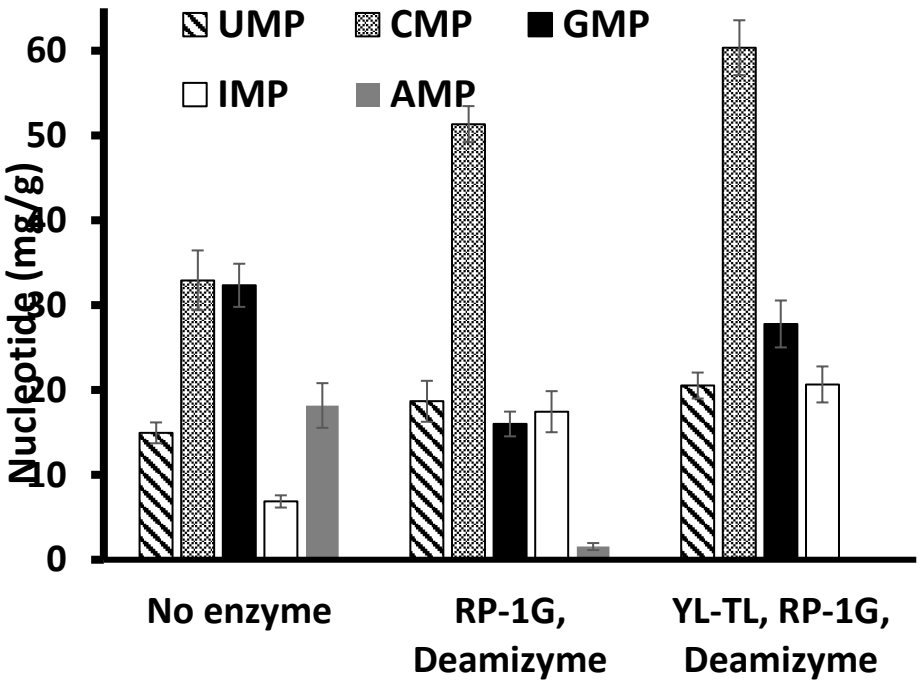


Figure 3.7 Average concentration of 5'-nucleotide before and after enzyme treatament. (No enzyme; 0.05% RP-1G for 24 h followed by 0.05% Deamizyme at pH 5.6, 55°C for 24 h; 2% YL-TL for 24 h followed by 0.05% RP-1G for 24 h followed by 0.05% Deamizyme pH 5.6,

55ºC for 24 h. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

3.3.5 Nuclease activity time course

After determining the effective concentration of nuclease required for liberation of 5'nucleotides (Section 3.3.4): we determined the optimal time of digestion for release of these nucleotides from wastewater. Assays using only RP-1G had a two-fold increase on the concentration of 5'-CMP while there were no changes in other nucleotides after 18h treatment (Figure 3.8) which were not significantly different from no enzyme controls ($P>0.05$). Assessing for the duration of digestion, it was observed that incubation over 2h yielded an 11 mg/g increase (CMP) and incubation for 18h led to a further 2 mg/g increase (Figure 3.8). Digestion with RP-1G for 2h saw the maximum increase in 5'-GMP with some decrease in 5'-GMP observed after this point, it was equivalent to less than 5.0 mg/g sample. The effects on 5'-AMP were also maximised after a 2h digestion. When compared to an untreated sample in terms of enzyme and heat, an increase in 5'-IMP could be observed at 2h. It was decided that 2h treatment with RP-1G was sufficient for maximum yield. In conclusion, prolonged digestion did increase yield of CMP, however, the majority of the increase occurred during the first 2h.

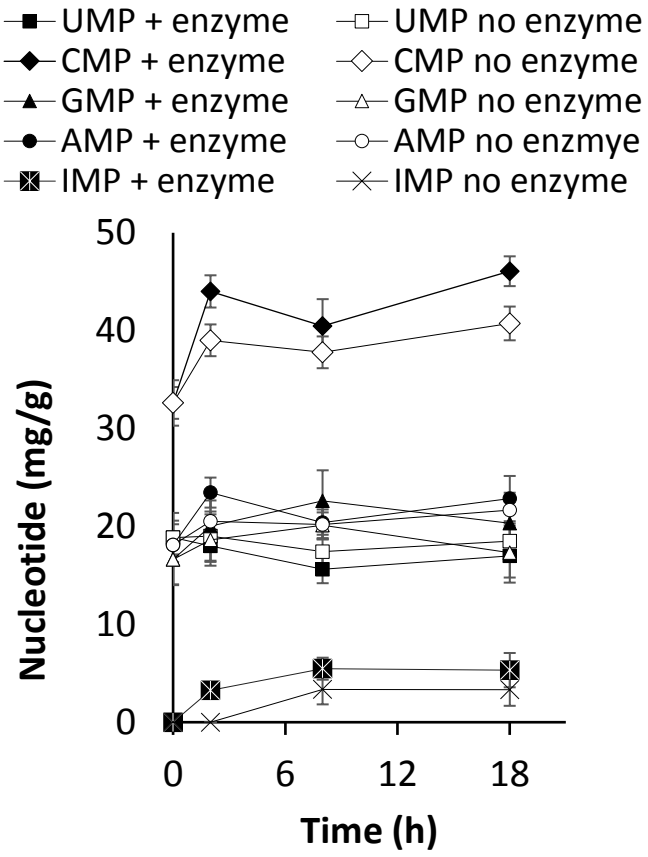


Figure 3.8 : Average concentration of 5'-nucleotides treated with 0.05% RP-1G with time over 18 h. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

Assays treated with 0.05% RP-1G followed by 1% YL-TL at various time led to an increase in concentrations of 5'-CMP (33.5%), 5'-GMP (115%), and 5'-AMP (23.7%) after incubation for 2h (Figure 3.9). Concentrations of GMP increased in no enzyme controls over the time period (22% increase) (Figure 3.9). There is no significant difference observed between the concentration of 5'-AMP increased in samples without enzyme treatment and in the presence of the enzymes ($P>0.05$).

Assessing for incubation period during a 1% YL-TL digestion it was observed that there was an increase in 5'-AMP yielding 4.3 mg/g sample after 2h; further digestion over 18h saw an approximately 50% increase in 5'-AMP. Greater

yields were obtained with prolonged digestion but the additional benefits of digesting for longer than 2h was not beneficial since the benefits are very small. Effects of YL-TL on 5'-GMP and 5'-AMP were maximised after 2h of treatment therefore, 2h was chosen as the best time point to be adopted for digestion using YL-TL.

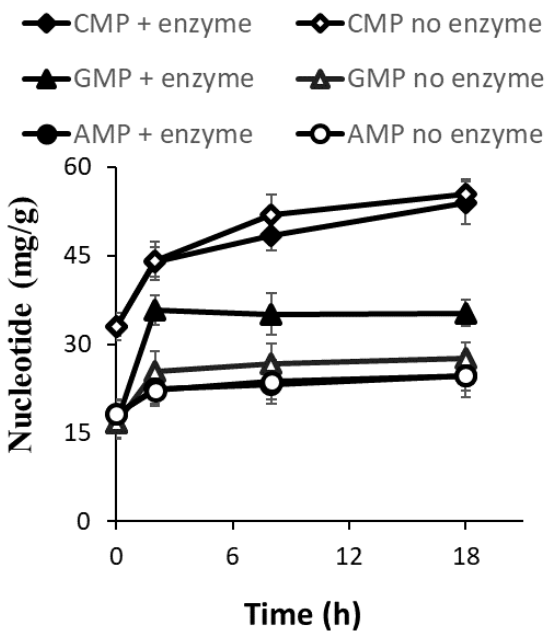


Figure 3.9 Average concentration of 5'-nucleotides treated with 2% YL-TL after 0.05% RP-1G enzyme treatment for 2 h with time as a variable for up to 18 h. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

After 2h incubation with enzyme Deamizyme, 32.4% of 5'-AMP was converted to 5'-IMP, however, after 8h there was a 100% conversion (Figure 3.10), reaching a total of 38 mg/g 5'-IMP.

Deamizyme was highly effective and full conversion of 5'-AMP to 5'-IMP within 8h was observed; this effect might be as a result of this enzyme's optimum pH recommended by Amano being close to the sample's pH giving the digestion optimal conditions.

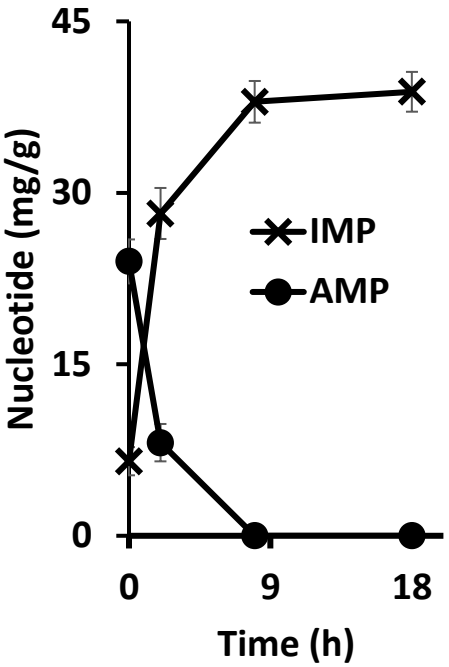


Figure 3.10 Average concentration of 5'-nucleotides treated with 0.05% Deamizyme pH 5.6, 55ºC with time as a variable to sample pre-treated with 0.05% RP-1G for 2h. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

In conclusion, a three step enzymic process for the liberation of taste active neucleotides from waste water has been developed and validated. Step one: Using YL-TL to release the intercellular contents to enhance the availability of the processor, Step two: RP-1G enzyme treatment to convert RNA into nucleotides (CMP, UMP, AMP, GMP), step three: further conversion of non-umami nucleotide AMP to the flavour active nucleotide IMP with Deamizyme.

3.3.6 Peptidase treatment with following variable enzyme treatment

To liberate the maximum concentration of amino acids, dried fermented waste was hydrated and digested using a blend of peptidase enzyme. An exopeptidase Flavourzyme (Flv) was used to cleave terminal peptide bonds releasing single

amino acids or dipeptides and was compared to the endopeptidase Alcalase (Alca), which cleaves internal peptide bonds of a polypeptide chain or protein, producing shorter peptide chains.

1% Flv treatment increased GLU and GLN by 3.5-fold and 280-fold, respectively. Optimisation assays with Alca revealed that incubation with 3% Alca was characterised by a 3-fold increase in GLU and a 74-fold increase in GLN following enzyme treatment compared to an untreated sample (Figure 3.11). 24h incubation was regarded as complete digestion and different dosages were applied to optimise the yields of GLU and GLN accordingly.

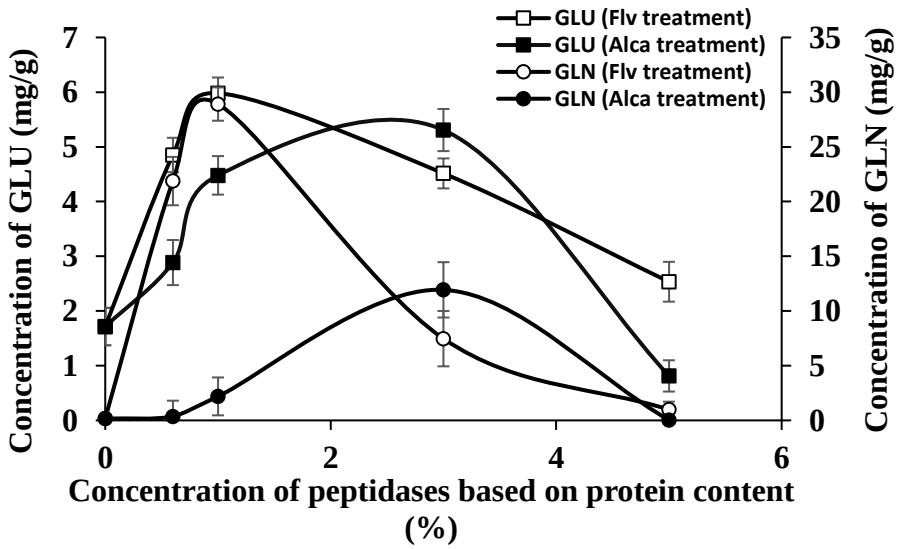


Figure 3.11 Average concentration of GLU and GLN with singular enzyme treatment for 18 h. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

Flv was superior to Alca in terms of release of GLU and GLN; this could be as a result of the sample not containing many large protein structures. Therefore, when treated with endopeptidases, not many internal peptide bonds were

available for cleavage but shorter peptide chains present could be easily cleaved by exopeptidases, releasing single amino acids (Waldron, 2009).

3.3.7 Effect of a two-step peptidase digestion on the yield of GLU and GLN

To evaluate if yields could be further improved by using a combination of endo and exo peptidates both individually and then in combination. Yield of GLN and GLU was determined after digestion with a combination of exopeptidases (Flv) and endopeptidases (Alca) and compared to singular enzyme digestion. Since Flv and Alca have different pH optima and temperatures, each enzyme was assayed for in different orders, using different dosage of individual enzymes to reveal the greatest amounts in the assay. Results from assays using enzymes in combination revealed that concentrations of GLU and GLN were dependent on the order and titre of enzymes added, such as higher concentrations of GLU (increase by 1.1-fold) and GLN (increase by 24.3-fold) in assays using 0.6% and 5% Flv before addition of Alca (Figure 3.12). Assays with 3% Alca initially then addition of Flv were characterised by increases in GLU (increase by 1.4-fold) and GLN (increase by 16-fold) ($P<0.05$). However, the yield of GLU or GLN in combined enzyme digestion did not surpass the yield using individual enzymes.

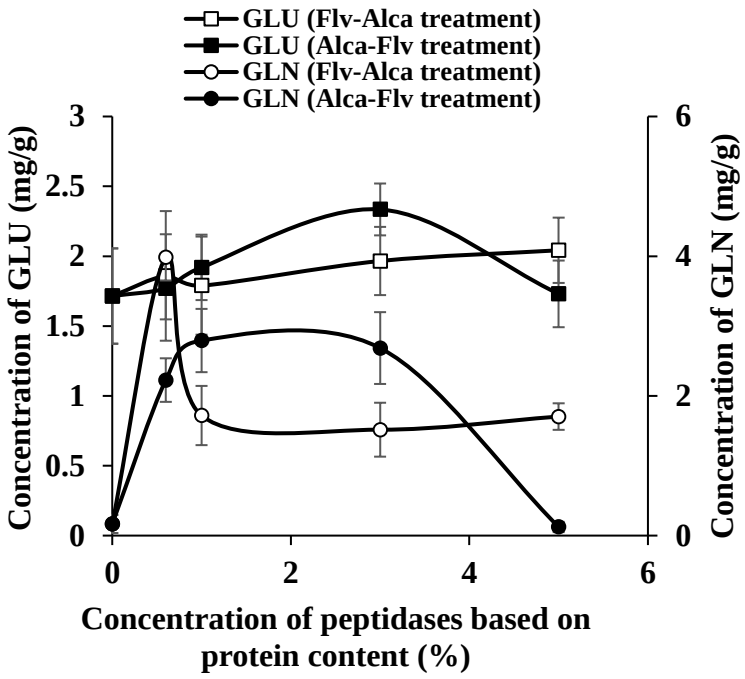


Figure 3.12 Average concentration of GLU and GLN with two-step enzyme treatment for 18h. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

In combined enzymatic treatment, there might be a better yield obtained when Alca was added initially then Flv (Alca-Flv), due to the modes of action for these enzymes. Initial cleavage of internal peptide bonds by Alca would leave more free ends for Flv's action. However, digestion with Flv alone was still superior (Figure 3.11), which could be influenced by the requirement for adjusting the pH multiple times and temperature for Alca treatment. When comparing 3% Alca with 3% Alca-Flv concurrently, GLN yield was higher in 3% Alca-Flv showing that addition of Flv did improve GLN yield but the pH change required for a digestion with Alca made this approach inferior to digestion with Flv individually. In conclusion, a 1% Flv digestion yielded the best results.

3.3.8 Time course of Flavourzyme activity

Figure 3.13 shows a significant increase of GLN from 2.1 mg/g to 27 mg/g through 22h treatment ($P<0.05$) while there was also a slight increase in the concentration of GLU from 2.6 mg/g to 4.3 mg/g. The increase trend indicates that the prolonged treatment of Flv were able to yield more GLU and GLN.

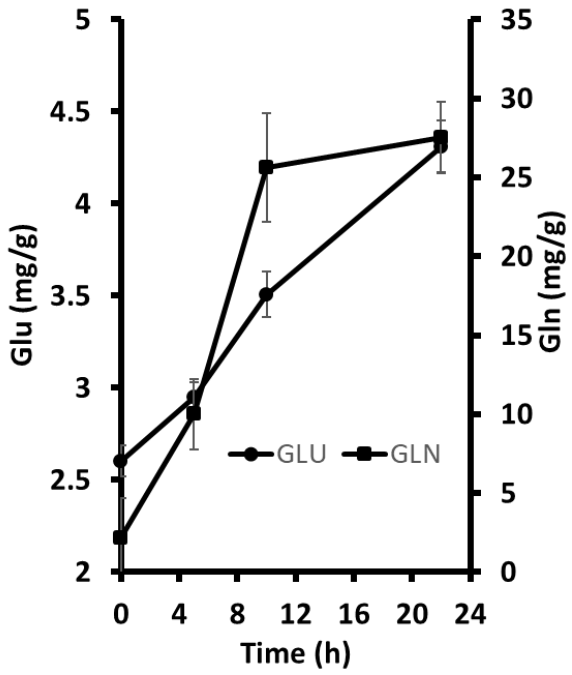


Figure 3.13 Average concentration of GLU and GLN with 1% Flavourzyme with time as a variable. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

3.3.9 Glutaminase digestion of GLN into GLU

GLN can be further convereted to umami flavour amino acid GLU and this process can be enhanced with Glutaminase treatment as shown in the Figure 3.14.

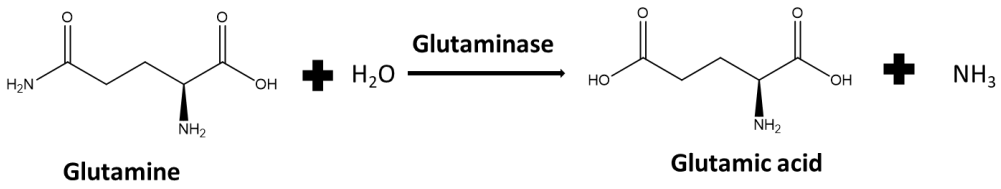


Figure 3.14 The chemical process of glutamine converted to glutamic acid

Glutaminase is an amidohydrolase enzyme which generates GLU from Gln; this enzyme was added after 1% Flv for a 24h incubation. It was observed that there was a decrease in GLN concentrations as Glutaminase concentrations increased (Figure 3.16), however, conversion of GLN into GLU was poor, as a 2×10^{-4} mol loss in GLN only resulted in a 1×10^{-5} mol increase in GLU, indicating that low activity of glutaminase or the degradation of the GLN instead of its' conversion to GLU. The GLN degradation may due to the heat treatment at high temperature (67 °C) and the Glutaminase treated at pH 6-7 may also have less stability during long-term treatment (Figure 3.15). Therefore, treating samples with glutaminase at lower temperature and pH may have better performance of converting GLN to GLU.

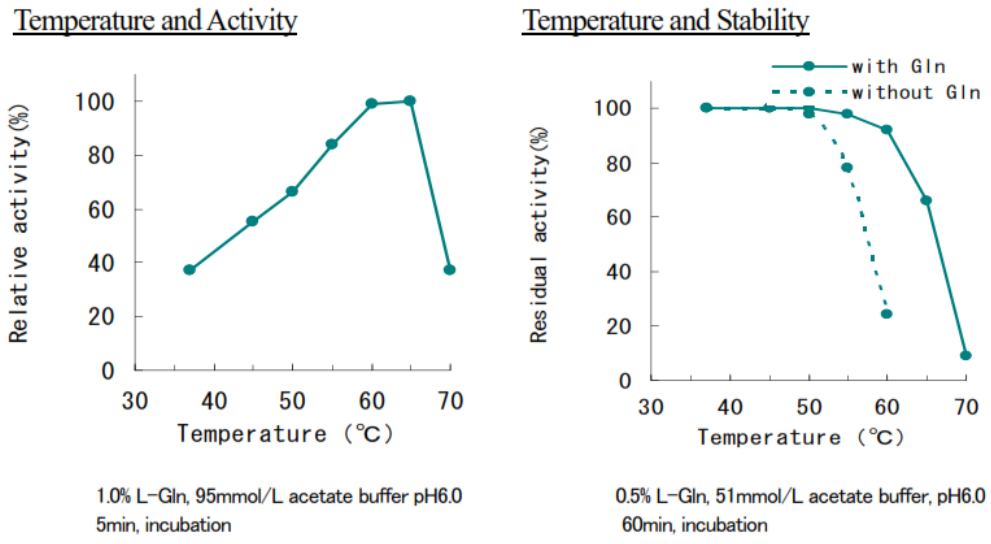


Figure 3.15 The enzyme performance characteristics results above were obtained by Amano Enzyme Europe Ltd for the Glutaminase SD-C100S

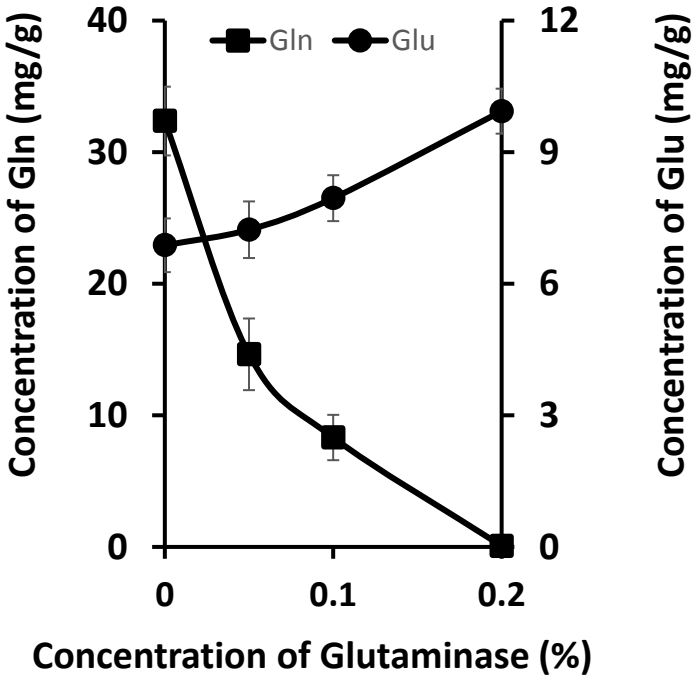


Figure 3.16 Average concentration of GLU and GLN with Glutaminase at different concentration for 24h after 1% Flavourzyme for 24h pretreatment. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

3.4 CONCLUSION

Optimisation of generation of umami components in the waste water sample was maximised using enzyme modifications and the best protocol has been determined for the production of the seasoning (Figure 3.1). There were two separate treatment streams for treating samples to enhance the umami flavour compound.

Stream A (Nuclease treatment):

1. YL-TL for releasing intercellular content
2. RP-1G for digesting RNA into nucleotides
3. Deamizyme for converting 5'-AMP to 5'-IMP

Stream B (Peptidase treatment)

1. Flavourzyme for digesting protein/peptide in the sample into amino acid
2. Glutaminase for converting Gln into Glu

Table 3.1 Optimised condition for individual enzyme digestion

Enzyme	Concentration (%)	Time (hours)	Conditions
Flavourzyme	1	24	pH 6.0, 50°C
Glutaminase	0.2	24	pH 6.0-7.0, 60°C
YL-TL	2	2	pH 5.0, 70°C
RP-1G	0.05	2	pH 8.0, 60°C
Deamizyme	0.05	8	pH 5.6, 55°C

However, future work may make the process more efficient and the efficiency of the process will depend on different food matrix, it is therefore critical that the enzymic process is evaluated on a solid matrix to evaluate the role of different RNA, protein profiles.

Further investigations will be required to optimise the umami components within the sample. pH and temperature modifications could be conducted concurrently since pH and temperature work synergistically. Temperature could also be a factor for further investigated since RNA degradation is temperature-dependent (Burka, 1969), which indicates that proper heating alone might disrupts the structure of RNA in order to release nucleotides even without enzyme. Moreover, the enzymic treatment time and dosage are also need to be further investigated based on matrix. Therefore, a response surface experimental model of these factors was proposed to be tested on buffalo worms (*Alphitobius diaperinus*).

