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Development of a Lactic Casein based Savoury Flavour Product

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Abstract

The aim of this project was to develop a casein-based hydrolysate formulation that has higher savoury flavour and is more cost effective than an existing commercial savoury hydrolysate. From the literature review, bovine casein protein has the most savoury flavour potential of all proteins due to its high glutamic acid and glutamine content. The symbol of savoury flavour is cheese which is made from casein protein in the western world. The main reaction resulting in cheese savoury flavour development is proteolysis where casein protein breaks down to peptides by protease and free amino acids by peptidases. Two different systems were designed to be based on those reactions in order to generate maximum free glutamic acid during the experiments. The enzyme substrate was a 10% lactic sodium caseinate slurry, which is the foundation of the experiments. With the first system, an enzyme preparation with protease functions was added first and followed by an enzyme preparation with peptidase functions. With the second system only one enzyme preparation with both protease and peptidase activity was added for each trial. From the results, it was found that none of the enzyme combinations from either system were able to achieve the same amount of free glutamic acid as the existing commercial product (31.95 mg/g of protein) within 24 hours. However, multiple options would have had equivalent free glutamic acid if the free glutamine content could be converted to L-glutamic acid using a glutaminase. Flavorzyme 1000L from system one was selected to be the option combining with glutaminase based on its cost, microbiology and chemistry process results. Two different dosages (0.25% and 0.5%) of Glutaminase C100SD were trialled with 2% of Flavorzyme 1000L. From the degree of hydrolysis, free amino acid content, molecular weight profile and residual glutamine results, there were almost no difference between the two trials. The final formulation of Flavorzyme 2% and 0.25% Glutaminase C100SD had 48% more free glutamic acid than the existing commercial control. It also achieved a 33% ingredient cost reduction. Most importantly, the final formulation resulted in a 22.5% final ingredient cost reduction per kilogram based on the same commercial cost model as the control. An informal sensory panel indicated that the new savoury hydrolysate was more savoury and less bitter than the existing commercial control.

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1 Introduction

Dairy flavours have become more and more important for the flavour industry consisting of 12% of US $20.2 billion in 2009 and it has been projected that 27,000 more tonnes of dairy flavours would be sold in 2014 (Anon, 2011). Industry leaders, consumers and retailers have followed six main flavour trends. Those trends are savoury flavours, premium quality, natural and authentic, health and wellness, ethical flavouring and costs (Anon, 2011). The Tatua Dairy Co-operative Company Limited is a dairy company that manufactures niche dairy products with premium quality. The company has extensive knowledge in enzyme-modified applications such as hydrolysis and lipolysis. The Tatua Dairy Co-operative Company Limited already manufactures a savoury protein hydrolysate. However, the hydrolysate takes four days to make and raw material costs are high. The product is also susceptible to out of specification losses due to microbiological contamination resulting from its lengthy hydrolysis time. There is a need to reduce cost so the product can be competitive in global markets. A new developed savoury dairy hydrolysate product should be more potent than the current product thus allowing lower levels of dosage in formulations. This product should be no more expensive than the current product so that gains in addition rate are not offset by increased costs. Additionally, a less complicated process with a shorter hydrolysis time is preferred.

2 Literature review

The goal of this thesis is to develop a cost effective bovine casein hydrolysate with a savoury flavour. The new savoury casein hydrolysate should be more potent than the current product to allow lower levels of dosage in food formulations. The purpose of this literature review is to investigate savoury flavour by understanding: what savoury is and how it is developed in foods. Because cheese is the symbol of savoury food in western diet, the review focuses on savoury flavour development from cheeses and identifies potential taste-active compounds contributing to the flavour. Savoury development mechanisms such as proteolysis during cheese maturation, and accelerated cheese ripening including enzyme modified cheese are covered. The molecular species responsible for savoury flavours are also reviewed. Other protein sources such as fish, soy and wheat are also considered in terms of research identifying savoury flavour compounds in their hydrolysed products.