4. THE LIBERATION OF UMAMI COMPOUNDS FROM *ALPHITOBIUS DIAPERINUS* FOLLOWING ENZYMATIC DIGESTION

4.1 INTRODUCTION

4.1.1 The value of *Alphitobius diaperinus* as umami flavour enhancer

In this study, the food grade insects *Alphitobius diaperinus* were provided by Snow Pony Ltd in Derby, UK. As can be seen from the table below of nutritional value and cost of various freeze-dried insects. The buffalo worm (*Alphitobius diaperinus)* has a relatively high content of protein while the price is much lower compared to crickets and locusts (Crunchycritters.com, 2017). The final decision was made to select the buffalo worms (*Alphitobius diaperinus)* as study object due to the low-fat content and high protein value of *Alphitobius diaperinus* as ultimately any final seasoning will have to be commercially viable.

Table 4.1 Price and nutrition value of various freeze-dried insects provided by Snow Pony Ltd

	Buffalo worms	Mealworms	Cricket
Price (£ per kg)	70	75	175
Energy (kcal/100g)	484	550	458
Fat (g/100g product) of which	24.7	37.2	18.5
Saturated fatty acids	8	9	7
Mono-unsaturated fatty acids	9.5	17.3	5.3
Poly-unsaturated fatty acids	7	10.9	6.4
Carbohydrates (g/100g product) of which	6.7	5.4	0.7
Sugars (g/100g product)	0	0	0
Fibre (g/100g product)	5.2	6.5	7.7
Protein (g/100g product)	56.2	45.1	69.1
Salt (g/100g product)	0.38	0.37	1.03

4.1.2 Hydrolysis approach to yield umami property compound from food-grade *Alphitobius diaperinus*

In this study, enzymatic hydrolysis was used to digest protein, DNA and RNA to generate umami compounds. In order to optimise the enzyme performance, several D-optimal designed experiments were carried using a response surface approach with Design-Expert software.

4.2 EXPERIMENTAL HYPOTHESES

The umami compound will be enhanced (umami active amino acid and nucleotide) by using different types of enzymes treating the *Alphitobius diaperinus*. With response surface modelling design for the enzyme treatment (pH, time, dosage, temperature), umami compound will be effectively released from *Alphitobius diaperinus* at optimum treatment condition.

4.3 RESULT

4.3.1 Amino acid profiling of *Alphitobius diaperinus* treated with various peptidase

Several peptidases listed below were used for digesting the protein in order to yield more amino acid to enhance flavour.

1. mixed peptidase containing endopeptidase and peptidase:

- Enzyme ProteAX by Amano Enzyme Europe Ltd (AX)
- Enzyme Protease A2SD by Amano Enzyme Europe Ltd (A2SD)
- Enzyme Flavourzyme by Novozyme Ltd (Flv)

2. Endopeptidase:

- Enzyme Alcalase 2.4 L FG by Alcalase Ltd (Alca)

3. Glutaminase:

- Enzyme Glutaminase SD-C100S by Amano Enzyme Europe Ltd (Glt)

4.3.1.1 The amino acid profile of Alphitobius diaperinus treated with peptidases

Freeze dried *Alphitobius diaperinus* was ground to a powder and suspended in reverse osmosis water, treated with three different peptidases AX, A2SD, Flv, respectively at 50˚C, pH 7 for 24 h in order to see their impact on amino acid profile.

The samples without enzymatic treatment had total amino acids content of 16 mg/g compared to with 0.55 mg/g GLU and 0.44 mg/g GLN.

As can be seen from the table below (Table 4.2), the total amount of amino acid increased substantially following treatment with three different peptidases. There are 8.7, 7.37 and 11.86-fold increase in total amino acid content with A2SD, AX and Flv compared to the control. For the amino acids with highest umami properties, glutamic acid (GLU), a 16, 12.5 and 26.4-fold increase was found in samples treated with A2SD, AX and Flv while for glutamine (GLN), 12.9, 12.15 and 19.4-fold increase was found in samples treated with A2SD, AX and Flv compared to the control. Therefore, when using these mixed peptidases,

Flv can yield the highest amount of total amino acid and the umami property amino acids GLU and GLN.

It is also worth pointing out that there was an significant increased level ($P<0.05$) of the bitterness amino acid (PHE, LEU, ILE, VAL and MET) apart from TYR found in the samples after the peptidase treatment, indicating the strong bitterness might be perceived along with the enhanced umami flavour. Since these amino acids all have hydrophobic side chain and the hydrophobic character plays the most significant role in creating a perception of bitter taste (Ishibashi et al., 1988), stimulating the human bitter receptors hTAS2Rs (Maehashi et al., 2008).

Table 4.2 Average amino acid concentration (mg/g solid content) for control and treated sample (A2SD, AX, Flv). Each value is the mean of three replicate measurements. The average of coefficient of variance (CV= standard deviation ÷mean) for all amino acid is 0.34.

		Control	1% A2SD	1%AX	1%Flv
4-Hydroxyproline	4HYP	0.03	0.04	0.03	0.06
α-Aminoadipic acid	AAA	0.01	0.02	0.01	0.02
α-Aminobutyric acid	ABA	0.0054	0.0055	0.0043	0.0078
Alanine	ALA	0.24	6.31	5.87	7.86
Asparagine	ASN	0.07	5.42	5.07	12.31
Aspartic acid	ASP	0.03	3.29	3.13	3.83
Cystine	C-C	Not Found (NF)	3.32	1.87	14.07
Glutamine	GLN	0.44	5.63	5.31	8.47
Glutamic acid	GLU	0.55	8.72	6.84	14.01
Glycine	GLY	0.12	3.17	2.6	3.9
Histidine	HIS	2.87	8.66	7.84	12.81
Isoleucine	ILE	0.37	5.65	5.28	6.57
Leucine	LEU	0.33	8.33	7.64	9.13
Lysine	LYS	1.3	8.66	8.27	10.99
Methionine	MET	NF	1.15	1.02	0.92
Ornithine	ORN	0.4	9.8	4.65	7.89
Phenylalanine	PHE	0.34	9.03	8.22	9.85
Proline	PRO	4.18	5.58	4.64	7.26
Serine	SER	0.15	11.22	8.59	18.71
Threonine	THR	0.03	8.51	8.13	11.32
Tryptophan	TRP	0.95	2.03	2.2	1.4
Tyrosine	TYR	2.63	9.18	8.26	12.57
Valine	VAL	1.03	6.88	5.72	10.16
	Total	16.07	130.59	111.19	184.12

4.3.1.2 The amino acid profile of Alphitobius diaperinus treated with peptidase and additional endopeptidase Alca

The endopeptidase Alcalase (Alca) was further added with peptidases to observe if additional amino acid could be generated.

Freeze dried *Alphitobius diaperinus* was ground into a powder and dissolved into reverse osmosis water, treated with three different peptidases AX, A2SD, Flv, respectively with the addition of endopeptidase Alca at 50˚C at pH 7 for 24 h in order to see their performance yielding amino acid.

By comparing Table 4.2 and Table 4.3, the addition of endopeptidase Alca yielded different amino acid profiles based on three peptidase SD, AX and Flv. The samples treated with both endopeptidase Alca and peptidase AX yielded more amino acid in total (1.4-fold increase) compared to samples treated only with AX peptidase. There were significant increases ($P<0.05$) in certain amino acid treated with AX and Alca such as glycine, lysine and cystine which is increased by 2.3, 3.4 and 2.6-fold, respectively compared to the one treated with AX alone. As for the umami property amino acid, GLU and GLN, there was an increase (1.5-fold) in GLU while a significant decrease (73%) in GLN ($P<0.05$).

Table 4.3 Average amino acid concentration (mg/g solid content) for control, peptidase and endopeptidase treated sample. Each value is the mean of three replicate measurements. The average of coefficient of variance (CV= standard deviation ÷ mean) for each amino acid is 0.28.

	2%Alca, 1%A2SD	2%Alca, 1%AX	2%Alca, 1%Flv
4HYP	0.02	0.03	0.03
AAA	0.02	0.02	0.02
ABA	0.0047	0.0065	0.0052
ALA	5.06	6.33	7.59
ASN	8.76	7.89	12.11
ASP	2.22	4.76	6.02
C-C	5.83	4.84	5.71
GLN	1.32	1.33	2.26
GLU	12.47	10.56	17.47
GLY	2.41	5.86	5.46
HIS	8.04	7.82	10.05
ILE	4.84	6.83	7.33
LEU	7.02	9.84	9.95
LYS	6.26	28.07	18.66
MET	0.60	1.45	1.40
ORN	7.39	8.70	9.03
PHE	6.77	11.75	12.23
PRO	5.09	4.43	4.92
SER	14.54	8.67	16.24
THR	8.18	8.52	14.04
TRP	1.08	3.27	2.92
TYR	8.41	9.40	12.76
VAL	6.95	6.70	7.76
Total	123.26	157.08	183.95

Samples treated with Alca and other two peptidase SD or Flv did not yield any increase in total amino acid compared to the sample without the addition of endopeptidase Alca, but still had significantly increase compared to the control

one ($P<0.05$). In terms of umami amino acid GLU, with the addition of endopeptidase based on three different peptidases A2SD, AX, Flv, all of them had increase in GLU concentration by 1.4, 1.5 and 1.25-fold, respectively.

By looking at the concentration of GLU and GLN among these treated samples, the combination of peptidase Flv and endopeptidase Alca yielded the most amount of GLU， although there was some loss in GLN with the addition of endopeptidase Alca. However, the increase of GLU (31-fold) concentration compared to the control sample exceeds the lost amount of GLN even by assuming the further enzymatic conversation rate from GLN to GLU is 100%.

Therefore, for the purpose of generating the most umami flavour amino acids to make the most umami activity out of the samples, peptidase Flv and endopeptidase Alca was finally selected.

4.3.1.3 The conversion of GLN to GLU of Alphitobius diaperinus treated with glutaminase (Glt)

The purpose of Glt used after peptidase and endopeptidase is to convert GLN to umami flavour amino acid GLU. 0.1% dosage of Glt was applied based on *Alphitobius diaperinus* solid content at 50°C, pH 7 for 24 hours. Figure 4.1 shows that after Glt treatment, the concentration of GLN decreases by 46%. However, there is no increase in Glutamic acid detected accordingly. The possible reason for that might be the decreased concentration of GLN was relatively small (1.2mg/g) compared to total concentration of GLU 17.5 mg/g in the sample treated with Alca and Flv. The small amount of GLN-to-GLU conversion has small impact on the increase in GLU concentration. Therefore, adding Glt appears to have no significant effect.

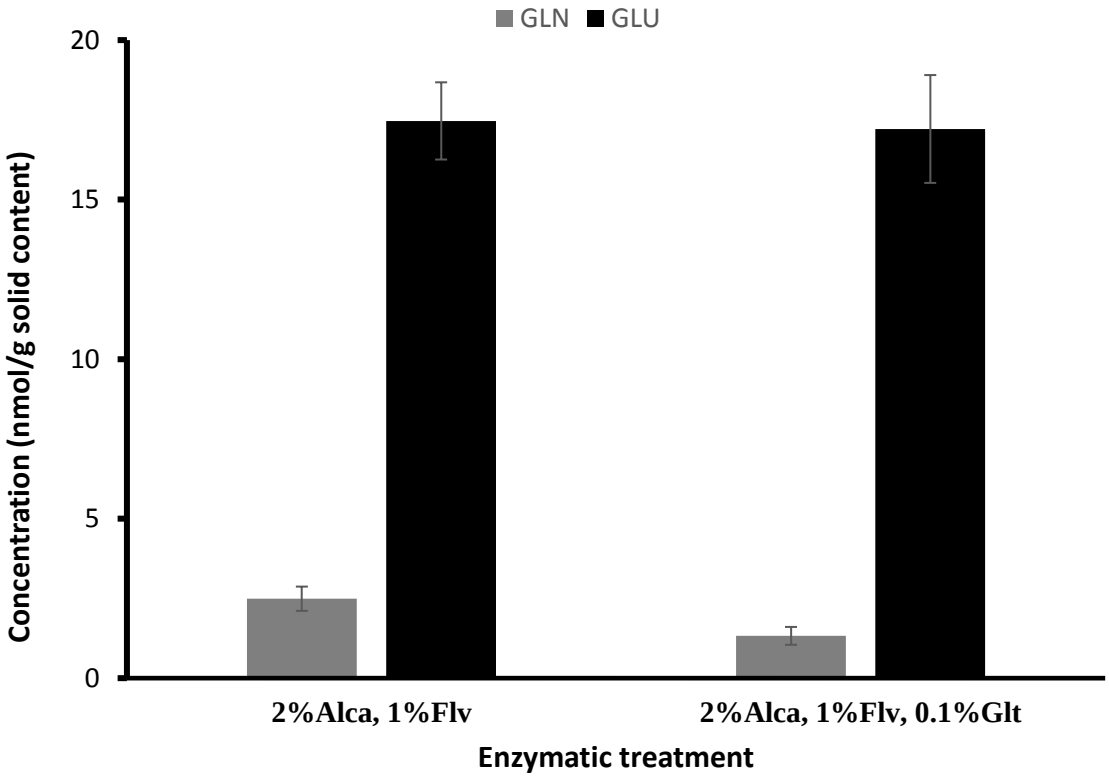


Figure 4.1 Average amino acid concentration (mg/g solid content) for samples treated before and after with Glt. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

4.3.1.4 Amino acid profile of Alphitobius diaperinus treated with peptidase and endopeptidase with time as a variable

After determining the effective endopeptidase and peptidase for liberation of amino acid, the treatment time was further optimised. Ground freeze dried *Alphitobius diaperinus* was treated with peptidase (1% Flv) and endopeptidase (2% Alca) for 0.5, 1, 2, 5, 12 and 24 h to see the total amino acid profile.

The prolonged treatment at 50°C made a slight decrease in total amino acid concentration as observed in controlled samples without any enzymatic treatment. (Table 4.4)

Table 4.4 Average amino acid concentration (mg/g solid content) for control samples with time as variable. Each value is the mean of three replicate measurements. The average of coefficient of variance (CV=standard deviation ÷mean) for all amino acid is 0.23

	Control 0.5h	Control 1h	Control 2h	Control 5h	Control 12h	Control 24h
4HYP	0.02	0.02	0.03	0.02	0.02	0.02
AAA	0.02	0.02	0.02	0.02	0.02	0.02
ABA	NF	NF	NF	NF	NF	NF
ALA	0.34	0.39	0.54	0.47	0.4	0.19
ASN	0.31	0.31	0.31	0.21	0.13	0.08
ASP	0.07	0.08	0.11	0.08	0.07	0.01
C-C	NF	NF	NF	NF	NF	NF
GLN	3.6	3.7	3.37	3.5	3.01	0.57
GLU	2.11	2.09	1.78	2.07	1.77	0.51
GLY	0.25	0.29	0.21	0.23	0.25	0.19
HIS	2.93	2.8	2.14	2.49	2.75	2.28
ILE	0.58	0.58	0.53	0.53	0.56	0.38
LEU	0.39	0.39	0.36	0.35	0.39	0.34
LYS	0.43	0.59	0.88	0.72	0.72	0.6
MET	0.01	0.01	0.01	0	0	0.01
ORN	0.01	0.01	0.02	0.02	0.02	1.07
PHE	0.52	0.53	0.5	0.44	0.58	0.52
PRO	6.2	6.26	5.37	6.03	6.15	5.81
SER	0.51	0.53	0.38	0.39	0.31	0.1
THR	0.17	0.18	0.15	0.15	0.14	0.03
TRP	1.25	1.42	1.61	1.25	1.59	1.29
TYR	1.46	1.75	1.95	1.72	2	1.9
VAL	0.9	0.93	0.98	0.96	0.91	0.63
Total	22.09	22.88	21.26	21.65	21.81	16.56

As can be seen from the Table 4.5, with the increase of the time up to 24 h, the total amino acid concentration increased significantly from 128.8 mg/g treated

for 0.5 h to 242.5 mg/g for 24 h ($P<0.05$). For the GLU, the concentration increased by 2.51-fold treated for 24 h compared to the 0.5 sample (Figure 4.2).

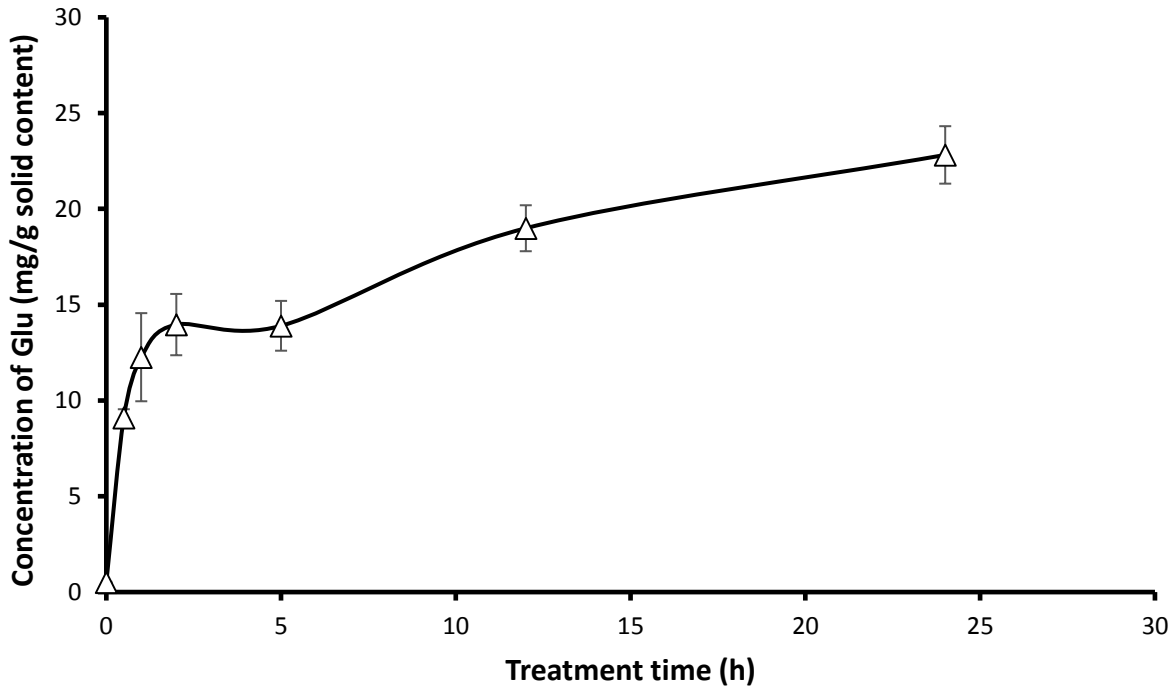


Figure 4.2 Average GLU concentration (mg/g solid content) after peptidase (1% Flv) and endopeptidase (2% Alca) treated sample with time over 24h. Each value is the mean of three replicate measurements. The error bars show the standard deviation.

Table 4.5 Average amino acid concentration (mg/g solid content) for control, peptidase (1% Flv) and endopeptidase (2% Alca) treated sample with time as variable. Each value is the mean of three replicate measurements. All coefficient of variance (CV= standard deviation ÷ mean) for each amino acid is less than 1

	Control 24h	Treated 0.5h	Treated 1h	Treated 2h	Treated 5h	Treated 12h	Treated 24h
4HYP	0.02	0.02	0.02	0.02	0.03	0.04	0.03
AAA	0.02	0.03	0.04	0.04	0.04	0.04	0.05
ABA	NF	0.01	0.01	0.01	0.01	0.01	0.01
ALA	0.19	3.92	6.08	6.59	7.52	9.18	10.02
ASN	0.08	7.69	11.67	12.94	13.03	14.29	16.66
ASP	0.01	2.73	5.32	6.09	6.94	8.52	9.66
C-C	NF	0.52	1.11	1.44	1.67	2.01	2.53
GLN	0.57	8.54	9.05	8.69	5.25	2.57	1.22
GLU	0.51	9.09	12.26	13.96	13.9	18.99	22.82
GLY	0.19	2.27	3.99	4.36	4.5	4.75	6.1
HIS	2.28	7.87	11.63	11.95	11.31	10.9	14.41
ILE	0.38	6.13	7.97	8.52	7.76	8.08	9.83
LEU	0.34	16.49	21.05	22.17	21.47	22.43	26.19
LYS	0.6	9.46	12.1	11.51	12.05	13.16	16.31
MET	0.01	1.47	2.56	2.21	2.83	2.31	3.36
ORN	1.07	0.02	0.09	0.1	0.08	0.12	0.15
PHE	0.52	8.98	12.08	13.08	12.46	12.91	15.89
PRO	5.81	6.31	7.11	7.51	7.67	7.44	9.13
SER	0.1	6.43	10.37	10.92	9.7	10.97	14.71
THR	0.03	8.53	16.2	17.98	16.12	17.65	21.83
TRP	1.29	3.5	3.94	3.51	4.46	3.87	5.75
TYR	1.9	10	13.78	15.59	15.08	17.17	20.42
VAL	0.63	8.77	12.34	13.04	12.31	12.96	15.42
Total	16.56	128.79	180.79	192.22	186.17	200.38	242.50

In conclusion, peptidase Flv can yield the most amino acid compared to other peptidases. By adding the peptidase (1% Flv) and endopeptidase (2% Alca) together can lead to a further increase in concentration of amino acid in samples.

The umami flavour amino acid GLU concentration had a significant increase by 44.7-fold compared to the control one ($P<0.05$). With prolonged treatment time up to 24 h, total amount of amino acid rises with the increase of treatment time. Therefore, this result suggests that with the peptidase treatment *Alphitobius diaperinus* can potentially yield more umami flavour.

4.3.1.5 Specificity of peptidases (A2SD, Flv, AX and Alca) on amino acid profiles

Although enzyme ProteAX (AX), Protease A 2SD (A2SD) provided by Amano and Flavourzyme (Flv) from Novozyme are all produced from *Aspergillus oryzae* fermentation, these are different peptidases that have unique EC (Enzyme Commission) number for their nomenclature.

According to the MSDS information of enzymes provided by companies. A2SD (EC 3.4.24.27) belongs to the category of metalloendopeptidases which specifically is a thermolysin peptidase that has preferential cleavage of leucine (LEU) and phenylalanine (PHE) (Rawlings and Salvesen, 2012). Metallopeptidase are among the hydrolases in which the nucleophilic attack on a peptide bond is mediated by a water molecule. In the case of thermolysin, a divalent metal cation zinc activates the water molecule. The metal ion is held in place by amino acid ligands, usually three in number (Rawlings and Salvesen, 2012).

AX and Flv (EC 3.4.11.1) are in the category of aminopeptidase also known as leucyl aminopeptidase which cleaves the N-terminal (P1) L-amino acid from a broad range of peptide substrate. It also has a cleavage preference for methionine or leucine at the N-terminal position (Rawlings and Salvesen, 2012).

Through personal communication with technical consultant of Amano and Novozyme, although enzyme A2SD's standardised activity is endopeptidase, it still contains significant levels of exopeptidase whereas AX and Flv are standardised on the exopeptidase, but contain a significant endopeptidase activity.

As seen from the Table 4.6, compared to the control samples without enzyme treatment, enzymatic treated samples with three different peptidases had significantly different amino acid profile ($P<0.05$).

All these three exopeptidase A2SD, AX and Flv have a main cleavage specificity for serine (SER) and threonine (THR). However, the proportion of cysteine (C-C), ornithine (ORN) and asparagine (ASN) are different among three peptidases' treatment. Samples treated with Flv had significantly more C-C ($P<0.05$), taking up 7.64% of the total amino acid profile compared to the sample treated with A2SD (2.54%) and AX (1.68%). Flv also produced a greater proportion of ASN (6.69 %) compared to A2SD (4.15%) and AX (4.56%). This indicates that the side activity of endopeptidase of enzyme Flv may have the preference for cleavage to liberate ASN and C-C. In the case of the amino acid ORN, there was considerably more in the samples treated with A2SD (7.5%) than AX (4.18%) and Flv (4.29%), suggesting that the side-activity of exopeptidase in A2SD might have specific cleavage leading to ORN. It also appears that although Flv and AX are the same type of aminopeptidase, they can still yield different amino acid profile due to the different production procedure of different companies.

Table 4.6 The percentage of amino acid of samples treated with various peptidases

Amino acid	Percentage			
	Control	**1% A2SD**	**1%AX**	**1%Flv**
4HYP	0.19	0.03	0.03	0.03
AAA	0.06	0.02	0.01	0.01
ABA	0.03	0.004	0.004	0.004
ALA	1.49	4.83	5.28	4.27
ASN	0.44	4.15	4.56	6.69
ASP	0.19	2.52	2.82	2.08
C-C	0.00	2.54	1.68	7.64
GLN	2.74	4.31	4.78	4.60
GLU	3.42	6.68	6.15	7.61
GLY	0.75	2.43	2.34	2.12
HIS	17.86	6.63	7.05	6.96
ILE	2.30	4.33	4.75	3.57
LEU	2.05	6.38	6.87	4.96
LYS	8.09	6.63	7.44	5.97
MET	0.00	0.88	0.92	0.50
ORN	2.49	7.50	4.18	4.29
PHE	2.12	6.91	7.39	5.35
PRO	26.01	4.27	4.17	3.94
SER	0.93	8.59	7.73	10.16
THR	0.19	6.52	7.31	6.15
TRP	5.91	1.55	1.98	0.76
TYR	16.37	7.03	7.43	6.83
VAL	6.41	5.27	5.14	5.52

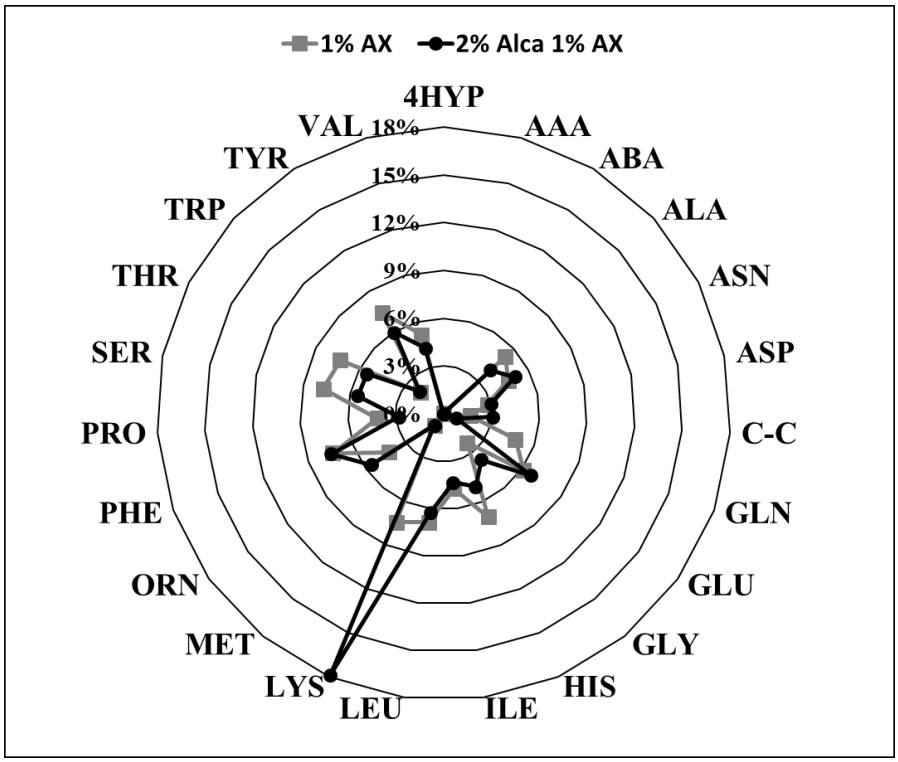
Alca (EC 3.4.21.62) is in the category of serine endopeptidases, also known as subtilisin produced from *Bacillus licheniformis* fermentation. It hydrolyses the protein with broad specificity for peptide bonds and has a preference for a large uncharged residue at the N-terminal. It has a catalytic triad in the order aspartic acid (ASP), histidine (HIS) and (SER) (Rawlings and Salvesen, 2012).

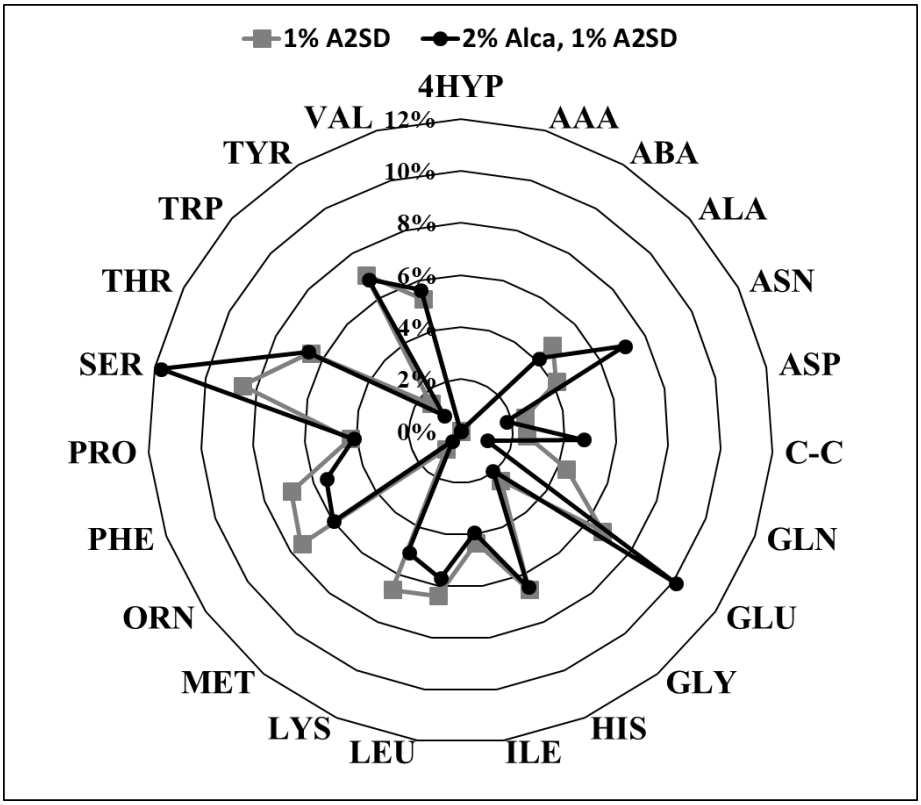
Table 4.7 shows the proportion of each amino acid in the samples treated with various peptidase and additional endopeptidase. In general, the secondary addition of endopeptidase Alca, changed the amino acid profile of the samples.

Table 4.7 The percentage of amino acid of samples treated with various peptidases and additional endopeptidase Alca

Amino acid	Percentage		
	2% Alca, 1% A2SD	**2% Alca, 1% AX**	**2% Alca, 1% Flv**
4HYP	0.02	0.02	0.02
AAA	0.02	0.01	0.01
ABA	0.004	0.004	0.003
ALA	4.11	4.03	4.13
ASN	7.11	5.02	6.58
ASP	1.80	3.03	3.27
C-C	4.73	3.08	3.10
GLN	1.07	0.85	1.23
GLU	10.12	6.72	9.50
GLY	1.96	3.73	2.97
HIS	6.52	4.98	5.46
ILE	3.93	4.35	3.98
LEU	5.70	6.26	5.41
LYS	5.08	17.87	10.14
MET	0.49	0.92	0.76
ORN	6.00	5.54	4.91
PHE	5.49	7.48	6.65
PRO	4.13	2.82	2.67
SER	11.80	5.52	8.83
THR	6.64	5.42	7.63
TRP	0.88	2.08	1.59
TYR	6.82	5.98	6.94
VAL	5.64	4.27	4.22

By comparing Table 4.6 and Table 4.7, the difference of amino acid profile among various enzymatic treatment can be illustrated through radar charts (Figure 4.5). There was significant increase of ASN, cysteine (C-C), GLU and SER with the addition of endopeptidase Alca compared to the amino acid profile treated with A2SD alone ($P<0.05$). In the case of AX and Flv with additional Alca treatment, LYS (Lysine) took the predominant proportion of amino acid profile instead of SER. the reason for the amino acid profile difference between A2SD + Alca treatment and AX + Alca might due to different predominant enzyme activity of AX (main exopeptidase activity) and A2SD (main endopeptidase activity). By comparing the amino acid profile of AX + Alca with Flv + Alca, although AX and Flv belong to the same category having exactly the same EC code. the considerable increased number of LYS proportion may due to the different endopeptidase specificity existed in the AX and Flv.


1% AX
2% Alca 1% AX
4HYP
AAA
ABA
ALA
ASN
ASP
C-C
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
ORN
PHE
PRO
SER
THR
TRP
TYR
VAL
18%
15%
12%
9%
6%
3%
0%


1% A2SD
2% Alca, 1% A2SD
4HYP
AAA
ABA
ALA
ASN
ASP
C-C
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
ORN
PHE
PRO
SER
THR
TRP
TYR
VAL
12%
10%
8%
6%
4%
2%
0%

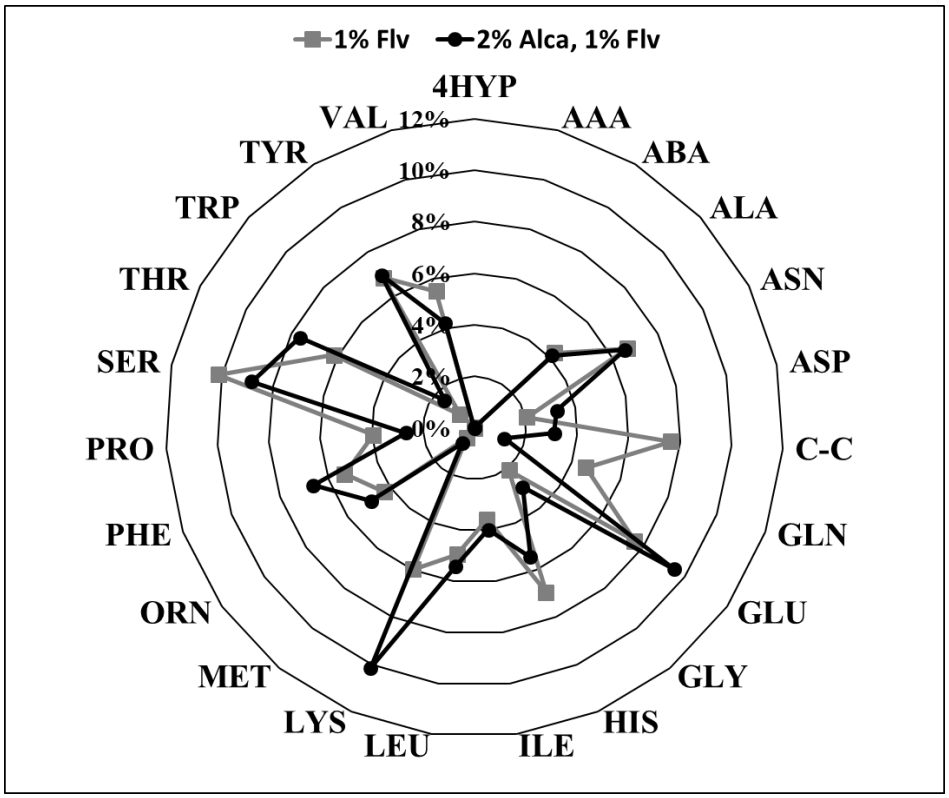


Figure 4.3 Radar charts illustrate the amino profile of samples treated with various peptidase and additional endopeptidase Alca.

4.3.2 Nucleotide profile of *Alphitobius diaperinus* after enzyme treatment

The total amount of free nucleotides present in the sample was evaluated first by heating the *Alphitobius diaperinus* in the water at 60°C for 10 h without enzymatic treatment. 6.1 mg/g of total nucleotide content was obtained, containing 1.7, 0.7, 0.6, 1 and 2.1 mg/g of UMP, CMP, GMP, IMP and AMP, respectively.

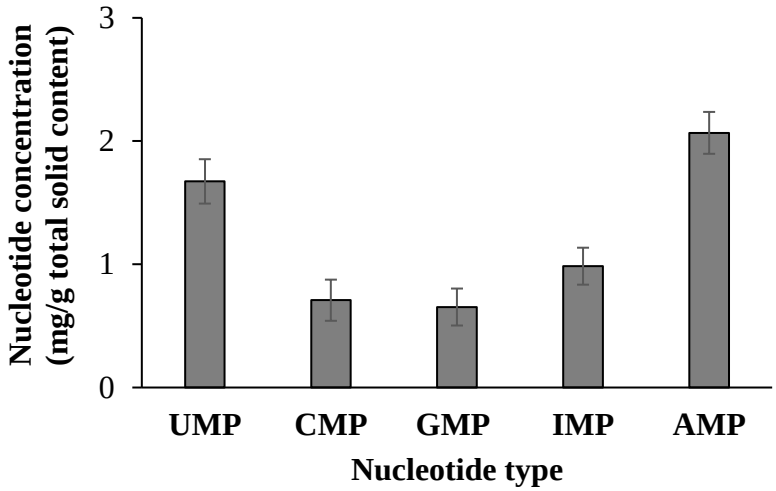


Figure 4.4 The nucleotide concentration of *Alphitobius diaperinus* after heating in the water at 60°C for 10 h. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

Then, there were two enzymes used for digesting DNA/RNA of *Alphitobius diaperinus* to yield nucleotides.

1. Enzyme RP-1G by Amano Enzyme Europe Ltd (RP-1G), a 5'-phosphodiesterase that can hydrolyse RNA/DNA to 5'-nucleotides.
2. Enzyme Deamizyme by Amano Enzyme Europe Ltd (Deamizyme), a 5'-Adenylic deaminase that converts 5'-Adenylic acid (AMP) into umami flavour nucleotide 5'-Inosinic acid (IMP).

Although Amano provides enzyme activity and thermo-stability data suggesting the potential optimum operating condition as shown in Figure 4.5 and Figure 4.6, the data provided is only in short-time treatment (15 or 30 min). The optima may vary for longer digestion. Therefore, a D-optimal designed experiment aims to determine the optimum treatment factors: treatment time, enzyme dosage, pH, temperature.

The pH-temperature D-optimal designed experiment was run first to decide the optimum pH and temperature point for best yield of umami property nucleotide (GMP, IMP, AMP). The time-dosage D-optimal designed experiment was then

performed using the optimum pH and temperature parameters determined previously.

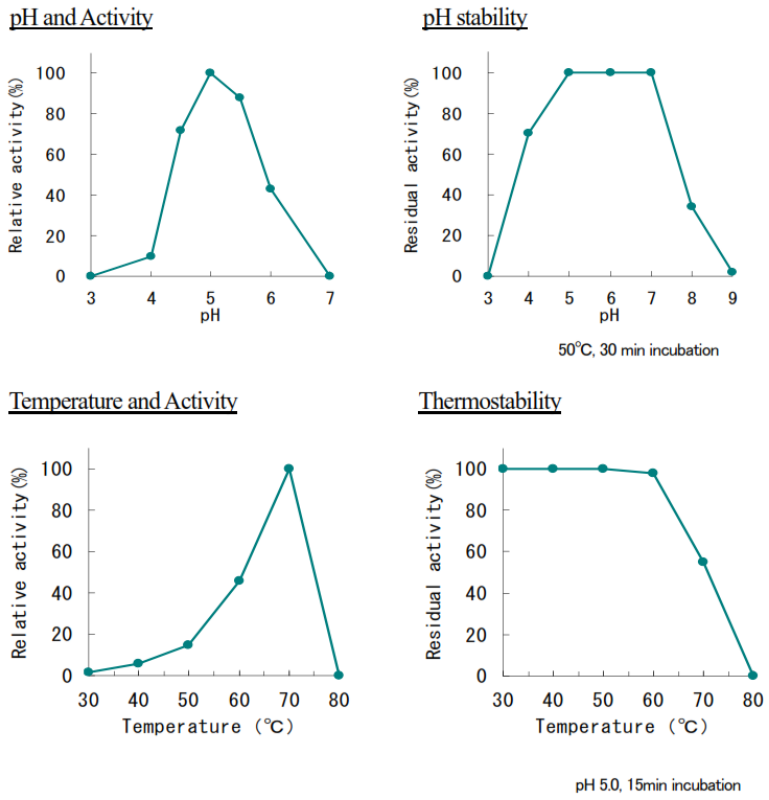


Figure 4.5 The enzyme performance characteristics results above were obtained by Amano Enzyme Europe Ltd for the RP-1G solution.

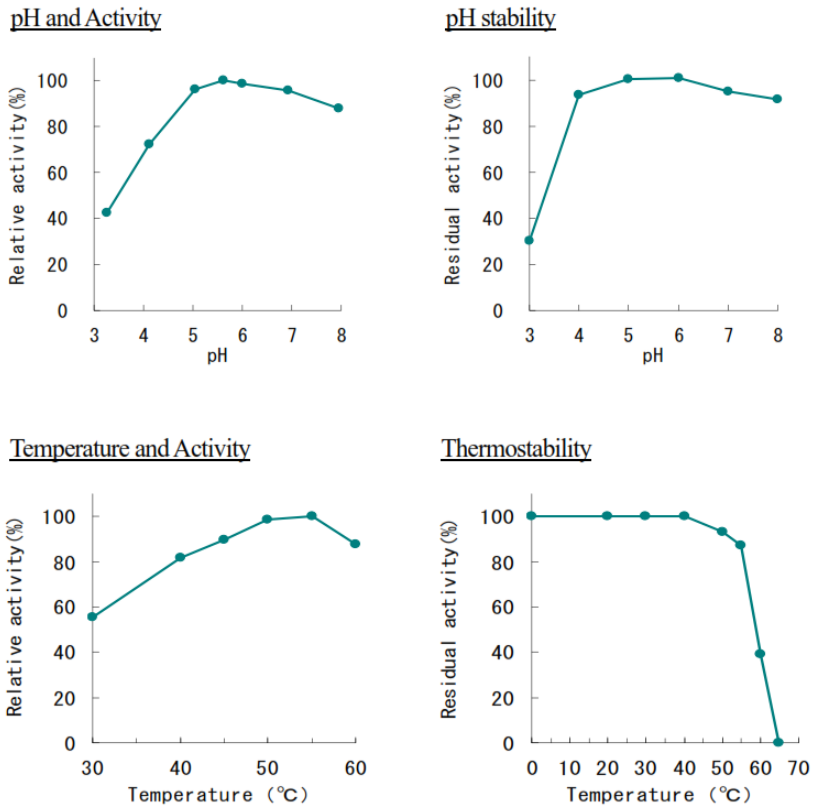


Figure 4.6 The enzyme performance characteristics results above were obtained by Amano Enzyme Europe Ltd for the Deamizyme solution in some buffers.

4.3.2.1 The buffer solution impact on nucleotide yield treated with enzyme RP-1G

For the pH-temperature D-optimal experiment, the pH (4, 4.5, 5, 5.5, 6, 6.5) and treatment temperature (40, 49, 50, 58, 62, 66, 75°C) were varied to produce 20 experimental design points with duplicates at the extremities and the edge of the temperature axis as shown in Figure 4.7. In this experiment, various buffer solutions were used to cover all pH range. Sodium citrate was used for pH 4, 4.5, 5.5 buffer solutions while sodium phosphate was used for pH 5, 6, 6.5. In Figure 4.7, design points are marked and the replicate design points are in bold font. Contours represent the concentration of nucleotide and each contour line links points with the same value on the plot. The significance of all the terms in the method was statistically evaluated and computed by the prob.>F at a confidence

level of <0.05. The model was recalculated based on backward deletion of terms that were insignificant.

Figure 4.7 shows that both pH and temperature has significantly effect on the response of these two nucleotides' concentration ($P<0.05$). Only AMP and IMP showed significant differences with pH and temperature ($P<0.05$). No significant difference was noted in other nucleotides such as UMP, GMP, CMP.

In general, the high temperature treatment at low pH could yield more AMP. Although RP-1G has no effect on IMP generation, the presence of IMP was also influenced by pH and temperature. Concentration of IMP decreased with the increase of pH and temperature (Figure 4.7).

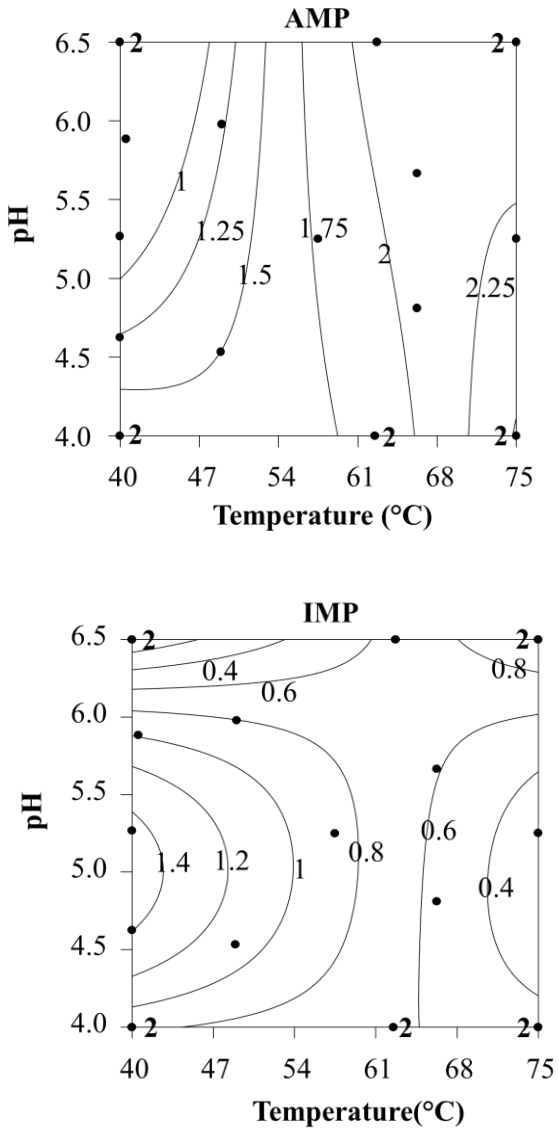


Figure 4.7 Two-dimensional contour plots derived from the generated models. Contours represent the concentration of nucleotide AMP and IMP (mg/g) as a function of the experimental factors –temperature (°C) and pH for a fixed treatment time (24h) and enzyme dosage (1% w/w).

The optimum temperature and pH was then determined based on maximum yield of AMP from the contour plot above. This was in the region of pH around 4.5 to 5 and 70°C to 75°C. These two experiment settings were used in two response surface experiments to further test dosage and time as experiment factor.

Figure 4.8 and Figure 4.9 show the GMP concentration varied through different time and dosage of RP-1G at two selected optimum pH-temperature conditions.

However, these two contour plots show two different responses. Figure 4.8 shows that GMP concentration was not influenced by the enzyme dosage and only had a slight increase through treatment time up to 20 h.

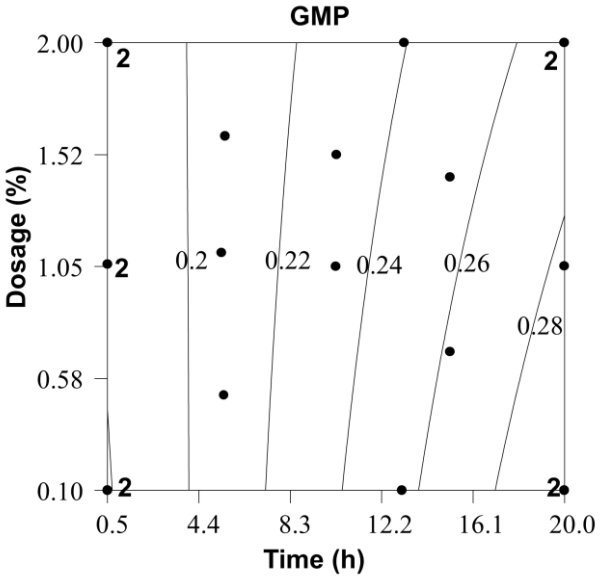


Figure 4.8 Two-dimensional contour plots derived from the generated models. Contours represent the concentration of nucleotide GMP (mg/g) as a function of the experimental factors – that is, the different time (h) and dosage (% w/w) for a fixed treatment pH (pH 4.5) and temperature (75 °C).

Figure 4.9 shows that the concentration of GMP was affected by both treatment time and enzyme dosage significantly ($P<0.05$), the concentration of GMP increased at higher enzyme dosager and longer treatment time. Since the pH and temperature points selected for these two plots are very close with 0.5 value difference in pH (pH 4.5 and 5) and 5°C difference in temperature (70 and 75 °C). They are expected to represent the similar trend in the time-dosage contour plots.