2.1 Definition of "savoury"

Gustatory sensation is a process where molecules or ions interact with taste receptors/channels of taste cells in the mouth, resulting in the perception of five basic tastes: sweet, salty, sour, bitter and umami (Kawai, Uneyama and Miyano, 2009 and Uneyama, Kawai, Sekine-Hayakawa and Torii, 2009). Umami is the now accepted Japanese word that describes savoury flavour. The molecules and ions interacting with taste receptors are generally hydrophilic compounds that are extracted from foods into the saliva and then act on the taste cells (Kawai et al., 1909).

Umami taste was determined relatively recently as a fifth sensation in 1908 by Dr. Kikunae Ikeda at the Tokyo Imperial University who discovered a taste active component that was salts of L - Glutamate from exacts of seaweed and the sensation of the taste was named "umami" (savoury taste) (Kawai et al., 2009 and Uneyama et al., 2009). Kawai et al. (2009) stated that because glutamic acid represents umami/savoury taste, the human instinct for protein nutrition is likely initiated by umami taste from foods. Tomato and cheese have been found to be rich sources of free glutamate, and L-glutamate contributes to the deliciousness of foods (Kawai et al., 1909 and Uneyama et al., 2009). Cheese is one of most popular manufactured protein rich dairy products and savoury flavour is generated from milk proteins during cheese maturation.

2.2 Flavour formation from milk protein in cheese

2.2.1 Flavour formation during cheese maturation overview

Cheese flavours are formed through proteolysis during the cheese maturation process as follows (McSweeney, 2011 and Visser, 1993): Milk proteins are suspended in milk by steric and electrostatic repulsion. This is due to the fact that C – terminal sequences of kappa casein with negative charge help to keep those proteins apart. Rennet, also called milk clotting enzyme, cleaves casein macropeptides (CMPs) from casein micelles that causes casein proteins to coagulate forming cheese curd. Milk clotting enzymes, natural milk proteinases and lactic bacteria proteinases then break curd protein into large peptides. During the maturation process, lactic bacteria (LAB) die and their cells lyse and release LAB peptidases. LAB proteinases and peptidases continue to break large peptides to small peptides. The small peptides are further degraded to free amino acids. The peptides and free amino acids produced contribute to cheese flavour. McSweeney (2011) and Solms (1969) stated that some free amino acids and peptides have savoury, sweet, sour and bitter taste. The production of free glutamic acid is one of most important contributors to savour flavour in cheese.

2.2.2 Factors involved in the proteolysis of cheese

2.2.2.1 Protein – substrate

There are two main protein types in the bovine milk: casein and whey respectively. The majority of whey proteins are drained from cheese curd during cheese manufacture. As a result, almost all the proteins in cheese are casein. The casein family of phosphoproteins that exist in the milk of all mammals are the main proteins in bovine milk; they account for over 80% of the total protein (Horne, 2011). Casein micelles are described as supra-molecules - that is, a system of two or more molecular entities held together and organized by means of intermolecular (non-covalent) binding interactions (International Union of Pure and Applied Chemistry, 1997). Nearly all the casein proteins interact with calcium phosphates in casein micelles. The orientation structure and solubility of casein protein will have a potential impact on enzyme accessibility during proteolysis, which means that a non-micellar casein system is more susceptible to hydrolysis compared to a micellar system. The casein proteins also have low solubility at pH 4.6 (isoelectric point). Therefore, solubility of the casein proteins during hydrolysis has to be considered. αs1-, αs2-, β- and κ-caseins are the four main casein fractions in bovine casein at an approximate proportion of 4:1:4.5:0.5 (Farrell et al., 2004). Native αs1-casein and αs2-casein were predicted to be unfolded and compact proteins with extended coil like conformations, whereas β- and κ-caseins would possess molten globule-like properties (Farrell, Qi and Uversky, 2006).

2.2.2.1.1 Amino acid compositions of bovine caseins

Farrell et al. (2004) summarised amino acid compositions of αs1-, αs2-, β- and κ-caseins. As it can be seen from Table 2, α_{s1}- and β- casein proteins combined represent 85% of total bovine casein proteins. α_{s2}- and κ-casein structures consist of 21 different amino acids. α_{s1}- and β- casein have 20 amino acids each as they do not have cysteine in their structures. Yildiz (2009) compared the total amino acid profile of casein protein with egg white, tuna, beef, chicken, whey, soy and yeast. As it can be seen in Table 2, bovine casein protein had the highest glutamic acid and proline content than the other protein sources. Therefore, casein may be expected to have more potential savoury compounds than the other protein sources. The high proline content might also affect final free glutamic acid content since the bond between proline and glutamine is difficult to be separated by enzymes due to its special orientation. Most common amino acids adjacent to proline are glutamine in bovine casein, which it can be converted to free glutamic acid under certain enzymatic and chemical conditions, which will be discussed later.