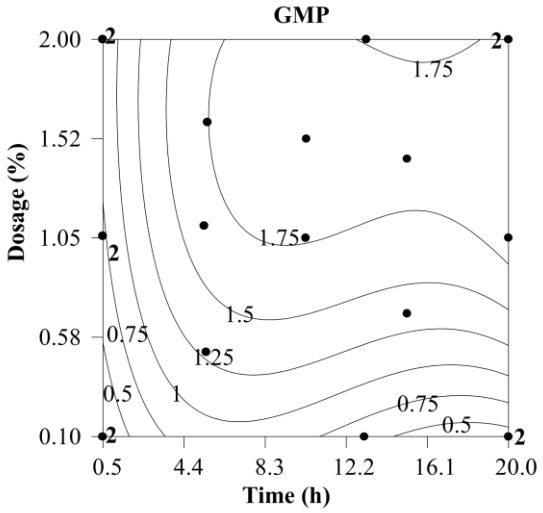


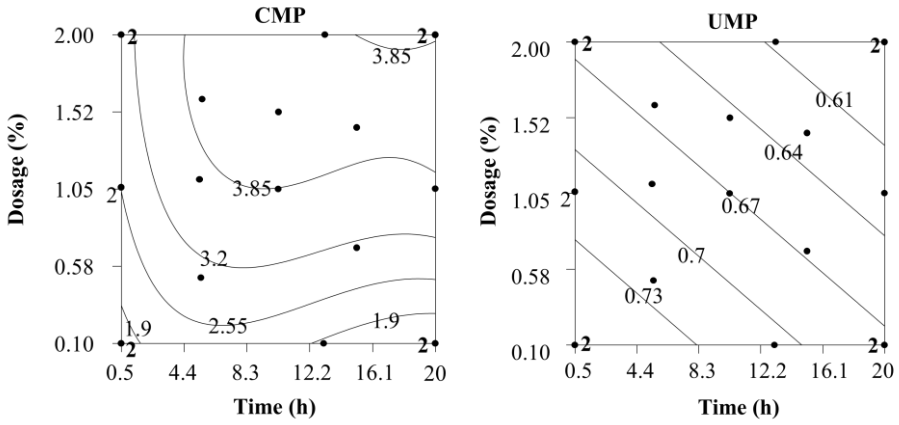
Figure 4.9 Two-dimensional contour plots derived from the generated models. Contours represent the concentration of nucleotide GMP (mg/g) as a function of the experimental factors – that is, the different time (h) and dosage (% w/w) for a fixed pH (pH 5) and temperature (70°C).

First pH-temperature response surface experiment showed that only AMP and IMP changed in responses to the enzyme treatment (Figure 4.7) while other nucleotides did not. Then, two optima points were selected for further time-dosage response surface experiment based on best yield of AMP from the result shown in Figure 4.7. However, the result of these two optima points are very different as can be seen in Figure 4.8 and Figure 4.9. One optima point was treated with citrate buffer and the other was treated with phosphate. Looking back at the detail of the data points from Figure 4.7, the data of points treated with citrate buffer differ from those with phosphate buffer. This was what was causing problem with previous analysis of pH-temperature response surface experiment. From Figure 4.8, the samples treated with RP-1G in sodium citrate buffer solution did not show any significant response of nucleotide concentration regarding factors such as pH, temperature, time and enzyme dosage while those suspended in sodium phosphate and sodium acetate both showed a significant activity. This indicates the cation citrate inhibited the RP-1G activity. As most

of the single-strand-specific nucleases are either metalloenzymes or metal requiring enzymes. Nuclease P_1 RP-1G from *Penicillium citrinum* is a zinc metalloprotein (Desai and Shankar, 2003). Citrate as a metal chelator directly inhibited RP-1G activity. Hence, knowing the inhibitors of enzymes must be taken into the consideration as one of the crucial criteria for identifying enzymes performance.

4.3.2.2 The optimum time and dosage for RP-1G treatment for best yield of umami property nucleotide

After the problem of buffer solution has been identified, the time-dosage response surface experiment treated with sodium phosphate buffer was selected for determining the optimum time and dosage.



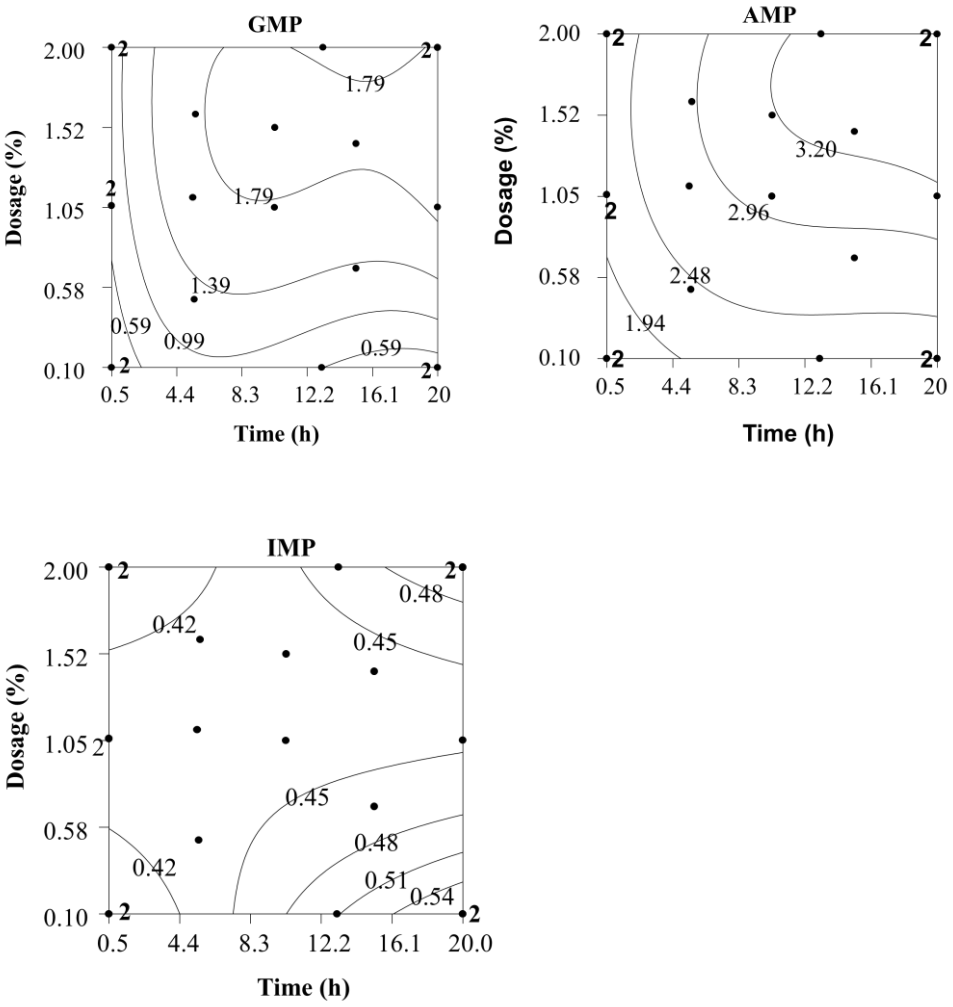


Figure 4.10 Two-dimensional contour plots derived from the generated models. Contours represent the concentration of five types of nucleotide UMP, CMP, GMP, AMP, IMP (mg/g) as a function of the experimental factors –time (h) and enzyme dosage for a fixed time (24h) and enzyme dosage (1% w/w).

As can be seen from the Figure 4.10, the factor time and dosage also have a significant effect on the yield of nucleotide ($P<0.05$) apart from UMP. There are interactions observed between pH and temperature that affect the yield of nucleotides. The optimum condition was then determined based on the prediction of maximized yield of GMP (1.97 mg/g) and AMP (3.41 mg/g).

Table 4.8 presents the specific optimum conditions for the defined concentration of certain nucleotides. The goal is on optimum conditions (time and enzyme dosage) to obtain a maximum concentration of AMP and GMP. The additional

parameters called importance and weight were further imposed on the responses. Importance parameter is designed for setting priorities to the responses. If all importance ratings are the same, then it has no impact. In this case. The weight parameter can be added to emphasize upper and lower bounds, affecting the shapes of the ramps and it gives more or less emphasis to the goal. When the weight = 1, the desirability goes up and down on linear ramp.

Table 4.8 Desirability specifications of numerical optimisation for D-optimal design

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
Time (h)	is in range	0.50	20.00	1	1	3
Dosage (% w/w)	is in range	0.10	2.00	1	1	3
UMP (mg/g)	is in range	0.56	0.86	1	1	3
CMP (mg/g)	is in range	1.30	4.13	1	1	3
GMP (mg/g)	maximize	0.24	1.89	1	1	3
IMP (mg/g)	is in range	0.38	0.65	1	1	3
AMP (mg/g)	maximize	1.43	3.41	1	1	3

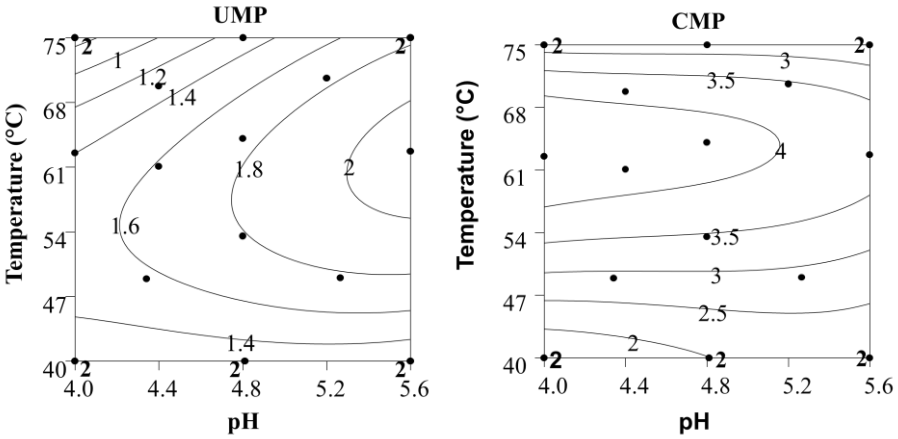
Table 4.9 Optimisation conditions, prediction and desirability of RP-1G model

		Concentration of nucleotide (mg/g)					
Time	Dosage Selection	UMP	CMP	GMP	IMP	AMP	Desirability
20 h Selected	2.0%	0.59	3.99	1.97	0.48	3.41	1
12 h	1.6%	0.61	4.12	1.88	0.44	3.24	0.9

Table 4.9 shows that all goals are joined into on desirability function which are based on various responses and factors. Thus, the optimum time (20 h) and dosage (2%) was determined to give maximum yield concentration of AMP, GMP. This was slightly higher than expected for samples treated with 1.6% dosage RP-1G for 12 h with yields of 1.88mg/g GMP and 3.24mg/g AMP.

4.3.2.3 The optimum pH and temperature for RP-1G treatment for best yield of umami property nucleotides with sodium acetate – acetic acid buffer solution

Sodium phosphate buffer solution can only cover the pH range from 5-8, therefore, sodium acetate buffer solution was used to cover the pH range from pH 4 to pH 5.6 in this experiment. Figure 4.11 shows contour plots of five nucleotides, in which there are significant interactions between factors (pH and temperature) affecting the nucleotide concentration (P<0.05).



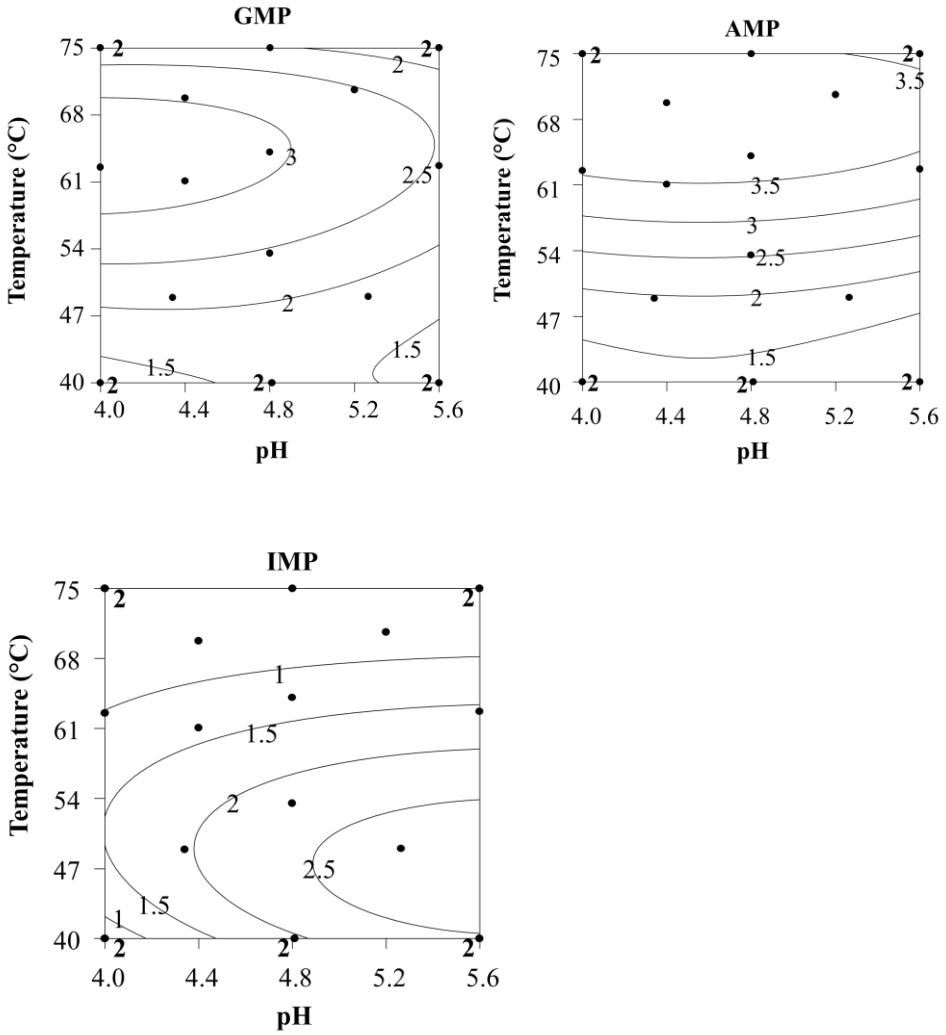


Figure 4.11 Two-dimensional contour plots derived from the generated models. Contours represent the concentration of five types of nucleotide UMP, CMP, GMP, AMP, IMP (mg/g) as a function of the experimental factors –temperature (°C) and pH for a fixed treatment pH (pH 5) and temperature (70°C).

By using software Design expert 10, the numerical optimisation finds a point that maximizes the desirability function.

Table 4.10 presents the specific optimum conditions for the defined concentration of certain nucleotides. The goal is at optimum conditions (pH and temperature) to obtain maximum concentration of AMP and GMP.

Table 4.10 Desirability specifications of numerical optimisation for D-optimal design

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
pH	is in range	4.00	5.60	1	1	3
Temperature (°C)	is in range	40	75.00	1	1	3
UMP (mg/g)	is in range	0.05	1.92	1	1	3
CMP (mg/g)	is in range	0.02	5.05	1	1	3
IMP (mg/g)	is in range	0.41	3.26	1	1	3
AMP (mg/g)	maximize	0.0	4.51	1	1	3
GMP (mg/g)	maximize	0.0	4.02	1	1	3

Table 4.11 Optimisation conditions, prediction and desirability of model

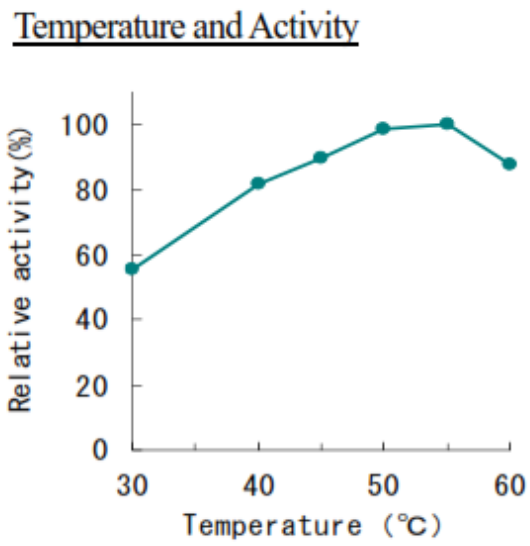
		Concentration of nucleotide (mg/g)					
pH	Temperature Selection	UMP	CMP	GMP	IMP	AMP	Desirability
4.0	65.52°C	1.35	4.20	3.21	0.88	3.89	0.83
Selected							

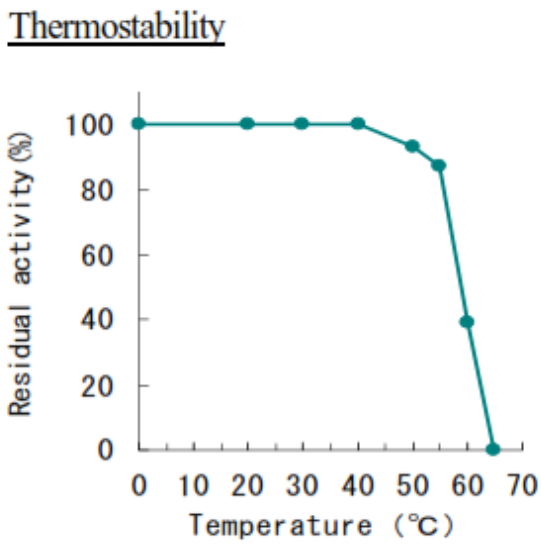
Table 4.11 shows that all goals are joined into on desirability function which are based on various responses and factors. Thus, the optimum pH (pH 4) and temperature (65°C) were predicted in the condition of maximum yield of AMP, GMP.

4.3.2.4 The optimum time and dosage for Deamizyme treatment for best yield of AMP converted from IMP.

Deamizyme is a 5-Adenylic deaminase which converts 5'-AMP acid into flavour enhancer 5'-IMP. The purpose of this study is to find the optimum treatment condition for Deamizyme for the conversion from AMP to IMP.

pH 5.6 is the optimum value provided on the datasheet by Amano Enzyme Europe Ltd (Figure 4.12) for treatment while the optimum pH for RP-1G treatment is pH4. It is important to see the real difference between those two pH conditions in terms of conversion rate from AMP to IMP so that the need for further pH adjustment can be determined.





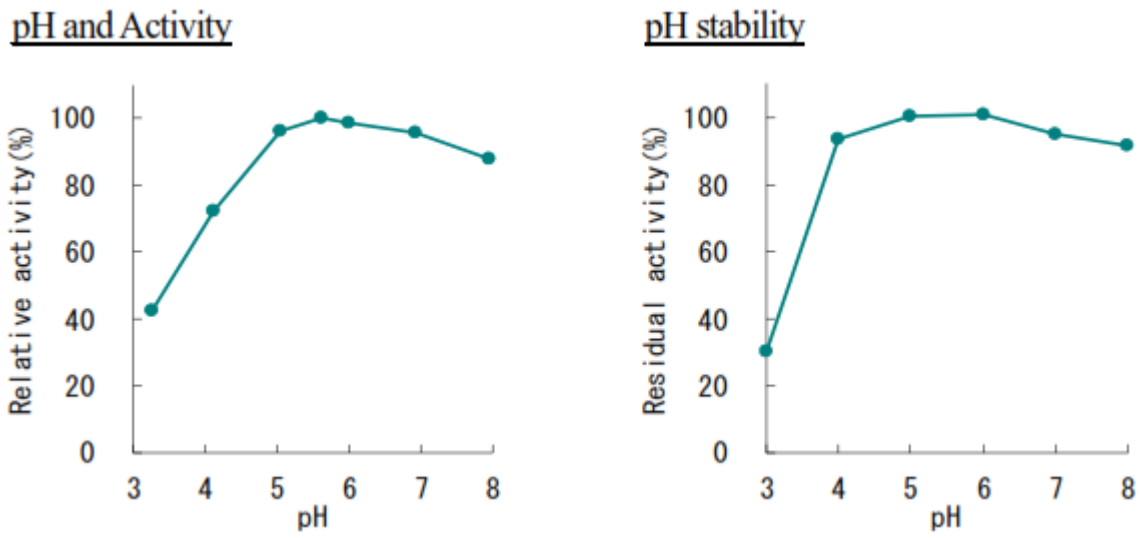


Figure 4.12 The enzyme performance characteristics results above were obtained by Amano Enzyme Europe Ltd for the Deamizyme solution in some buffers.

Figure 4.13 shows that the conversion rate from AMP to IMP is higher at pH 4 than pH 5.6 ($P<0.05$) which is different from the optimum pH value suggested by Amano. Therefore, sodium acetate solution at pH 4 would be used for both RP-1G and Deamizyme treatment.

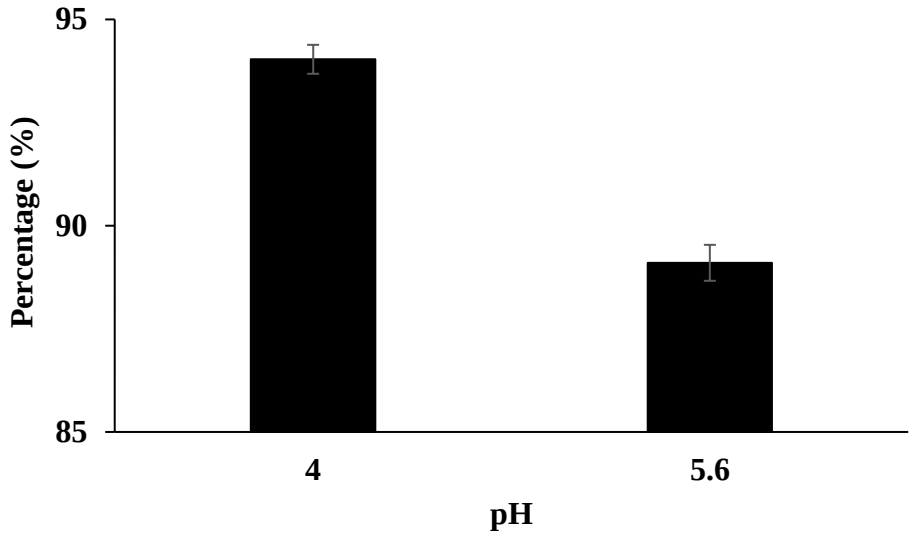


Figure 4.13 Conversation rate comparison between pH 4 and pH 5.6 under 2% RP-1G treatment of Alphitobius diaperinus at pH 4 for 10 h followed by 0.1% Deamizyme for 24 h ($P<0.05$). Sodium acetate solution was used as treating buffer.

After the treatment pH was decided, three factors (time, temperature, dosage) were modelled in design-expert cubic response surface model to estimate the

possible optimum condition. 30 points are scattered for building the model throughout time, temperature and dosage (Figure 4.14).

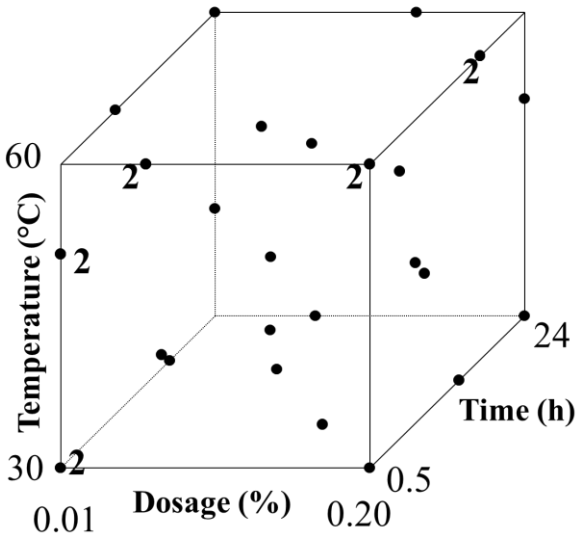


Figure 4.14 Graphical representation of the sample space to model the influence of Deamizyme dosage, treatment temperature and time on the yield of nucleotide concentration.

Figure 4.15 shows that factor temperature interacted with Deamizyme dosage significantly affecting the conversion of AMP to IMP ($P<0.05$). Design expert gave the prediction based on best yield of IMP in the model, a 0.2% dosage of Deamizyme at 60˚C for 23 h. This can convert the maximum amount of AMP to IMP (4.8 mg/g solid content). Alternatively, 0.02% dosage at temperature 30 ˚C for 16 h can reach the same IMP yield.

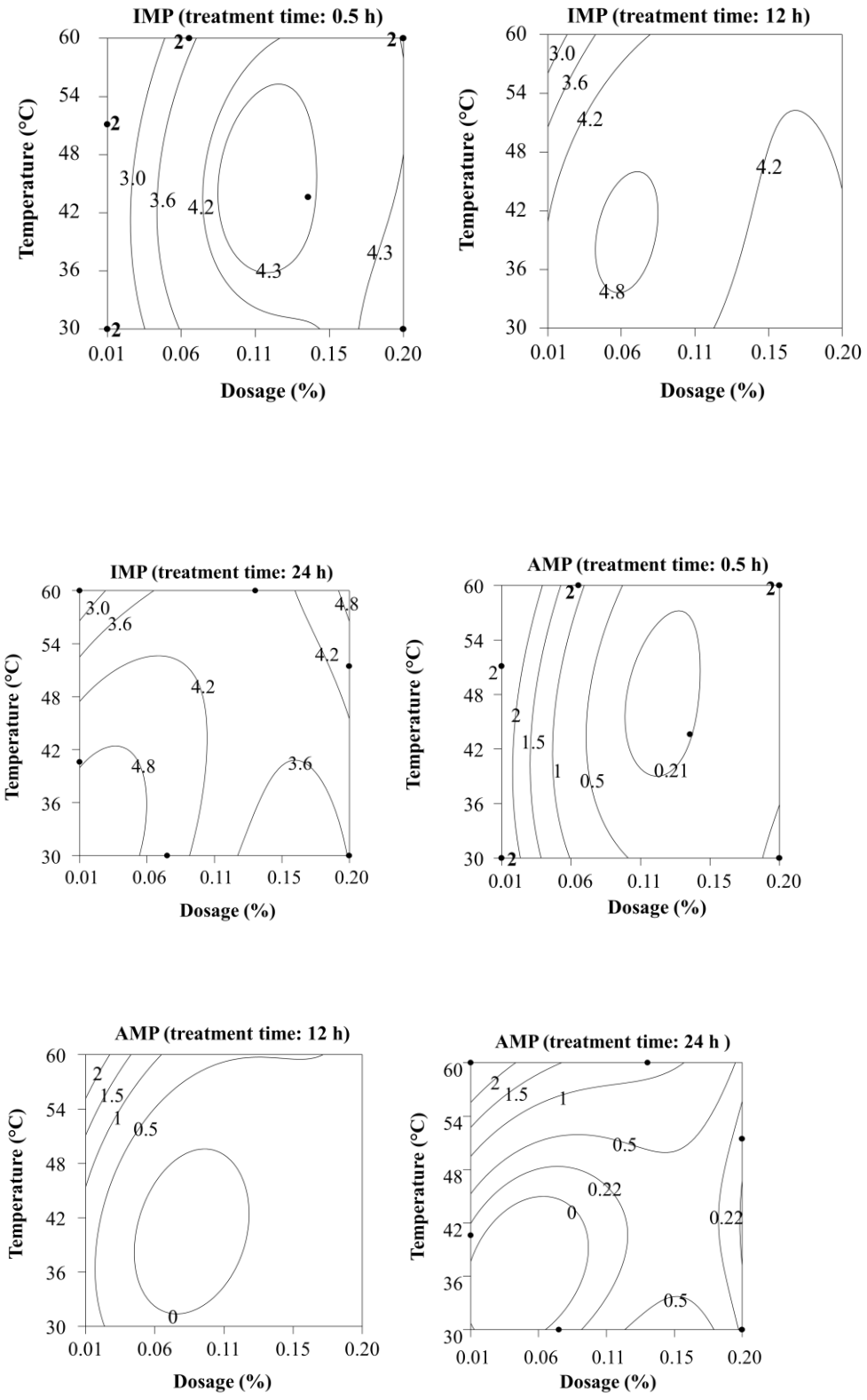


Figure 4.15 Two-dimensional contour plots derived from the generated models. Contours represent the concentration of five types of nucleotide AMP, IMP (mg/g) as a function of the experimental factors – time (h), enzyme dosage (%), and temperature (°C) for a fixed pH (pH 4).

A series of enzymatic treatments were then carried out based on the determined optimum conditions for RP-1G and Deamziyme, which is 2% dosage of RP-1G based on total solid content at pH 4, 65˚C for 20 h followed by the treatment of 0.2% dosage of Deamizyme based on total solid content at pH 4, 60˚C for 24 h. For the comparison, the control sample had the same condition of pH, temperature, treatment time apart from the addition of enzymes. The final result can be seen in Figure 4.16. Umami flavour nucleotide GMP and IMP increased significantly by 3.5-fold and 7.8-fold ($P<0.05$), respectively.

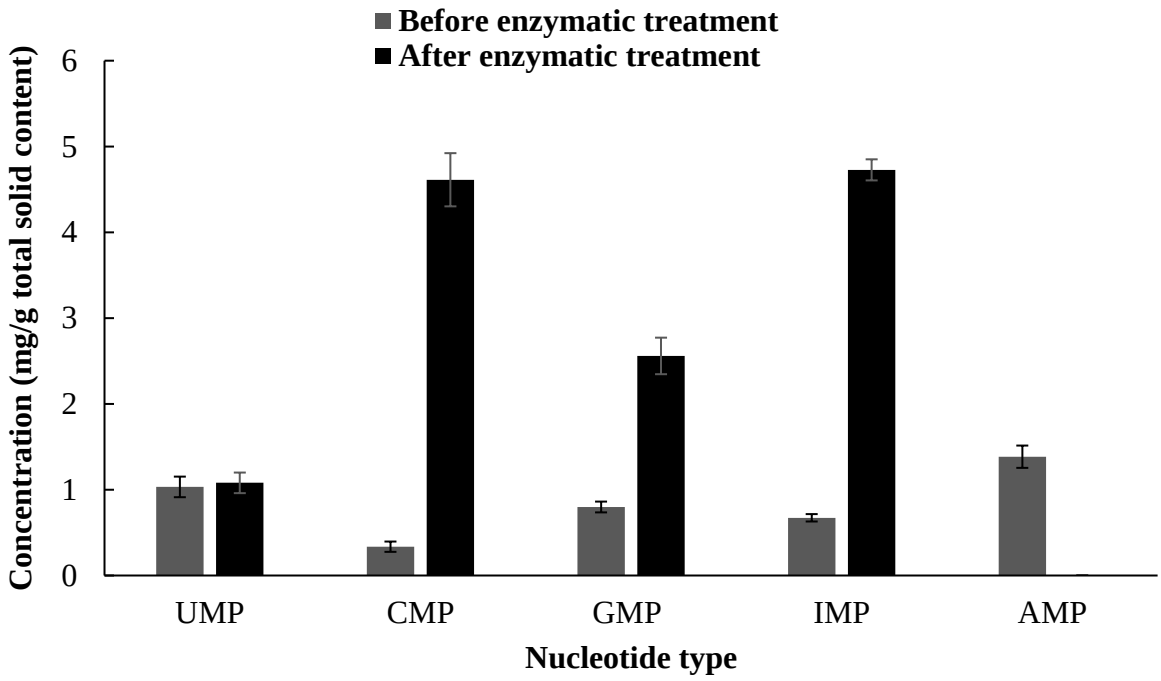


Figure 4.16 The comparison of nucleotide concentration with and without enzymatic treatment of 2% RP-1G and 0.2% Deamizyme. Each value is the mean of three replicate measurements. The error bars show the standard deviation error.

4.4 CONCLUSION

The results suggest that the umami flavour of *Alphitobius diaperinus* can be liberated resulting in a considerable increase of umami flavour amino acid (GLU)

and nucleotides (GMP, IMP). This can be achieved having enzymatic treatment at appropriate treatment condition (pH, enzyme dosage, temperature and time).

5. FURTHER DISCUSSION

5.1 INTRODUCTION

In this chapter, the mechanism of some enzymes based on the results of chapter 3 and 4 will be discussed in detail, to evaluate the influential factors for the liberation and enhancement of taste active amino acids and nucleotides.

Before the further discussion, here is the review of the key findings in chapter three and chapter four.

1. There is significant increase ($P<0.05$) in the yield of umami compound of *Fusarium venenatum* fermented water and *Alphitobius diaperinus* treated with nuclease and peptidase.

- In the *Fusarium venenatum* study, a 4.8-fold increase was observed in the concentration of GLU while the 20mg/g yield of IMP was generated from none in non-enzymatically treated samples.
- In the *Alphitobius diaperinus* study, there was a 44.7, 3.5 and 7.8-fold increase in the concentration of GLU, GMP and IMP, respectively.

2. In the Alphitobius diaperinus study, different amino acid profiles were generated in the samples treated with endopeptidases and exopeptidases.

3. The nucleotide yield of samples treated with nuclease was inhibited by the buffer solution sodium citrate

4. In the *Alphitobius diaperinus* study, D-optimal response surface model shows the multi-factor interaction between pH, temperature, time and enzyme dosage in terms of nucleotide yield, which demonstrates the capability of the model to

find the best enzymatic treatment condition for *Alphitobius diaperinus* as food matrix.

5.2 WHY PEPTIDASE AND NUCLEASE CANNOT BE TREATED TOGETHER

In this whole research, enzyme digestions by peptidase and nuclease were always being treated separately and cannot be mixed together. This is due to the presence of phosphatases, also known as phosphoric monoester hydrolase enzymes, which are able to hydrolyse the terminal phosphoryl group of nucleotides to cause the formation of free inorganic phosphates and nucleosides (Nagodawithana and Reed, 2013). The phosphatase is present in almost all tissues and microorganisms (Nagodawithana and Reed, 2013). For instance, the enzymes used in the study such as 5'-phosphodiesterase RP-1G and 5'-Adenylic deaminase Deamizyme contain such phosphatases.

Phosphatases can be categorized into two groups: non-specific phosphatases and specific phosphatases. As the term implies, the enzymes included in the first category are known to hydrolyse all phosphomonesters without distinguishing between the groups esterifying the phosphoryl residue (Nagodawithana and Reed, 2013) while the other specific ones are being very specific in the nature of the substituent on the phosphoryl group during hydrolysis (Enzyme nomenclature. Recommendations (1984) of the nomenclature committee of the international union of biochemistry, 1985). Among this series of enzymes, nucleosidases are considered destructive to the processing of the flavour-enhancing nucleotides, the best known of which are 5'-nucleotidases that are characterized by the position at which the phosphate group is hydrolysed on the respective nucleotides. For instance, the 5'-nucleotidases are known for their

activity in splitting flavour-enhancing 5'-nucleotides to their corresponding nucleosides and orthophosphates, causing a decline in the flavour-enhancing ability of the final product (Figure 5.1).

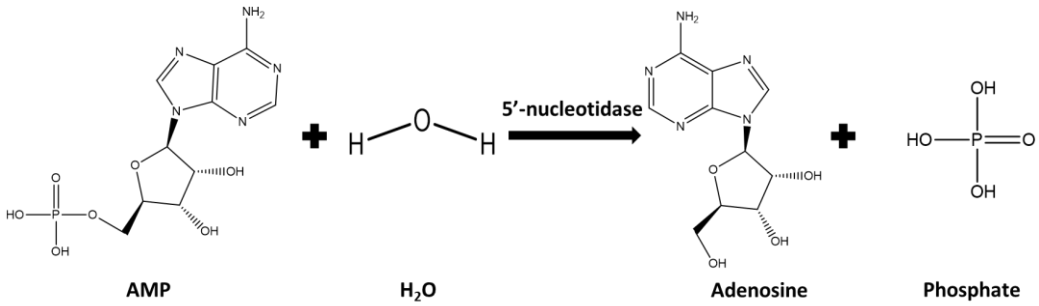


Figure 5.1 The 5'-nucleotidase catalysed reaction of an AMP nucleotide to adenosine nucleoside and phosphate

These nonspecific phosphatases and nucleotidases are distributed widely in bacteria, fungi, potatoes and gramineous seeds. In this study, nucleases being used were RP-1G and Deamizyme, and are produced from the fermentation of *Penicillium citrinum* and *Aspergillus melleus,* while exopeptidases such as AX, A2SD, Flv and endopeptidase Alca are produced from the *Aspergillus oryzae* and *Bacillus licheniformis* fermentation, which all belong to the fungi kingdom.

Apart from phosphatase, enzymes called nucleosidases can also destroy desirable flavour-enhancing nucleotides formed in the enzymatic treatment. In detail, the nucleosidase has the ability to hydrolyse N-glycosyl compounds thereby liberating free bases as a result of the hydrolysis of the β-glycosidic bond. In general, these reactions involve single step conversion, for instance, the conversion of 5'-IMP to hypoxanthine and D-ribose-5'-phosphate in the presence of adenosine nucleosidase (Nagodawithana and Reed, 2013).

$$5'\text{-IMP} + H_2O \xrightarrow[\text{nucleosidase}]{\text{Inosinate}} \text{hypoxanthine} + \text{D-ribose-phosphate}$$

These enzymes are likely to be present in high concentrations in many of the materials employed in the production of 5'-phosphodiesterase enzymes (RP-1G) used in flavour-nucleotide production such as *Penicillium citrinum.*

Therefore, the precaution must be taken to eliminate or minimise such competing enzymes. For peptidases being used in the study, the temperature optima are around 45 to 50°C, the secondary activity of phosphatase at these temperature associated with peptidases would potentially dephosphorylate nucleotides, if treatments were carried out together with nucleases. In the case of samples treated with nucleases such as RP-1G and deamizyme, the temperature optima however can reach up to 70°C at which the phosphatase and nucleosidase can be inactive.

5.3 MECHANISM OF ENZYMES TREATED IN THE STUDY

5.3.1 Nuclease P_1 (RP-1G)

The commercial nuclease enzyme RP-1G used in the study that produce 5'-phosphomonoester compounds as a result of its activity on RNA or DNA is derived from microbial origin – *Penicillium citrinum,* which also belong to nuclease P_1 category. This enzyme shows a preference for cleaving phosphodiester bonds in single-strand fragments of nucleic acids yielding 5'-nucleotides. For instance, in the case of dinucleotide monophosphates, X-Y, this nuclease showed a higher preference when X is adenosine or deoxyadenosine (Nagodawithana and Reed, 2013). Its base preference may depend on X rather than Y when the nuclease acts on substrates that are typically of X-Y type. In order to recognize the substrate, the critical structure such as nucleoside 3-phosphate portion is required and the RP-1G preferential breakdown the di-ester

bond taking place between the 3'-hydroxyl group of adenosine/deoxy-adenosine and the 5'-phosphoryl group of the succeeding nucleotide in the chain (Nagodawithana and Reed, 2013). This can indicate the high yield of 5'-AMP nucleotide after RP-1G treatment.

Although RP-1G can be placed among the endonuclease family, it is classified as nuclease P_1 as it also has exo-nucleolytic activity, which can simultaneously generate 5'-mononucleotides (Fujimoto et al., 1974). It can attack a variety of nucleic acids regardless of the type of sugar or base, the size of molecule, or the presence or absence of the terminal phosphate group in the nucleic acid chain. Therefore, nuclease P_1 like RP-1G is one of the most useful and ideal enzymes available for the commercial production of 5'-nucleotide rich flavour compound from substrates rich in RNA, DNA.

Moreover, the frequency of appearance of each mononucleotide from RNA/DNA is also different.

RNA is shown to be: AMP > GMP > UMP > CMP, while the rate for denatured DNA is:

dAMP > dTMP > dCMP > dGMP. According to the information of molar ratios of 5'-nucleotides released during partial hydrolysis of RNA, AMP, GMP, CMP and UMP accounts for 37.7%, 24.6%, 17.1% and 20.6%, respectively (Fujimoto et al., 1974).

However, from the result of chapter three and four, the increased amount of nucleotide generated after being treated with RP-1G does not match the ratio provided above by Fujimoto (Table 5.1). In the case of *Fusarium venenatum fermented* water study, since nucleotides were already present in the samples,

the impact of nuclease RP-1G on releasing certain nucleotides such as UMP, GMP and AMP was not remarkable compared to CMP. This may due to the fermentation processing that had already hydrolysed the RNA already into considerable amount of nucleotides. Although the concentration of GMP increased with the addition of YL-TL, as YL-TL released more intercellular contents as substrates for RP-1G to digest. However, the amount of GMP decreased with the increasing time during enzymatic treatment.

Table 5.1 Concentration of increased nucleotide of different samples compared to non-enzymatic ones

	Changes in concentrations of nucleotide (mg/g)			
Sample type	**UMP**	**CMP**	**GMP**	**AMP**
Alphitobius diaperinus	0.05	4.3	1.8	2.67
Fusarium venenatum water	-4.4	27.5	-4.5	2.5

For *Alphitobius diaperinus,* apart from UMP, other nucleotides' concentration (CMP, GMP and AMP) had a significant increase compared to the ones without enzymatic treatment ($P<0.05$). The reason for no increased concentration of UMP may due to the poor UMP liberation performance by RP-1G.

5.3.2 Yeast-lytic enzyme (YL-TL)

Enzyme YL-TL is a yeast lytic enzyme from *Bacillus stearothermophilus*. It was therefore only used in the study of *Fusarium venenatum*, since *Fusarium venenatum* and yeast both belong to the fungi kingdom. It is assumed that the effect of YL-TL may apply only on *Fusarium venenatum* but not *Alphitobius diaperinus* which does not have cell walls.

5.3.2.1 The cell wall of fungi

The cell wall of fungi is a highly dynamic structure that is responsible for protecting the cell from rapid changes in external osmotic potential. The cell wall is composed mostly mannoprotein and fibrous β (1→3) glucans with branches of β (1→6) glucans (Figure 5.2). The glucans are essential components, responsible for the mechanical strength, shape and elasticity of the cell well. The major constituent of the inner wall is β (1→3) glucan-chitin complex that forms the fibrous scaffold of the wall. On the outer surface of the wall are the mannoproteins, which are densely packed and limit wall permeability to solutes. β (1→6) glucan links the components of the inner and outer walls. All these components are covalently linked to form macromolecular complexes that are assembled in unit modules, each built around a molecule of β (1→3) glucan. A minority of modules has chitin, a β (1→4) linked polymer of N-acetylglucosamine, chains attached to β (1→3) or β (1→6) glucan. The modules are associated by noncovalent interactions in the glucan-chitin layer and by covalent cross-links in the mannoprotiein layer, including disulfide bond between mannoproteins.

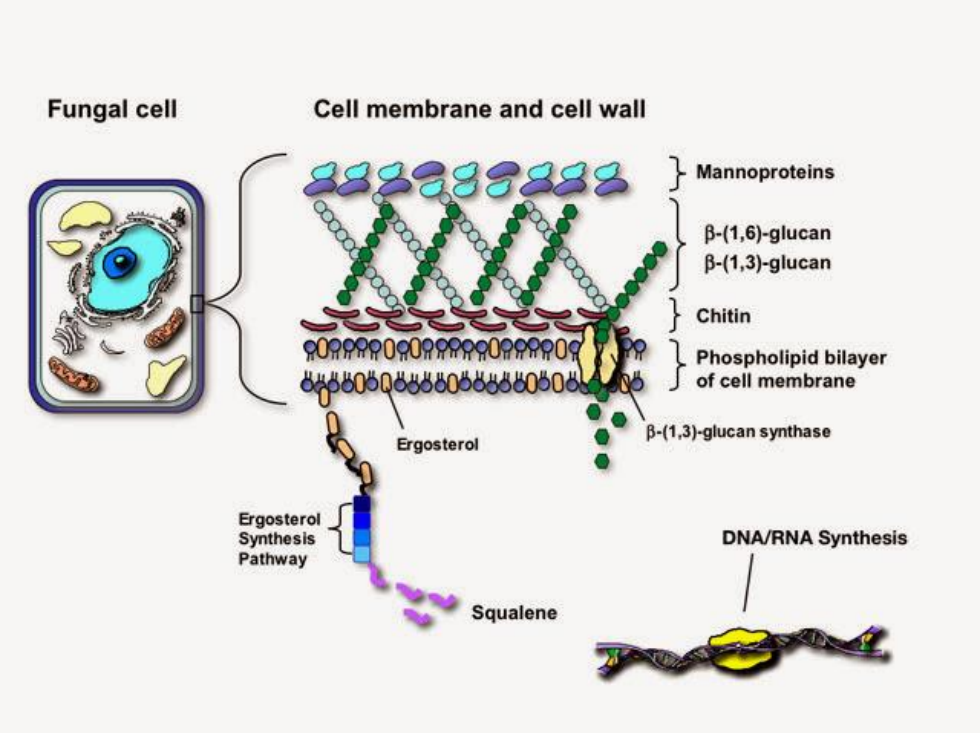


Figure 5.2 The illustration of fungi cell wall structure (Mejia, Mejia and profile, 2017)

5.3.2.2 Lysing action mechanism

Enzyme systems for yeast cell lysis are usually a mixture of several different enzymes such as β (1→3) glucanase (lytic and non-lytic), peptidase, β (1→6) glucanase, mannanase and chitinase, which act synergistically for lysing the cell wall.

The enzymatic lysis of fungi begins with binding of the lytic peptidase to the outer manoprotein layer of the wall. Afterwards, the protein structure is opened up by the peptidase, releasing wall proteins and mannans and exposing the glucan surface. The glucanase then attacks the inner wall and solubilises the glucan. When the combined action of the peptidase and glucanase has opened a sufficiently large hole in the cell wall, the plasma membrane and its content are extruded as protoplast while intercellular contents such as DNA, RNA and proteins are released from the wall.

5.3.3 Nucleodeaminase (Deamizyme)

In the study of *Alphitobius diaperinus* and *Fusarium venenatum* water, all of the 5'-AMP generated has been fully converted to 5'-IMP with Deamizyme treatment. Nucleodeaminases preferentially act on C-N bonds other than peptide bonds in cyclic amidines, liberating free ammonia by deamination. This reaction can occur in compounds that contain purine or pyrimidine bases. In a typical reaction, adenine deaminase converts adenine to hypoxanthine and ammonia

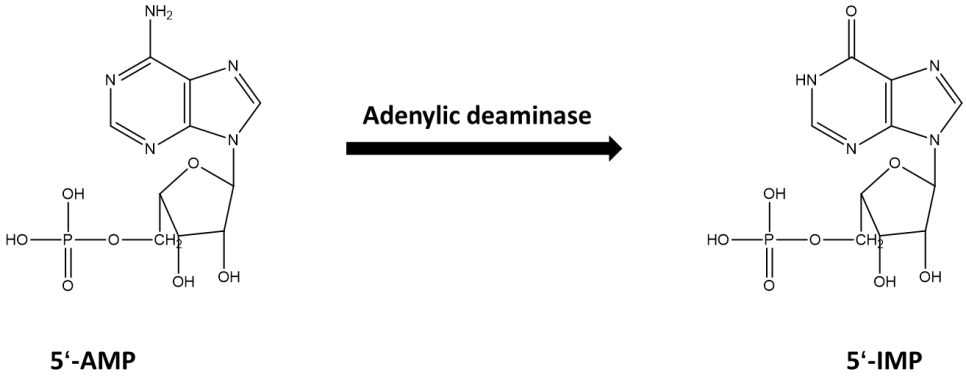


Figure 5.3 Conversion of 5'-AMP to 5'-IMP by reaction of the enzyme adenlyic deaminase

Adenosine phosphate deaminase is a well-known enzyme in this category for the production of umami flavour nucleotides. Like the 5'-phosphodiesterase enzymes (RP-1G), adenosine phosphate deaminase, also known as adenylic deaminase, has become an important enzyme for the production of 5'-nucleotide rich extracts. The function of it is to convert 5'-AMP, which offers no flavour-enhancing properties, to 5-IMP, a product that has umami flavour-enhancing properties in food systems.

Among the four 5'-nucleotides (CMP, UMP, GMP, AMP) formed during the hydrolysis of RNA and DNA by the action of 5'-phosphodiesterase enzyme (RP-1G), 5′ -GMP is the only one that can provide the umami flavour-potentiating

properties. Nonetheless, the 5'-AMP produced, which has no flavour-enhancing properties, can be converted to 5'-IMP, which has flavour-enhancing properties like those of 5'-GMP, by applying a second enzyme, adenine deaminase (Deamizyme). Both compounds then have flavour-enhancing properties, although GMP is approximately twice as effective as IMP.

5.4 CONCLUSION

The purpose of this study is to develop a systematic approach to make a low-value food system into a higher-value one that has enhanced umami flavour. The experiment subject of this whole research was based on low-value food systems such as fermented waste water and insects. By exploring their protein and nucleotide content with various assays in preliminary experiment stage, we now understand the potential capability of different raw material to liberate umami compound.