Table 1: Amino acid compositions of α_{s1}-, α_{s2}-, β- and κ-caseins (Farrell et al., 2004)

Casein Proteins		**α_{s1}-casein**	**α_{s2}-casein**	**β-casein**	**κ-casein**
Reference Proteins		CN B-8P	A-11P	A2-5P	A-1P
Ratio		40%	10%	45%	5%
Aspartic Acid	Asp	7	4	4	4
Asparagine	Asn	8	14	5	8
Threonine	Thr	5	15	9	15
Serine	Ser	8	6	11	12
Post-translational phosphorylation site	Ser-P	8	11	5	1
Glutamic Acid	Glu	25	24	19	12
Glutamine	Gln	14	16	20	14
Proline	Pro	17	19	35	20
Glycine	Gly	9	2	5	2
Alanine	Ala	9	8	5	14
Cysteine	Cys	0	2	0	2
Valine	Val	11	14	19	11
Methionine	Met	5	4	6	2
Isoleucine	Ile	11	11	10	12
Leucine	Leu	17	13	22	8
Tyrosine	Tyr	10	12	4	9
Phenylalanine	Phe	8	6	9	4
Lysine	Lys	14	24	11	9
Histidine	His	5	3	5	3
Tryptophan	Trp	2	2	1	1
Arginine	Arg	6	6	4	5
Total Amino Acids		199	207	209	169

Table 2: Percentage (%) by Weight of Amino Acid in a Food Protein (Yildiz, 2009)

Amino Acid (% composition)	Protein Type							
	Egg white	**Tuna**	**Beef**	**Chicken**	**Whey**	**Casein**	**Soy**	**Yeast**
Alanine	6.6	6.0	6.1	5.5	5.2	2.9	4.2	8.3
Argnine	5.6	6.0	6.5	6.0	2.5	3.7	7.5	6.5
Aspartic acid	8.9	10.2	9.1	8.9	10.9	6.6	11.5	9.8
Cystine	2.5	1.1	1.3	1.3	2.2	0.3	1.3	1.4
Glutamic acid	13.5	14.9	15.0	15.0	16.8	21.5	19.0	13.5
Glycine	3.6	4.8	6.1	4.9	2.2	2.1	4.1	4.8
Histidine	2.2	2.9	3.2	3.1	2.0	3.0	2.6	2.6
Isoleucine	6.0	4.6	4.5	5.3	6.0	5.1	4.8	5.0
Leucine	8.5	8.1	8.0	7.5	9.5	9.0	8.1	7.1
Lysine	6.2	9.2	8.4	8.5	8.8	3.8	6.2	6.9
Methionine	3.6	3.0	2.6	2.8	1.9	2.7	1.3	1.5
Phenylalanine	6.0	3.9	3.9	4.0	2.3	5.1	5.2	4.7
Proline	3.8	3.5	4.8	4.1	6.6	10.7	5.1	4.0
Serine	7.3	4.0	3.9	3.4	5.4	5.6	5.2	5.1
Threonine	4.4	4.4	4.0	4.2	6.9	4.3	3.8	5.8
Tryptophan	1.4	1.1	0.7	1.2	2.2	1.3	1.3	1.6
Tyrosine	2.7	3.4	3.2	3.4	2.7	5.6	3.8	5.0
Valine	7.0	5.2	5.0	5.0	6.0	6.6	5.0	6.2

2.2.2.1.2 Solubility of the caseins in different pH and temperatures

A key feature enabling proteolysis is enzyme accessibility to the substrate and also the degree of enzyme activity which is dependent on both temperature and pH. Enzyme accessibility is related to not only tertiary and quaternary structures but also overall protein solubility. Therefore, it is important to understand the relationship between hydrolysis conditions (i.e. pH and temperature) and the state of the protein substrate.