With enzymatic treatment with various peptidase and nuclease on fermented waste water, we concluded that the umami flavour compound can be considerably enhanced. Furthermore, we applied the developed approach on the insect (*Alphitobius diaperinus)* and analysed the enzyme performance and optimised it to yield maximum umami compounds based on four factors (pH, temperature, time and dosage). The results indicate that opportunities still exist to reduce the cost of enzyme treatment of these low-value food system. From the result, to yield the maximum yield of interested umami compound, it is not necessary to follow the enzyme treatment condition given by the company. Optima parameters like shorter treatment time, lower enzyme dosage, less demanding pH ranges and lower temperatures proves that efficiency can be further improved through a targeted experiment design. However, through

personal communication with some of the enzyme companies, decreasing the temperature and heating it constant at industrial scale may be practically challenging. Treatment temperature of nucleases such as RP-1G and Deamizyme must be under critical consideration in order to yield the most valuable taste active, whilst inactivating the phosphatases for attacking the nucleotides. Moreover, different peptidases may attack each other and inhibit enzymic activity once they are treated together. Therefore, for possible commercial application, industry needs to balance the weight of processing energy (heating temperature, time) and enzymic activity.

Apart from the umami compound, we can also find that the amino acid profile varied with different peptidase treatment in the study of *Alphitobius diaperinus*. Although individual amino acids have their own taste, the mixture of various amino acids may have a place in an integrated flavour picture, which is well-proved in some meat flavour studies (Solms, 1969). A recent study also suggested that each basic taste of amino acids appears to be dependent on its own characteristic such as hydrophobicity, size, charge, functional groups on the side chain, and chirality of the alpha-carbon (Kawai et al., 2012). Moreover, the overall taste of amino acid was found to be the combination of basic taste according to the partial structure. For instance, hydrophilic non-charged middle-sized amino acids elicited sweetness, and L-enantiomeric hydrophilic middle-sized structure was necessary for umami taste. Take serine for example, L-serine was found to have mainly sweet and minor umami taste while D-sesrine had sweet taste alone (Kawai et al., 2012). Whilst, it may not be a convincing practice to predict the potential flavour by judging the amino acid profile without any sensory study, but regarding it as a reference to understand the peptidases'

specificity to release certain amino acids. Nevertheless, umami as a taste that can enhance the overall flavour, by yielding considerable amount of umami property compound (GLU, GMP and IMP) into the low-value food system that do not own the appealing flavour, the positive effect of taste enhancement may be achieved without doubt.

6. FUTURE WORK

6.1 SENSORY STUDY

Opportunities still exist to maximize the potential value of low-value system without failing consumers' taste expectation. Sensory tests are an important tool in research and quality control. Tests such as triangle and paired comparison test can assess the personal response (preference or acceptance) of current or potential customers to a product, a product idea, or specific product characteristics (Civille, Carr and Meilgaard, 2015). Although sensory evaluation of umami flavour has been carried by past researchers using different methodology, there is little research is available to understand the acceptance and overall flavour of enzymatic treated flavour enhancer from low-value food system such as waste water and insects, thus this future study seeks to address this gap in the current research. The economic benefit of using low-value food system is based on the fact that it allows for decreased resource cost and construction while still can produce flavour enhancer. It will be interesting to find out that if applying the idea on a well-chosen existing food product which possesses neutral flavour such as rice, couscous, cracker to identify if these novel seasonings can enhance palatability.

6.2 LOOKING FOR BETTER ENZYMES

Moreover, it is also worth looking for another more productive nuclease for yielding 5'-nucleotides that possesses umami property. Since current commercial strains do not yield more than 10-12% RNA (Nagodawithana and Reed, 2013).

Although protein engineering is still in its infancy with respect to application to food technology, there seems to be an almost limitless potential to develop more

active, stable and tailored enzymes that can perform specific process and yield certain interesting amino acid profiles to benefit the flavour. However, the application of tailored amino acid profile modification is mainly dependent on the area of the peptidase research and development.

6.3 KOKUMI, A NEW CONCEPT OF UMAMI SYNSERGY?

A new word kokumi appears in some Japanese literature at the beginning of 21st century (UEDA et al., 1990). However, the various definition of kokumi from different literature adds the difficulty for people to understand and it hardly can be translated by one word. Moreover, the translation from Japanese work on Kokumi into English version even adds more confusion to its definition. If looking into those descriptions being used for Kokumi: fullness, long-lasting, balance, continuity, deliciousness, depth, duration, growth, full body, mouth fullness, roundness, etc., it is quite similar to the sensation of umami yet indeed a complex and positive sensation.

In one paper, it was translated as ‘enhancing the five primary tastes’, which indicates the further umami enhancement with the presence of kokumi. Although the human receptor is the final solid proof for previous umami controversy argued in the second half of the 20 centuries, there is no certain receptor for kokumi confirmed yet. Many Japanese publications are dedicated to spot some ‘delicious’ peptides like y-glutamyl-peptide as proof of the existence of kokumi (Figure 6.1). Moreover, the isolation of these certain peptide such as putting them into plain water result in a very high threshold while the threshold concentration in complex food matrix is lower, which make people to think the

kokumi might only have some synergy effect on umami under certain context of other taste molecules.

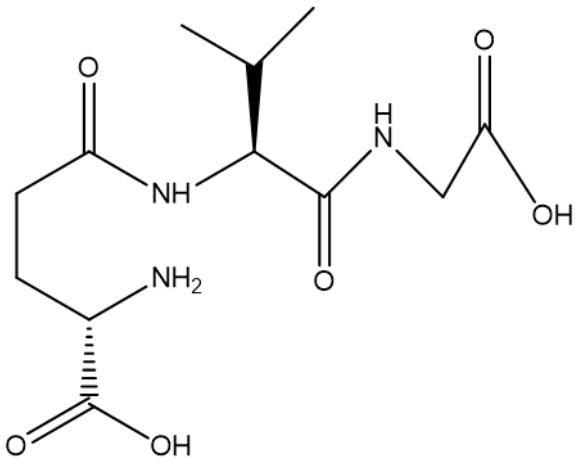


Figure 6.1 The structure of kokumi peptide: y-GLU-VAL-GLY

It is worth noting that only Ajinomoto company has seriously invested the interaction of kokumi molecules with specific receptors. Their research claims that the calcium sensing receptor (CaSR) could be kokumi receptor in their experiment performed in mouse taste cell (Ohsu et al., 2009) (Maruyama et al., 2012). However, the research was carried with limited y-glutamyl diepetides and remains to be proven for human taste perception. If calcium receptor like CaSR is such a critical co-factor to kokumi sensation, then another calcium receptor T1R3 studied in one research by Monell scientists (Tordoff et al., 2012) needs to be noticed since T1R3 is also the part of the sweet and the umami receptor yet does not co-occur with CaSR. In conclusion, it is still too early to determine whether the Kokumi as an umami enhancement factor but a new and exciting research topic.

REFERENCE

Ajinomoto.com. (2017). Ajinomoto Group | Features | Encyclopedia of Amino Acids | How Versatile Amino Acids Are! | Food and Amino Acids. [online] Available at: http://www.ajinomoto.com/features/amino/how/food/.

Amadi, E.N. and Kiin Kabari, D.B. (2016). Nutritional Composition and Microbiology of Some Edible Insects Commonly Eaten in Africa, Hurdles and Future Prospects: A Critical Review. Journal of Food: Microbiology, Safety & Hygiene, 01(01).

Arii K. et al. (1999) Degradation kinetics of L-glutamine in aqueous solution. Eur J Pharm Sci. 9: 75-78.

Badawy, A., Morgan, C. and Turner, J. (2007). Application of the Phenomenex EZ:faast™ amino acid analysis kit for rapid gas-chromatographic determination of concentrations of plasma tryptophan and its brain uptake competitors. Amino Acids, 34(4), pp.587-596.

Barrett, A. and McDonald, J. (1986). Nomenclature: protease, proteinase and peptidase. Biochemical Journal, 237(3), pp.935-935.

Bellisle, F., Dartois, A. and Broyer, M. (1992). Two studies on the acceptability of calcium glutamate as a potassium-free sodium substitute in children. Journal of Renal Nutrition, 2(3), pp.42-46.

Ben-Menachem, E. (2011). Mechanism of action of vigabatrin: correcting misperceptions. Acta Neurologica Scandinavica, 124, pp.5-15.

Beynon, R. and Bond, J. (2001). Proteolytic Enzymes: A Practical Approach (Practical approach series). Oxford University Press, pp.1–21.

Burka E. R. (1969) Characteristics of RNA degradation in the erythroid cell. J Clin Invest. 48: 1266-1272.

Caparros Megido, R., Sablon, L., Geuens, M., Brostaux, Y., Alabi, T., Blecker, C., Drugmand, D., Haubruge, É. and Francis, F. (2013). Edible Insects Acceptance by Belgian Consumers: Promising Attitude for Entomophagy Development. Journal of Sensory Studies, 29(1), pp.14-20.

Chae, H., Joo, H. and In, M. (2001). Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. Bioresource Technology, 76(3), pp.253-258.

Charalambous, G. and INGLETT, G. (1982). Chemistry of foods and beverages. New York: Academic Press, pp.181-194.

Charlton, A., Dickinson, M., Wakefield, M., Fitches, E., Kenis, M., Han, R., Zhu, F., Kone, N., Grant, M., Devic, E., Bruggeman, G., Prior, R. and Smith, R. (2015). Exploring the chemical safety of fly larvae as a source of protein for animal feed. Journal of Insects as Food and Feed, 1(1), pp.7-16.

Chaudhari, N., Landin, A. and Roper, S. (2000). A metabotropic glutamate receptor variant functions as a taste receptor. Nature Neuroscience, 3(2), pp.113-119.

Chaudhari, N. and Roper, S. (2010). The cell biology of taste. The Journal of Cell Biology, 190(3), pp.285-296.

Choudhary, A. (2012). tRNA contains modified bases. [online] Molecularstudy.blogspot.co.uk. Available at: http://molecularstudy.blogspot.co.uk/2012/10/trna-contains-modified-bases.html.

Christensen, D., Orech, F., Mungai, M., Larsen, T., Friis, H. and Aagaard-Hansen, J. (2006). Entomophagy among the Luo of Kenya: a potential mineral source? International Journal of Food Sciences and Nutrition, 57(3-4), pp.198-203.

Civille, G., Carr, B. and Meilgaard, M. (2015). Sensory Evaluation Techniques, Fifth Edition. Boca Raton: CRC Press.

Crunchycritters.com. (2017). Nutrition | Crunchy Critters. [online] Available at: https://www.crunchycritters.com/nutrition/.

Desai, N. A. and Shankar, V. (2003) 'Single-strand-specific nucleases', FEMS Microbiol Rev, 26(5), pp.457-91.

Desor, J., Maller, O. and Turner, R. (1973). Taste in acceptance of sugars by human infants. Journal of Comparative and Physiological Psychology, 84(3), pp.496-501.

Diffen.com. (n.d.). Image:dna-vs-rna.png - Difference and Comparison | Diffen. [online] Available at: http://www.diffen.com/difference/Image:dna-vs-rna.png.

Eilenberg, J., Vlak, J., Nielsen-LeRoux, C., Cappellozza, S. and Jensen, A. (2015). Diseases in insects produced for food and feed. Journal of Insects as Food and Feed, 1(2), pp.87-102.

Elser, W. J., Thomas, Ruth A., and Steffen, G. I. 1935 The desiccation of sera and other biological products (including microorganisms) in the frozen state with the preservation of the original qualities of products so treated. J. Immunol., 28, 433-473.

Enzyme nomenclature. Recommendations (1984) of the nomenclature committee of the international union of biochemistry. (1985). Analytical Biochemistry, 147(2), p.547.

Fao.org. (2017). Insects for food and feed. [online] Available at: http://www.fao.org/edible-insects/en/.

Fujimoto, M., Fujiyama, K., Kuninaka, A. and Yoshino, H. (1974). Mode of Action of Nuclease P1on Nucleic Acids and Its Specificity for Synthetic Phosphodiesters. Agricultural and Biological Chemistry, 38(11), pp.2141-2147.

Fuke, S. and Ueda, Y. (1996). Interactions between umami and other flavor characteristics. Trends in Food Science & Technology, 7(12), pp.407-411.

Getchell, T. (1991). Smell and taste in health and disease. New York: Raven Press, pp.405-416.

Gibson, R. (2015). Dietary-induced Zinc Deficiency in low Income Countries. Nutrition Today, 50(1), pp.49-55.

Guan Y. et al. (2013) Utilization of Candida utilis cells for the production of yeast extract: effects of enzyme types, dosages and treatment time. Adv J Food Sci Technol. 5(5): 551-556.

Gulliksen, H. (1956). A least squares solution for paired comparisons with incomplete data. Psychometrika, 21(2), pp.125-134.

Guschlbauer, W. (1976). Nucleic Acid Structure. New York, NY: Springer New York, pp.26-28.

Hanboonsong, Y., Jamjanya, T. and Durst, P. (2013). Six-legged livestock: edible insect farming, collection and marketing in Thailand. Food and Agriculture Organizati.

House, J. (2016). Consumer acceptance of insect-based foods in the Netherlands: Academic and commercial implications. Appetite, 107, pp.47-58.

Ikeda K. (1909) New seasonings. Translated from the Japanese, by Ogiwara Y. and Ninomiya Y. Chem Senses. 27: 847-849.

Ikeda, K. (2002). New Seasonings. Chemical Senses, 27(9), pp.847-849.

Ishibashi, N., Ono, I., Kato, K., Shigenaga, T., Shinoda, I., Okai, H. and Fukui, S. (1988). Studies on flavoured peptides. Part III. Role of the hydrophobic amino acid residue in the bitterness of peptides. Agricultural and Biological Chemistry, 52(1), pp.91-94.

Jansson, A. and Berggren, Å. (2015). Insects as Food – Something for the Future? SLU, Future Agriculture – Livestock, Crops and Land Use.

Kawai, M., Sekine-Hayakawa, Y., Okiyama, A. and Ninomiya, Y. (2012). Gustatory sensation of l- and d-amino acids in humans. Amino Acids, 43(6), pp.2349-2358.

Keller, T., McGee, H., Murata, Y. and Saito, A. (2014). Umami. Tokyo: Japan Publications Trading Co., Ltd.

Khan, K. and Elia, M. (1991). Factors affecting the stability of L-glutamine in solution. Clinical Nutrition, 10(4), pp.186-192.

Kodama S. (1913) On a procedure for separating inosinic acid. J Tokyo Chem Soc. 34: 751.

Koshland, D. and Springhorn, S. (1956). Mechanism of action of 5'-nucleotidase. Journal of biological chemistry, 221, pp.469-476.

Kuninaka A. (1960) Studies on taste of ribonucleic acid derivatives. J Agric Chem Soc Jpn. 34: 487-492.

Kurihara K. (2009) Glutamate: from discovery as a food flavour to role as a basic taste (umami). Am J Clin Nutr. 90(3): 719S-722S.

Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M. and Adler, E. (2002). Human receptors for sweet and umami taste. Proceedings of the National Academy of Sciences, 99(7), pp.4692-4696.

Maehashi, K., Matano, M., Wang, H., Vo, L., Yamamoto, Y. and Huang, L. (2008). Bitter peptides activate hTAS2Rs, the human bitter receptors. Biochemical and Biophysical Research Communications, 365(4), pp.851-855.

Maruyama, Y., Yasuda, R., Kuroda, M. and Eto, Y. (2012). Kokumi Substances, Enhancers of Basic Tastes, Induce Responses in Calcium-Sensing Receptor Expressing Taste Cells. PLoS ONE, 7(4), p.e34489.

McLean, E., Cogswell, M., Egli, I., Wojdyla, D. and de Benoist, B. (2008). Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993–2005. Public Health Nutrition, 12(04), p.444.

McLennan A. et al. (2012) Molecular Biology. 4th ed. New York: Garland Science.

Mejia, M., Mejia, M. and profile, V. (2017). Unique features of Fungi. [online] Oh93independentstudyonfungi.blogspot.co.uk. Available at: http://oh93independentstudyonfungi.blogspot.co.uk/2013/11/unique-features-of-fungi.html.

Morikawa, K., Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakanishi, S. and Jingami, H. (2000). Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature, 407(6807), pp.971-977.

Nadathur, S., Wanasundara, J. and Scanlin, L. (2016). Sustainable protein sources. Academic Press, pp.305–325.

Nagodawithana, T. and Reed, G. (2013). Enzymes in food processing. 3rd ed. San Diego: Academic Press, pp.401-421.

Nature.com. (2010). Gene Expression | Learn Science at Scitable. [online] Available at: https://www.nature.com/scitable/topicpage/gene-expression-14121669.

Nguyen, T., Palcic, M. and Hadziyev, D. (1988). Characterization of cell-wall-bound nuclease and ribonuclease from potato tuber. Agricultural and Biological Chemistry, 52(4), pp.957-965.

Ohsu, T., Amino, Y., Nagasaki, H., Yamanaka, T., Takeshita, S., Hatanaka, T., Maruyama, Y., Miyamura, N. and Eto, Y. (2009). Involvement of the Calcium-sensing Receptor in Human Taste Perception. Journal of Biological Chemistry, 285(2), pp.1016-1022.

Okiyama, A. and Beauchamp, G. (1998). Taste dimensions of monosodium glutamate (MSG) in a food system: role of glutamate in young American subjects. Physiology & Behavior, 65(1), pp.177-181.

Phillips, G. and Williams, P. (2011). Handbook of food proteins. Cambridge, UK: Woodhead Publishing, pp 335–352.

Prescott, J. (1998). Comparisons of taste perceptions and preferences of Japanese and Australian consumers: overview and implications for cross-cultural sensory research. Food Quality and Preference, 9(6), pp.393-402.

Raliou, M., Wiencis, A., Pillias, A., Planchais, A., Eloit, C., Boucher, Y., Trotier, D., Montmayeur, J. and Faurion, A. (2009). Nonsynonymous single nucleotide polymorphisms in human tas1r1, tas1r3, and mGluR1 and individual taste sensitivity to glutamate. American Journal of Clinical Nutrition, 90(3), pp.789S-799S.

Raper, K. B. and Alexander, Dorothy F. 1945 Preservation of molds by the lyophile process. Mycologia, 37, 499-525.

Rawlings, N. and Salvesen, G. (2013). Handbook of proteolytic enzymes. Boston: Academic Press.

Reineccius, G. (2005). Flavor Chemistry and Technology, Second Edition. Hoboken: CRC Press, pp.323-324.

Rumpold, B. and Schlüter, O. (2013). Nutritional composition and safety aspects of edible insects. Molecular Nutrition & Food Research, 57(5), pp.802-823.

Saenger, W. (1995). Principles of nucleic acid structure. New York: Springer.

San Gabriel, A., Maekawa, T., Uneyama, H., Yoshie, S. and Torii, K. (2007). mGluR1 in the fundic glands of rat stomach. FEBS Letters, 581(6), pp.1119-1123.

Sen, D. (2005). Advances in fish processing technology. New Delhi: Allied Publishers, pp.127-128.

Solms, J. (1969). Taste of amino acids, peptides, and proteins. Journal of Agricultural and Food Chemistry, 17(4), pp.686-688.

Steiner, J., Glaser, D., Hawilo, M. and Berridge, K. (2001). Comparative expression of hedonic impact: affective reactions to taste by human infants and other primates. Neuroscience & Biobehavioral Reviews, 25(1), pp.53-74.

Sun, Q., Zhao, Z. and Guo, S. (2016). Research on the production technology of umami flavoring from tofu wastewater protein. Food Industry, 10, pp.139-143.

Taylor, A. and Hort, J. (2008). Modifying flavour in food. Boca Raton: CRC, pp.95-105.

Tordoff, M., Alarcón, L., Valmeki, S. and Jiang, P. (2012). T1R3: A human calcium taste receptor. Scientific Reports, 2(1).

Ueda, Y., Sakaguchi, M., Hirayama, K., Miyajima, R. and Kimizuka, A. (1990). Characteristic flavor constituents in water extract of garlic. Agricultural and Biological Chemistry, 54(1), pp.163-169.

van der Spiegel, M., Noordam, M. and van der Fels-Klerx, H. (2013). Safety of Novel Protein Sources (Insects, Microalgae, Seaweed, Duckweed, and Rapeseed) and Legislative Aspects for Their Application in Food and Feed Production. Comprehensive Reviews in Food Science and Food Safety, 12(6), pp.662-678.

van Huis, A. (2015). Edible insects contributing to food security? Agriculture & Food Security, 4(1).

van Huis, A., Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G. and Vantomme, P. (2013). Edible insects Future prospects for food and feed security. food and agriculture organization of the united nations.

Waldron, K. (2009). Handbook of waste management and co-product recovery in food processing. Cambridge: Woodhead Publishing.

Wrap.org.uk. (2016). Estimates of Food and Packaging Waste in the UK Grocery Retail and Hospitality Supply Chains| WRAP UK. [online] Available at: http://www.wrap.org.uk/about-us/what-we-do/key-publications.

Yamaguchi S. (1967). The Synergistic Taste Effect of Monosodium Glutamate and Disodium 5'-Inosinate. Journal of Food Science, 32(4), pp.473-478.

Yamaguchi, S., Yoshikawa, T., Ikeda, S. and Ninomiya, T. (1968). Synergistic Taste Effects of Some New Ribonucleotide Derivatives. Agricultural and Biological Chemistry, 32(7), pp.797-802.

Yi, L., Lakemond, C., Sagis, L., Eisner-Schadler, V., van Huis, A. and van Boekel, M. (2013). Extraction and characterisation of protein fractions from five insect species. Food Chemistry, 141(4), pp.3341-3348.

Zhang, F., Klebansky, B., Fine, R., Xu, H., Pronin, A., Liu, H., Tachdjian, C. and Li, X. (2008). Molecular mechanism for the umami taste synergism. Proceedings of the National Academy of Sciences, 105(52), pp.20930-20934.

Appendix I (Thermofisher.com, 2017)

INSTRUCTIONS

Thermo SCIENTIFIC

Pierce™ BCA Protein Assay Kit

23225 23227 1296.9

Number	Description
23225	**Pierce BCA Protein Assay Kit**, sufficient reagents for 500 test-tube or 5000 microplate assays
23227	**Pierce BCA Protein Assay Kit**, sufficient reagents for 250 test-tube or 2500 microplate assays

Kit Contents:

BCA Reagent A, 1000mL (in Product No. 23225) or 500mL (in Product No. 23227), containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide

BCA Reagent B, 25mL, containing 4% cupric sulfate

Albumin Standard Ampules, 2mg/mL, 10 × 1mL ampules, containing bovine serum albumin (BSA) at 2mg/mL in 0.9% saline and 0.05% sodium azide

Storage: Upon receipt store at room temperature. Product shipped at ambient temperature.

Note: If either Reagent A or Reagent B precipitates upon shipping in cold weather or during long-term storage, dissolve precipitates by gently warming and stirring solution. Discard any kit reagent that shows discoloration or evidence of microbial contamination.

Table of Contents

Introduction 1
Preparation of Standards and Working Reagent (required for both assay procedures) 2
Test Tube Procedure (Sample to WR ratio = 1:20) 3
Microplate Procedure (Sample to WR ratio = 1:8) 3
Troubleshooting 4
Related Thermo Scientific Products 5
Additional Information 5
References 6

Introduction

The Thermo Scientific™ Pierce™ BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid.[1] The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000µg/mL). The BCA method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA.[2] Studies with di-, tri- and tetrapeptides suggest that the extent of color formation caused by more than the mere sum of individual color-producing functional groups.[2] Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration are prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve. If precise quantitation of an unknown protein is required, it is advisable to select a protein

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/pierce

standard that is similar in quality to the unknown; for example, a bovine gamma globulin (BGG) standard (see Related Thermo Scientific Products) may be used when assaying immunoglobulin samples.

Two assay procedures are presented. Of these, the Test Tube Procedure requires a larger volume (0.1mL) of protein sample; however, because it uses a sample to working reagent ratio of 1:20 (v/v), the effect of interfering substances is minimized. The Microplate Procedure affords the sample handling ease of a microplate and requires a smaller volume (10-25µL) of protein sample; however, because the sample to working reagent ratio is 1:8 (v/v), it offers less flexibility in overcoming interfering substance concentrations and obtaining low levels of detection.

Preparation of Standards and Working Reagent (required for both assay procedures)

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1mL ampule of 2mg/mL Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume for three replications of each diluted standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Dilution Scheme for Enhanced Test Tube Protocol (Working Range = 5–250µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	700	100 of Stock	250
B	400	400 of vial A dilution	125
C	450	300 of vial B dilution	50
D	400	400 of vial C dilution	25
E	400	100 of vial D dilution	5
F	400	0	0 = Blank

B. Preparation of the BCA Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

   Example: for the standard test-tube procedure with 3 unknowns and 2 replicates of each sample:

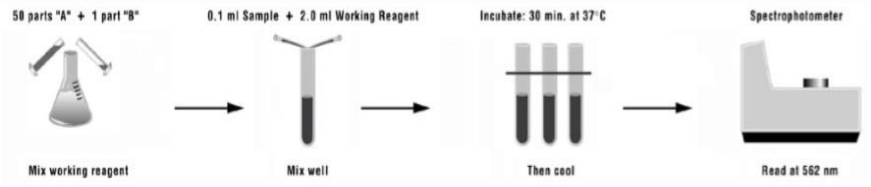
   (9 standards + 3 unknowns) × (2 replicates) × (2mL) = 48mL WR required

   Note: 2.0mL of the WR is required for each sample in the test-tube procedure, while only 200 µl of WR reagent is required for each sample in the microplate procedure.

2. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50mL of Reagent A with 1mL of Reagent B.

   Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

Procedure Summary (Test-tube Procedure, Standard Protocol)



Test-tube Procedure (Sample to WR ratio = 1:20)

1. Pipette 0.1mL of each standard and unknown sample replicate into an appropriately labeled test tube.
2. Add 2.0mL of the WR to each tube and mix well.
3. Cover and incubate tubes at selected temperature and time:
   - Standard Protocol: 37°C for 30 minutes (working range = 20-2000µg/mL)
   - RT Protocol: RT for 2 hours (working range = 20-2000µg/mL)
   - Enhanced Protocol: 60°C for 30 minutes (working range = 5-250µg/mL)

   Notes:
   - Increasing the incubation time or temperature increases the net 562nm absorbance for each test and decreases both the minimum detection level of the reagent and the working range of the protocol.
   - Use a water bath to heat tubes for either Standard (37°C incubation) or Enhanced (60°C incubation) Protocol. Using a forced-air incubator can introduce significant error in color development because of uneven heat transfer.
4. Cool all tubes to RT.
5. With the spectrophotometer set to 562nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

   Note: Because the BCA assay does not reach a true end point, color development will continue even after cooling to RT. However, because the rate of color development is low at RT, no significant error will be introduced if the 562nm absorbance measurements of all tubes are made within 10 minutes of each other.
6. Subtract the average 562nm absorbance measurement of the Blank standard replicates from the 562nm absorbance measurement of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Microplate Procedure (Sample to WR ratio = 1:8)

1. Pipette 25µL of each standard or unknown sample replicate into a microplate well (working range = 20-2000µg/mL) (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041).

   Note: If sample size is limited, 10µL of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2000µg/mL.
2. Add 200µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT. Measure the absorbance at or near 562nm on a plate reader.

   Notes:
   - Wavelengths from 540-590nm have been used successfully with this method.
   - Because plate readers use a shorter light path length than cuvette spectrophotometers, the Microplate Procedure requires a greater sample to WR ratio to obtain the same sensitivity as the standard Test Tube Procedure. If higher 562nm measurements are desired, increase the incubation time to 2 hours.
   - Increasing the incubation time or ratio of sample volume to WR increases the net 562nm measurement for each well and lowers both the minimum detection level of the reagent and the working range of the assay. As long as all standards and unknowns are treated identically, such modifications may be useful.

5. Subtract the average 562nm absorbance measurement of the Blank standard replicates from the 562nm measurements of all other individual standard and unknown sample replicates.
6. Prepare a standard curve by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

   Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

Troubleshooting

Problem	Possible Cause	Solution
No color in any tubes	Sample contains a copper chelating agent	Dialyze, desalt or dilute sample Increase copper concentration in working reagent (e.g., use 50:2, Reagent A:B) Remove interfering substances from sample using Product No. 23215
Blank absorbance is OK, but standards and samples show less color than expected	Strong acid or alkaline buffer, alters working reagent pH	Dialyze, desalt, or dilute sample
	Color measured at the wrong wavelength	Measure the absorbance at 562nm
Color of samples appears darker than expected	Protein concentration is too high	Dilute sample
	Sample contains lipids or lipoproteins	Add 2% SDS to the sample to eliminate interference from lipids[3]
		Remove interfering substances from sample using Product No. 23215
All tubes (including blank) are dark purple	Buffer contains a reducing agent	Dialyze or dilute sample Remove interfering substances from sample using Product No. 23215
	Buffer contains a thiol	
	Buffer contains biogenic amines (catecholamines)	
Need to measure color at a different wavelength	Spectrophotometer or plate reader does not have 562nm filter	Color may be measure at any wavelength between 540nm and 590nm, although the slope of standard curve and overall assay sensitivity will be reduced

A. Interfering substances

Certain substances are known to interfere with the BCA assay including those with reducing potential, chelating agents, and strong acids or bases. Because they are known to interfere with protein estimation at even minute concentrations, avoid the following substances as components of the sample buffer:

Ascorbic acid	EGTA	Iron	Impure sucrose
Catecholamines	Impure glycerol	Lipids	Tryptophan
Creatinine	Hydrogen peroxide	Melibiose	Tyrosine
Cysteine	Hydrazides	Phenol Red	Uric acid

Other substances interfere to a lesser extent with protein estimation using the BCA assay, and these have only minor (tolerable) effects below a certain concentration in the original sample. Maximum compatible concentrations for many substances in the Standard Test Tube Protocol are listed in Table 2 (see last page of Instructions). Substances were compatible at the indicated concentration in the Standard Test Tube Protocol if the error in protein concentration estimation caused by the presence of the substance was less than or equal to 10%. The substances were tested using WR prepared immediately before each experiment. Blank-corrected 562nm absorbance measurements (for a 1000µg/mL BSA standard + substance) were compared to the net 562nm measurements of the same standard prepared in 0.9% saline. Maximum compatible concentrations will be lower In the Microplate Procedure where the sample to WR ratio is 1:8 (v/v).

Furthermore, it is possible to have a substance additive affect such that even though a single component is present at a concentration below its listed compatibility, a sample buffer containing a combination of substances could interfere with the assay.

B. Strategies for eliminating or minimizing the effects of interfering substances

The effects of interfering substances in the Pierce BCA Protein Assay may be eliminated or overcome by one of several methods.

- Remove the interfering substance by dialysis or gel filtration.
- Dilute the sample until the substance no longer interferes. This strategy is effective only if the starting protein concentration is sufficient to remain in the working range of the assay upon dilution.
- Precipitate the proteins in the sample with acetone or trichloroacetic acid (TCA). The liquid containing the substance that interfered is discarded and the protein pellet is easily solubilized in ultrapure water or directly in the alkaline BCA WR.[4] A protocol detailing this procedure is available from our website. Alternatively, Product No. 23215 may be used (see Related Thermo Scientific Products).
- Increase the amount of copper in the WR (prepare WR as 50:2 or 50:3, Reagent A:B), which may eliminate interference by copper-chelating agents.

Note: For greatest accuracy, the protein standards must be treated identically to the sample(s).

Related Thermo Scientific Products

15041	**Pierce 96-Well Plates,** 100/pkg.
15075	**Reagent Reservoirs,** 200/pkg.
15036	**Sealing Tape for 96-Well Plates,** 100/pkg.
23209	**Albumin Standard Ampules, 2mg/mL**, 10 × 1mL ampules, containing bovine serum albumin (BSA)
23208	**Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set**, 7 × 3.5mL
23212	**Bovine Gamma Globulin Standard, 2mg/mL**, 10 × 1mL ampules
23213	**Pre-Diluted Protein Assay Standards**, (BGG) Set, 7 × 3.5mL aliquots
23235	**Pierce Micro BCA Protein Assay Kit**, working range of 0.5-20µg/mL
23236	**Coomassie Plus™ (Bradford) Assay Kit**, working range of 1-1500µg/mL
23215	**Compat-Able™ Protein Assay Preparation Reagent Set**
23250	**Pierce BCA Protein Assay Kit–Reducing Agent Compatible**

Additional Information

A. Please visit our website for additional information including the following items:

- Tech Tip #8: Eliminate interfering substances from samples for BCA Protein Assay

B. Alternative Total Protein Assay Reagents

If interference by a reducing substance or metal-chelating substance contained in the sample cannot be overcome, try the Thermo Scientific Coomassie Plus (Bradford) Assay Kit (Product No. 23236), which is less sensitive to such substances.

C. Cleaning and Re-using Glassware

Exercise care when re-using glassware. All glassware must be cleaned and given a thorough final rinse with ultrapure water.

D. Response characteristics for different proteins

Each of the commonly used total protein assay methods exhibits some degree of varying response toward different proteins. These differences relate to amino acid sequence, pI, structure and the presence of certain side chains or prosthetic groups that can dramatically alter the protein's color response. Most protein assay methods use BSA or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined (Figure 1). However, if great accuracy is required, prepare the standard curve from a pure sample of the target protein.

Typical protein-to-protein variation in color response is listed in Table 3. All proteins were tested at 1000µg/mL using the 30-minute/37°C Test Tube Protocol. The average net color response for BSA was normalized to 1.00 and the average net color response of the other proteins is expressed as a ratio to the response of BSA.

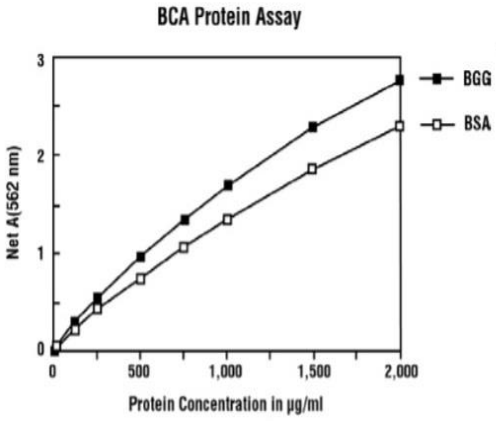


Figure 1: Typical color response curves for BSA and BGG using the Standard Test Tube Protocol (37°C/30-minute incubation).

Table 3. Protein-to-protein variation. Absorbance ratios (562nm) for proteins relative to BSA using the Standard Test Tube Protocol.

Ratio = (Avg "test" net Abs.) / (avg. BSA net Abs.)

Protein Tested	Ratio
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.85
α-Chymotrypsinogen, bovine	1.14
Cytochrome C, horse heart	0.83
Gamma globulin, bovine	1.11
IgG, bovine	1.21
IgG, human	1.09
IgG, mouse	1.18
IgG, rabbit	1.12
IgG, sheep	1.17
Insulin, bovine pancreas	1.08
Myoglobin, horse heart	0.74
Ovalbumin	0.93
Transferrin, human	0.89
	1.02
Standard Deviation	**0.15**
Coefficient of Variation	**14.7%**

Cited References

1. Smith, P.K., *et al.* (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:**76-85.
2. Wiechelman, K., *et al.* (1988). Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal Biochem* **175**:231-7.
3. Kessler, R. and Fanestil, D. (1986). Interference by lipids in the determination of protein using bicinchoninic acid. *Anal Biochem* **159:**138-42.
4. Brown, R., *et al.* (1989). Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal Biochem* **180:**136-9.

Product References

Adilakshami, T. and Laine, R.O. (2002). Ribosomal protein S25 mRNA partners with MTF-1 and La to provide a p53-mediated mechanism for survival or death. *J Biol Chem* **277:**4147-51.

Fischer, T., *et al.* (1999). Clathrin-coated vesicles bearing GAIP possess GTPase-activating protein activity in vitro. *Proc Nat Acad Sci* **96:**6722-7.

Prozialeck, W.C., *et al.* (2002). Chlamydia trachomatis disrupts N-cadherin-dependent cell-cell junctions and sequester β-catenin in human cervical epithelial cells. *Infection and Immunity* **70:**2605-13.

Roberts, K.P., *et al.* (2002). A comparative analysis of expression and processing of the rat epididymal fluid and sperm-bound forms of proteins D and E. *Biology of Reproduction* **67:**525-33.

Products are warranted to operate or perform substantially in conformance with published Product specifications in effect at the time of sale, as set forth in the Product documentation, specifications and/or accompanying package inserts ("Documentation"). No claim of suitability for use in applications regulated by FDA is made. The warranty provided herein is valid only when used by properly trained individuals. Unless otherwise stated in the Documentation, this warranty is limited to one year from date of shipment when the Product is subjected to normal, proper and intended usage. This warranty does not extend to anyone other than Buyer. Any model or sample furnished to Buyer is merely illustrative of the general type and quality of goods and does not represent that any Product will conform to such model or sample.
NO OTHER WARRANTIES, EXPRESS OR IMPLIED, ARE GRANTED, INCLUDING WITHOUT LIMITATION, IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR ANY PARTICULAR PURPOSE, OR NON INFRINGEMENT. BUYER'S EXCLUSIVE REMEDY FOR NON-CONFORMING PRODUCTS DURING THE WARRANTY PERIOD IS LIMITED TO REPAIR, REPLACEMENT OF OR REFUND FOR THE NON-CONFORMING PRODUCT(S) AT SELLER'S SOLE OPTION. THERE IS NO OBLIGATION TO REPAIR, REPLACE OR REFUND FOR PRODUCTS AS THE RESULT OF (I) ACCIDENT, DISASTER OR EVENT OF FORCE MAJEURE, (II) MISUSE, FAULT OR NEGLIGENCE OF OR BY BUYER, (III) USE OF THE PRODUCTS IN A MANNER FOR WHICH THEY WERE NOT DESIGNED, OR (IV) IMPROPER STORAGE AND HANDLING OF THE PRODUCTS.

Unless otherwise expressly stated on the Product or in the documentation accompanying the Product, the Product is intended for research only and is not to be used for any other purpose, including without limitation, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses, or any type of consumption by or application to humans or animals.

Current product instructions are available at www.thermoscientific.com/pierce. For a faxed copy, call 800-874-3723 or contact your local distributor.

© 2013 Thermo Fisher Scientific Inc. All rights reserved. Triton is a trademark of The Dow Chemical Company. Brij, Tween and Span are trademarks of Croda International PLC. Zwittergent is a trademark of Merck KGaA. All (other) trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries. Printed in the USA.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/pierce

Table 2. Compatible substance concentrations in the Thermo Scientific Pierce BCA Protein Assay (see text for details).[§]

Substance	Compatible Concentration
Salts/Buffers	
ACES, pH 7.8	25mM
Ammonium sulfate	1.5M
Asparagine	1mM
Bicine, pH 8.4	20mM
Bis-Tris, pH 6.5	33mM
Borate (50mM), pH 8.5 (# 28384)	undiluted
B-PER™ Reagent (#78248)	undiluted
Calcium chloride in TBS, pH 7.2	10mM
Na-Carbonate/Na-Bicarbonate (0.2M), pH 9.4 (# 28382)	undiluted
Cesium bicarbonate	100mM
CHES, pH 9.0	100mM
Na-Citrate (0.6M), Na-Carbonate (0.1M), pH 9.0 (# 28388)	1:8 dilution*
Na-Citrate (0.6M), MOPS (0.1M), pH 7.5 (#28386)	1:8 dilution*
Cobalt chloride in TBS, pH 7.2	0.8mM
EPPS, pH 8.0	100mM
Ferric chloride in TBS, pH 7.2	10mM
Glycine•HCl, pH 2.8	100mM
Guanidine•HCl	4M
HEPES, pH 7.5	100mM
Imidazole, pH 7.0	50mM
MES, pH 6.1	100mM
MES (0.1M), NaCl (0.9%), pH 4.7 (#28390)	undiluted
MOPS, pH 7.2	100mM
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted
Nickel chloride in TBS, pH 7.2	10mM
PBS; Phosphate (0.1M), NaCl (0.15M), pH 7.2 (# 28372)	undiluted
PIPES, pH 6.8	100mM
RIPA lysis buffer; 50mM Tris, 150mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0	undiluted
Sodium acetate, pH 4.8	200mM
Sodium azide	0.2%
Sodium bicarbonate	100mM
Sodium chloride	1M
Sodium citrate, pH 4.8 or pH 6.4	200mM
Sodium phosphate	100mM
Tricine, pH 8.0	25mM
Triethanolamine, pH 7.8	25mM
Tris	250mM
TBS; Tris (25mM), NaCl (0.15M), pH 7.6 (# 28376)	undiluted
Tris (25mM), Glycine (192mM), pH 8.0 (# 28380)	1:3 dilution*

Substance	Compatible Concentration
Detergents**	
Brij™-35	5.0%
Brij-56, Brij-58	1.0%
CHAPS, CHAPSO	5.0%
Deoxycholic acid	5.0%
Octyl β-glucoside	5.0%
Nonidet P-40 (NP-40)	5.0%
Octyl β-thioglucopyranoside	5.0%
SDS	5.0%
Span™ 20	1.0%
Triton™ X-100	5.0%
Triton X-114, X-305, X-405	1.0%
Tween™-20, Tween-60, Tween-80	5.0%
Zwittergent™ 3-14	1.0%
Chelating agents	
EDTA	10mM
EGTA	--------
Sodium citrate	200mM
Reducing & Thiol-Containing Agents	
N-acetylglucosamine in PBS, pH 7.2	10mM
Ascorbic acid	--------
Cysteine	--------
Dithioerythritol (DTE)	1mM
Dithiothreitol (DTT)	1mM
Glucose	10mM
Melibiose	--------
2-Mercaptoethanol	0.01%
Potassium thiocyanate	3.0M
Thimerosal	0.01%
Misc. Reagents & Solvents	
Acetone	10%
Acetonitrile	10%
Aprotinin	10mg/L
DMF, DMSO	10%
DMSO	10%
Ethanol	10%
Glycerol (Fresh)	10%
Hydrazides	--------
Hydrides (Na_2BH_4 or $NaCNBH_3$)	--------
Hydrochloric Acid	100mM
Leupeptin	10mg/L

* Diluted with ultrapure water.
** Detergents were tested using high-purity Thermo Scientific Surfact-Amps Products, which have low peroxide content.
-- Dashed-line entry indicates that the material is incompatible with the assay.

[§] For a more extensive list of substances, download Tech Tip # 68: Protein assay compatibility table from our website. This Tech Tip includes compatible substances for all of our protein assays and enables easy comparisons.

Appendix II (Thermofisher.com, 2017)

Protein Peptide Hydrolysis Protocol: acid hydrolysis using vacuum hydrolysis tubes.

Materials Required:

- Hydrochloric Acid (6N), Constant Boiling
- Vacuum Hydrolysis Tubes
- Reacti-Therm Heating Module, outfitted with appropriate Heating Block

A. Preparation

Unscrew the PTFE Plug from the vacuum hydrolysis tube.

Using a small diameter glass pipette (disposable Pasteur) or syringe, introduce the sample into the bottom reservoir of the tube. Avoid leaving any residue of the sample in the upper portion of the tube, particularly in the area of the threads, and at the top of the reservoir where the PTFE Plug makes a seal.

Add the specially purified constant boiling hydrochloric acid up to the desired reaction level.

NOTE: For best results, the total volume of sample and hydrochloric acid should not exceed 1/3 of the stated reservoir volume. This will help prevent loss during the application of vacuum, when bumping and foaming may occur. To prevent excessive bumping and foaming during the vacuum process, the sample can be frozen prior to the application of vacuum.

B. Application of Vacuum

Insert the inert PTFE Plug and screw it down just enough to leave a small passageway between the plug and the glass at the stricture point.

Secure the tube and affix the vacuum source to the side arm. It is helpful to use vacuum tubing to connect the hydrolysis tubes to a three-way stopcock. The stopcock should be connected to a vacuum source and a supply of purified nitrogen or argon. This will allow you to alternate between vacuum and an inert gas, which ensures the complete removal of oxygen from the sample.

Additionally, this set-up will facilitate the controlled release of the vacuum after the hydrolysis is complete.

C. Hydrolysis

When the desired vacuum is reached, seal the unit by slowly screwing the plug down until it is flush with the glass surface at the stricture. Do not overtighten! Applying too much pressure to the hydrolysis tube is the most common cause of damage to the tube or PTFE Plug. Pierce Vacuum Hydrolysis Tubes are designed to withstand temperatures up to 200°C for 48 hours.

NOTE: At 150°C, specially purified hydrochloric acid allows rapid (6-hour) hydrolysis of proteins. (Standard protein hydrolysis conditions are 105-110°C for 16-24 hours.)

After the tubes have cooled, release the PTFE Plug just enough to create a small passageway between the plug and the glass stricture. The three-way stopcock set-up, described earlier, facilitates this procedure and allows you to break the vacuum without allowing air/oxygen into your sample.

D. Sample Concentration

After securely fastening the tube, your sample can be concentrated (via vacuum) with or without heating the tube. Alternatively, the hydrolysis reagent may be removed by lyophilization, or via a stream of nitrogen/argon directed to the surface of the sample.

At the appropriate point in your procedure, use a small diameter pipette to remove your sample. The hydrolysed sample can easily be re-dissolved in the sodium citrate sample dilution buffer.

Completely remove the PTFE Plug and use a small pipette to add the appropriate quantity of buffer to the dried sample.

Allow the sample to dissolve and remove it with a pipette.

Appendix III

Part A: Mass spectra of amino acids

Spectra obtained from the chromatogram of standards, library spectra provided with the kit could not be opened with the software available. Correct peak assignments were determined using the listings of major ion for each compound on pages 2 and 3 of the user's manual.