Post, Arnold, Weiss and Hinrichs (2010) investigated the solubility of α_s- and β-caseins in different pHs and temperatures. At pH values above the isoelectric point, the caseins are negatively charged and the aggregation is retarded due to electrostatic repulsion (Liu and Guo, 2008; Madadlou, Mousavi, Emam-Djomeh, Sheehan and Ehsani, 2009). The result showed that the temperatures (2°C and 20°C) had no effect on the α_s-Casein solubility in demineralised water at pH 2.0 to 11.0 whereas the pH values had a major impact on the solubility of α_s-Casein. Strange, Van Hekken and Holsinger (1994) determined that α_{s1} – caseins were almost completely soluble in demineralised water between pH 2.0 and 3.5. β-caseins were more soluble at low temperature (2°C) and the solubility was strongly temperature dependent close to the isoelectric point of the casein micelles. De Kruif and Grinberg (2002) noted that decreasing the temperature weakens the strength of hydrophobic attraction and shifts the monomer/micelle equilibrium

in β-caseins solutions towards monomer side at temperatures below 15°C. Huppertz, Grosman, Fox, and Kelly (2004) and Yong and Foegeding (2010) also found that a decrease in pH can dissociate casein micelle through decreasing the stability of the κ-CN layer at low temperature. This could potentially increase casein protein solubility and promote enzyme accessibility during hydrolysis.

2.2.2.2 ***Enzymes***

Enzymes are important to cheese maturation because they break down fatty acids and proteins generating flavours and changing texture. There are three different sources of enzyme in cheese; those from animal source (calf rennet), bovine milk and lactic acid bacteria and each has a significant role in the proteolysis of cheese.

2.2.2.3 ***Rennet***

Calf rennet is widely used in cheeses as the milk clotting enzyme and there are two types of enzymes in calf rennet. The first enzyme is called chymosin (EC 4.3.23.4) with 88 – 94% of the milk clotting activity (MCA) (Sousa et al., 2001), a main enzyme component of calf rennet (Visser, 2003 and Sousa et al., 2001); it prefers to break down the Phe_{105} – Met_{106} bond of κ-caseins (Visser, 1993 and Fox, 2000) but it also hydrolyses the Phe_{23} – Phe_{24} bond of α_{s1}-casein and Leu_{192} – Tyr_{193} bond of β-casein (Visser, 1993). Chymosin is an aspartic acid proteinase with the highest activity in a lower pH range around 4.5 to 6.5 (Visser, 1993). The second enzyme is bovine pepsin (EC 3.4.23.1), which has 6 – 12% of the MCA (Sousa et al., 2001). The pepsin is more pH sensitive than chymosin so the activity of the pepsin is dependent on pH of cheese curd (Fox and McSweeney, 1996). Creamer (1975) and Fox (1970) described that the rate of hydrolysis of α_{s1}-caseins and β-caseins is affected by the accessibility of the substrate molecules as any orientation or structure changes such as aggregation between the molecules can prevent the peptide bonds from being cleaved. Visser (1993) also reported that rennet induced hydrolysis was more effective on α_{s1}-caseins than β-caseins whereas α_{s2}-caseins and κ-caseins are more resistant to rennet enzyme. However, the majority of rennet enzyme activities are lost with the whey stream during the de-wheying process; only around 0 to 15% of the activity remains in cheese curd (Guinee and Wikinson, 1992) although this depends in the curd pH at cutting. In the initial stage of cheese ripening, the role of rennet enzyme is very dominant, especially in cheese made with low cooking temperatures (Visser, 1993).