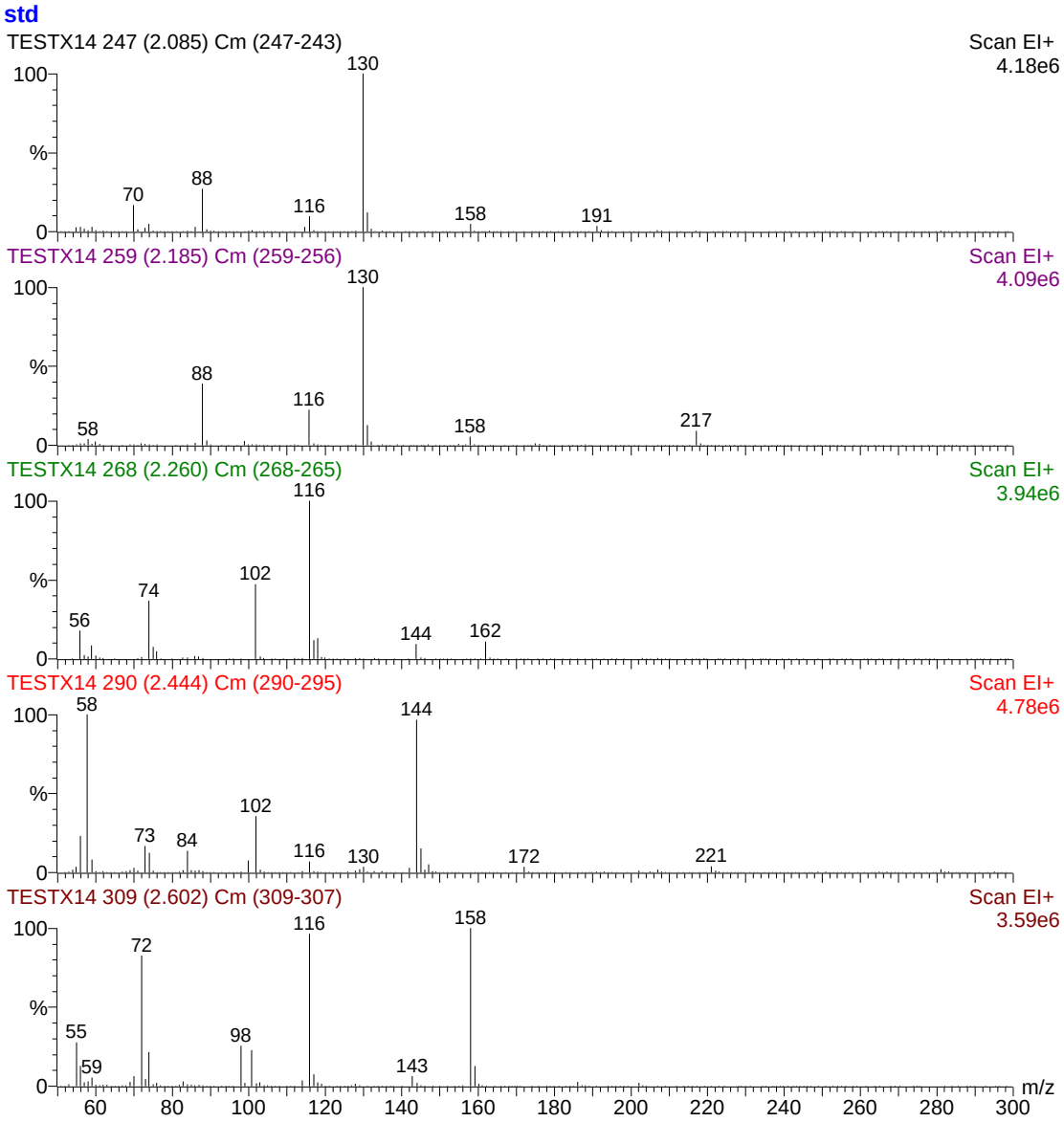


Figure A1: Mass spectra of ALA, SAR, GLY, ABA, VAL (from top to bottom)

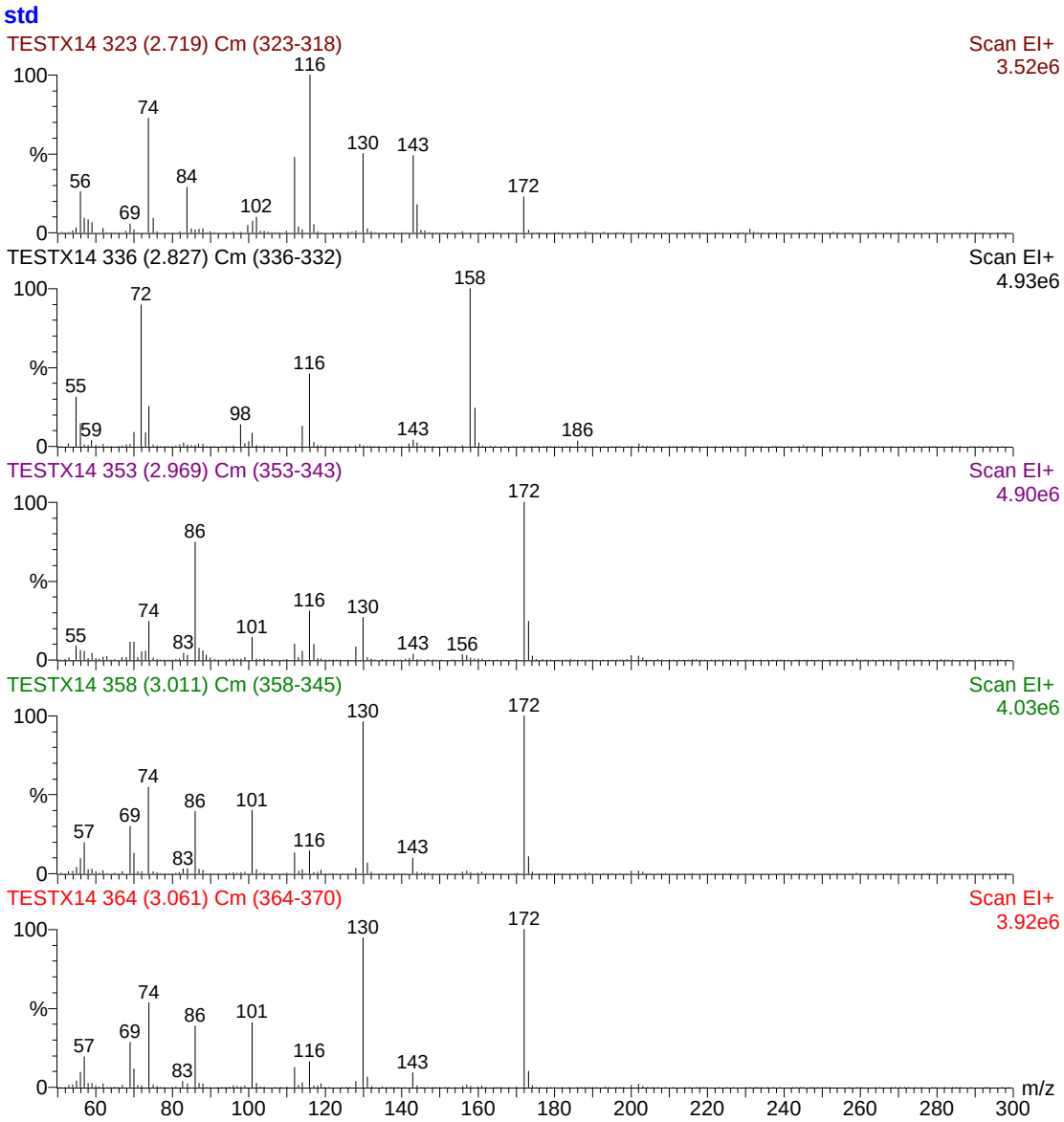


Figure A2: Mass spectra of β-AIB, NOR (IS), LEU, AILE, ILE (from top to bottom)

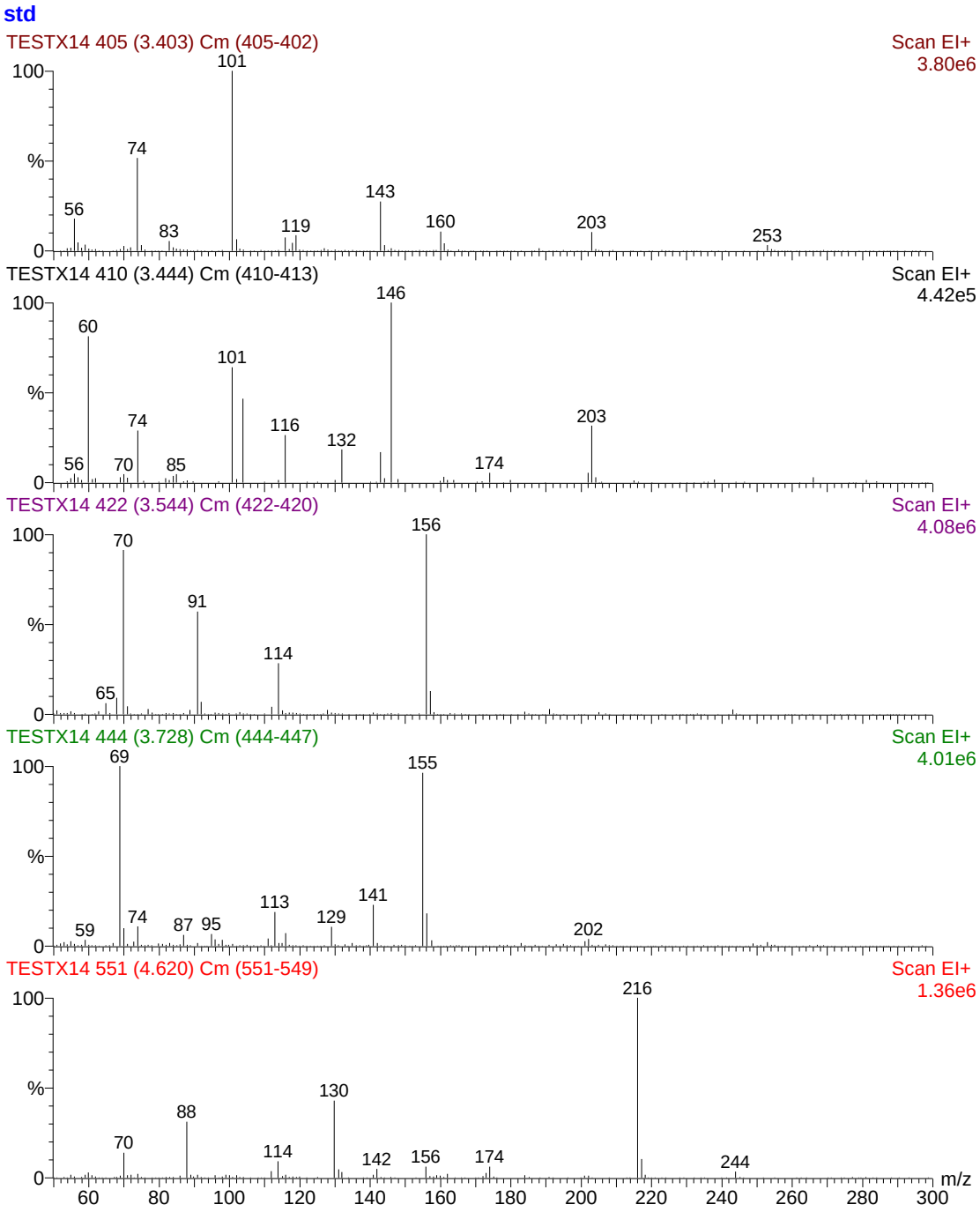


Figure A3: Mass spectra of THR, SER, PRO, ASN, ASP (from top to bottom)

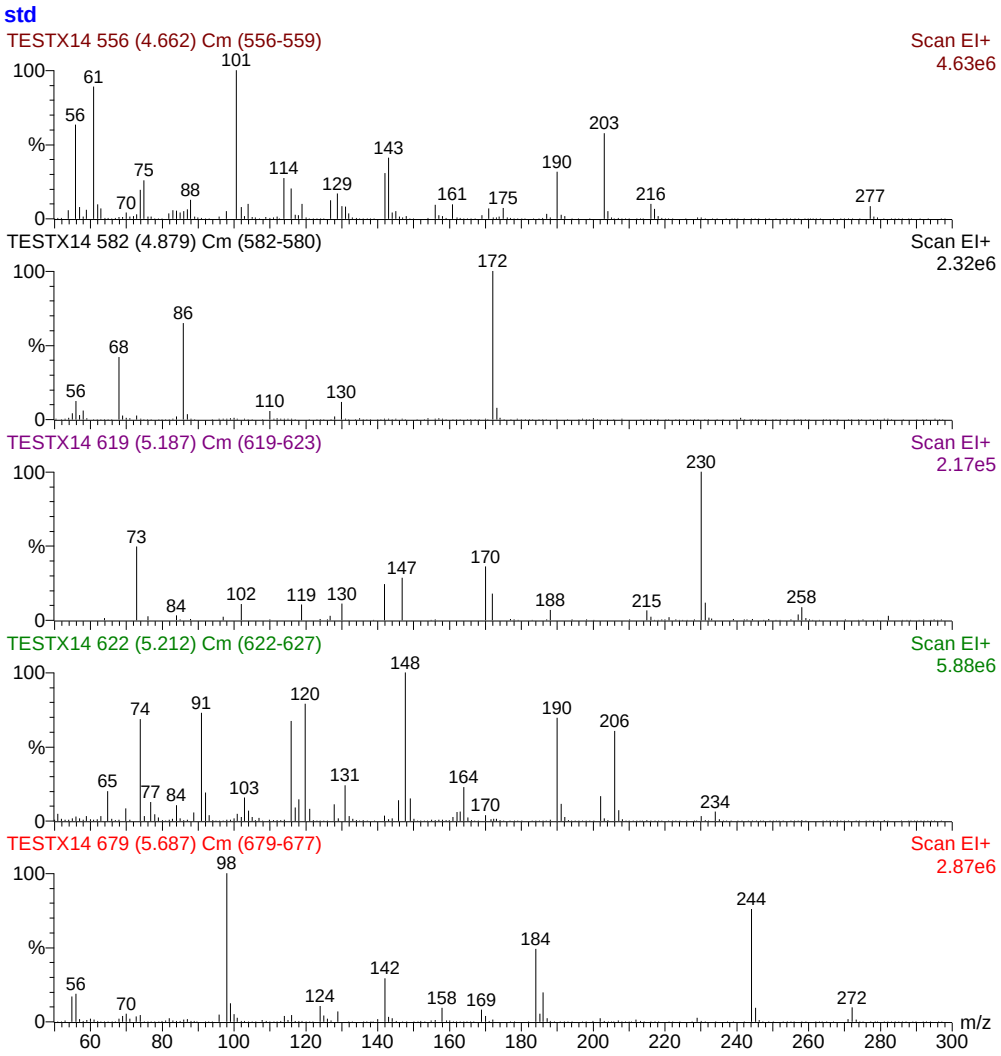


Figure A4: Mass spectra of MET, 4HYP, GLU, PHE, AAA (from top to bottom)

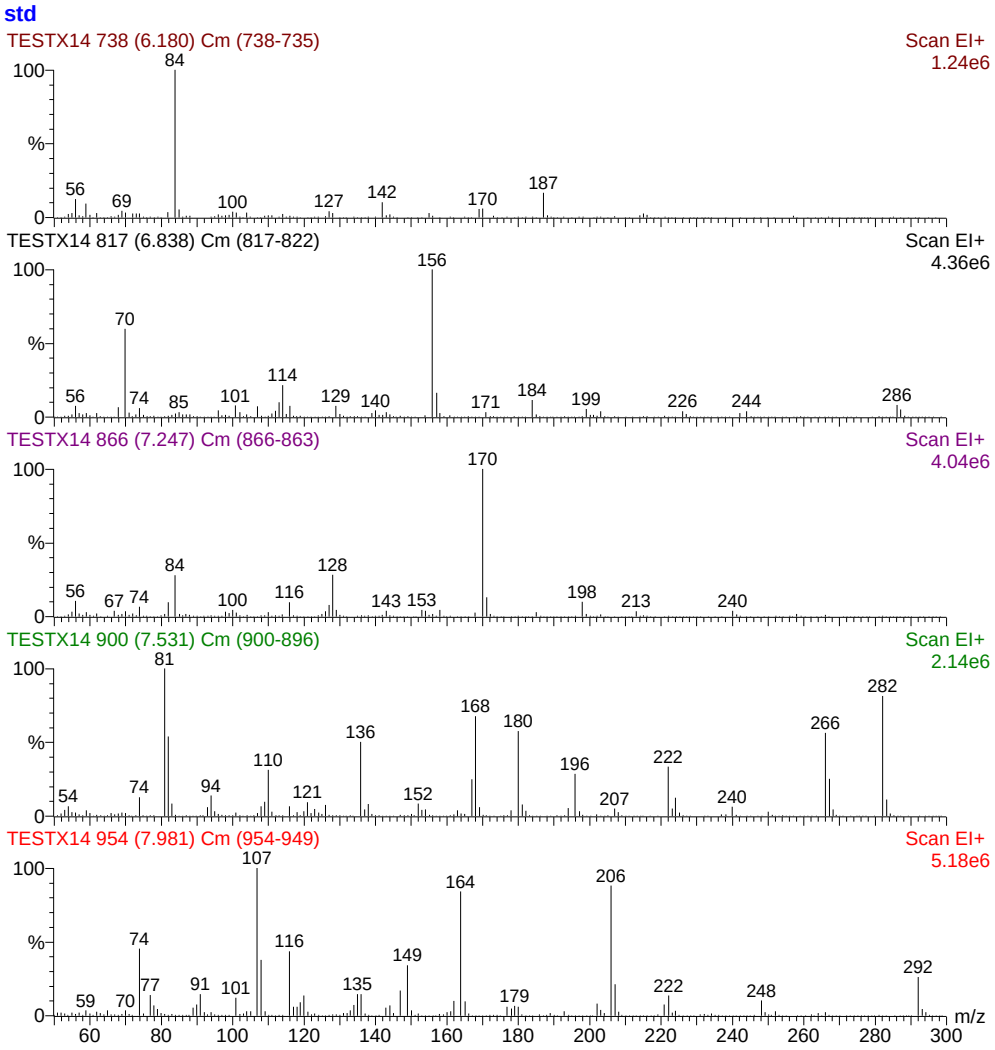


Figure A5: Mass spectra of GLN, ORN, LYS, HIS, TYR (from top to bottom)

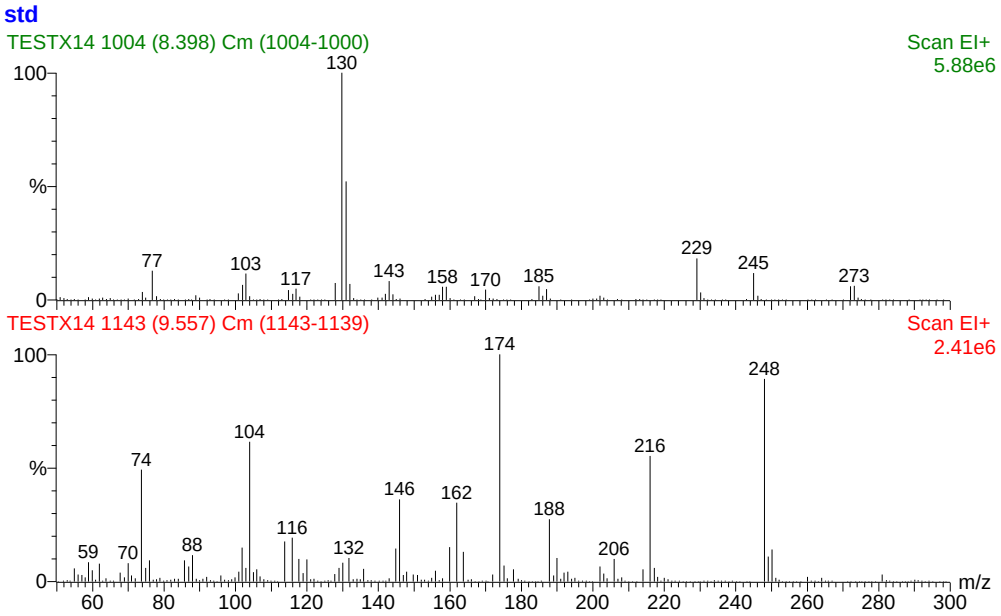


Figure A6: Mass spectra of TRP, C-C (from top to bottom)

Part B: Compounds, Retention time and ions used for their detection.

Table B1: Amino acid retention times and ions for quantification

Amino acid	Retention time	Ion for Quan	Alternative ions
ALA	2.09	130	158/114
SAR	2.19	130	LOW
GLY	2.26	144	101/114
ABA	2.44	144	130
VAL	2.60	158	144/172
B-AIB	2.71	116	LOW
NOR (IS)	2.83	158	116/144
LEU	2.97	172	130/156
AILE	3.00	172	LOW
ILE	3.05	172	116/156
THR	3.40	101	144
SER	3.46	146	180
PRO	3.54	156	243
ASN	3.73	155	116/130
ASP	4.63	130	244
MET	4.66	101	116
4HYP	4.88	172	130
GLU	5.20	172	NA
PHE	5.21	146	NA
AAA	5.69	184	244
GLN	6.18	84	101/156
ORN	6.84	156	114/101
LYS	7.25	116	172
HIS	7.54	180	116/155
TYR	7.98	116	158
TRP	8.41	130	158/244
C-C	9.56	248	146/155

Low- not detected in current runs

NA none available – these compounds must be on-scale for analysis, no other ions can be used to resolve peaks and detect compounds.

Table B2: Ion windows for SIR detection and ions to monitor

Time window	Start- Stop times*	Ions
1	0.0 - 2.35	101, 114, 130, 144, 158
2	2.35 – 2.9	116, 130, 144, 158, 172
3	2.9-3.23	116, 130, 156, 172
4	3.23 - 3.64	101, 144, 146, 156, 180, 243
5	3.64 - 4.18	116, 130, 155
6	4.18 - 5.45	101, 116, 130, 146, 172, 244
7	5.45 – 7.04	84,101, 114, 156, 184, 244
8	7.04 – 7.76	116, 155, 172, 180
9	7.76 – 9.00	116, 130, 158, 244
10	9.00 – 11.00	146, 155

* Note times of windows may vary depending on chromatographic conditions. Preliminary runs with standards should be performed with all ions and then the exact times of windows set for the main analyses.

Adjust times by retention time shifts relative to values in Table B1.

Part C Monitoring ions through specific time window

Due to the number of ions monitored (12) and the narrow peak widths (0.05min, or, 3s) the number of data points per peak was low and could do with improvement i.e. higher temporal resolution. This can be achieved by monitoring ions over specific time periods. This will improve quantitation through better peak shape and providing a greater data density for the peak picking algorithm.

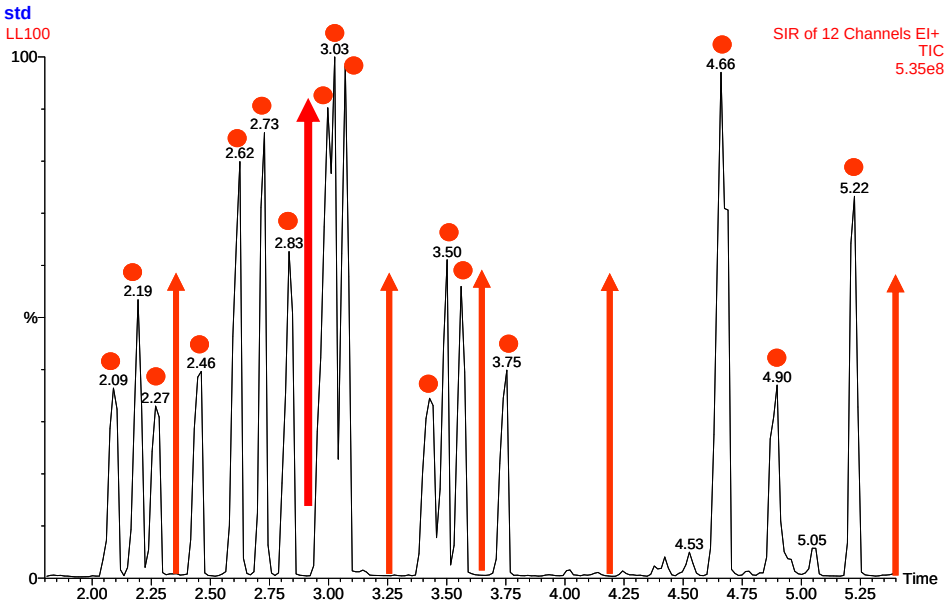


Figure C1: Proposed division of chromatogram into sections for Selected Ion analysis to minimise number of ions monitored per unit time. Dots denote amino acids, arrows transition points in SIR files. 200nmol/ml amino acid standard. 1.8 to 5.4min of chromatogram

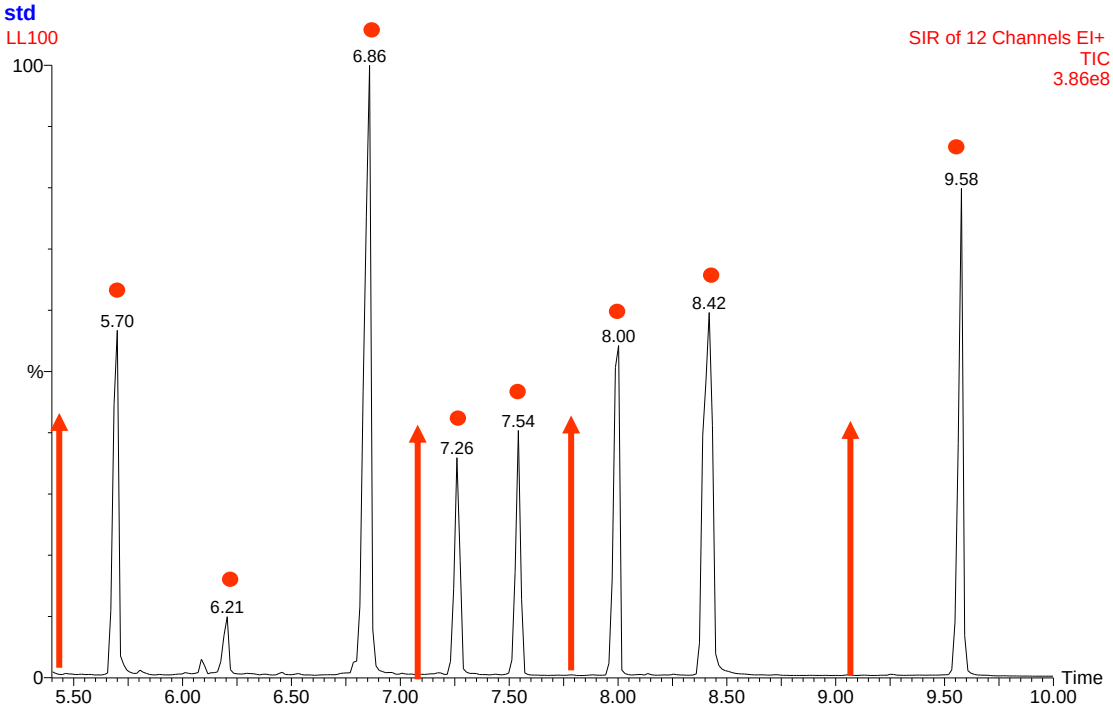


Figure C2: Proposed division of chromatogram into sections for Selected Ion analysis to minimise number of ions monitored per unit time. Dots denote amino acids, arrows transition points in SIR files. 200nmol/ml amino acid standard. 5.4 to 10.0min of chromatogram