2.2.2.3.1 Natural milk enzymes

Plasmin (EC 3.4.21.7), a serine proteinase, is a dominant native milk proteinase in milk (McSweeney, 2004, Sousa et al., 2001; Visser, 1993,). Plasmin preferentially attacks peptide bonds at the C-terminal of lysyl, and to a lesser extent arginyl, residues in bovine casein proteins; the enzyme hydrolyses the casein proteins in the following order: β-casein = α_{s2}-casein > α_{s1}-casein > κ-caseins. McSweeney, (2004), Visser (2003) and Sousa et al. (2001) stated that κ-casein is more resistant to proteolysis than the rest of caseins. The three main cleavage sites that Plasmin enzyme hydrolyses are Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108} producing γ-CN (β-CN f29-209), γ_2 –CN (β-CN f106-209), γ_3 –CN (β-CN f108-209), protease peptone PP8 fast (β-CN f29-209), PP8 slow (β-CN f29-105 and f29-107) and PP5 (β-CN f1-105 and 1-107) (Farrell, 2004; McSweeney, 2004). γ-CN components represent large C-terminal parts of β -caseins such as f29-209, f106-209 and f108-209 (Visser,1993). α_{s2}-caseins are very sensitive to plasmin action and they often disappear during cheese ripening whereas κ-caseins seem to be more resistant to the action of plasmin (McSweeney, 2004; Sousa, 2001). Plasmin cleaves eight sites of α_{s2}-casein structure compared to three sites of β-casein (Sousa, 2001). Plasmin has optimal activity around pH 8 but is still active under milk clotting and cheese ripening conditions (Kaminogawa et al., 1972). According to McSweeney (2004) and Visser (1993), higher cooking temperature enhances the ripening activity during cheese ripening as plasminogen activator inhibitors are deactivated by heat that promotes more plasminogen activators converting plasmingen to plasmin, which help to increase degradation rate of the casein proteins.

2.2.2.3.2 Starter proteinases and peptidases from lactic acid bacteria

In New Zealand, all raw milk is pasteurised prior to cheese manufacturing, commercial starter cultures are the only source to provide lactic bacteria. Starter cultures consisting of lactic acid bacteria are widely used in cheese manufacturing to break down casein proteins to peptides and free amino acids. Proteinases and peptidases systems have been extensively studied and reviewed recently (Exterkate, 1995; Kunji, Mierau, Hagting, Poolman and Koings, 1996; Visser, 1993). Olson (1990) identified that four main functions have an impact on processes and they were cheese flavour development and ripening, digesting milk sugar lactose as its energy source, reduction of the redox potential for growth, citrate fermentation and degradation of casein. The degradation of casein has an important role in texture and flavour development as some peptides are generated contributing to the formation of flavours whereas certain peptides gives bitter tasting flavour (Exterkate, 1995; Kunji et al., 1996, Sousa et al., 2001; Visser, 1993). The degradation process is started by a cell envelope associated proteinase (CEP, PrtP and lactocepin) and followed by intracellular proteinases and peptidases such as endopeptidases (PepO and PepF) and exopeptidase that include aminopeptidase, carboxy (PepN, PepC, PepA, PCP) peptidase, proline-specific peptidases (PepX, PepI, PepR, PepQ, PepP), dipeptidases (PepV, PepD, PepDA) and tripeptidase (PepT) (Exterkate, 1995; Kunji et al., 1996; Visser, 1993).

Endopeptidase is a type of peptidase that can break down peptide bonds within an amino acid chain. Exopeptidase only can break down peptide bonds from either N-terminal (aminopeptidase) or C-terminal side (carboxypeptidase) of an amino acid chain. Dipeptidases only recognise and hydrolyse peptides consisting of two amino acids whereas tripeptidases only recognise peptides with three amino acids. Proline-specific peptidases hydrolyse peptide bonds around proline amino acids. All these proteases and peptidases break intact casein proteins down to various lengths of peptides and free amino acids.

2.3 Savoury flavour development during maturation of cheese

The peptides and amino acids released by the enzymes are a complex mixture. Multiple studies have been conducted to determine which free amino acids and peptides contributed to savoury taste in cheeses by extracting the water soluble portion from cheeses. Different types of cheeses have been considered such as Cheddar, Comté, Gouda and Parmesan-type cheeses (Anderson, Ardo and Berdie, 2010; Mayer and Fiechter, 2013; Molina, Ramos, Alonso, and Lopez-Fandio, 1999; Salles, Septier, Roudot-Algaron, Guillot and Etiévant, 1995; Subramanian, Alvarez, Harper and Rodriguez-Saona, 2011; Toelstede, Dunkel, and Hofmann, 2009; Toelstede and Hofmann, 2009. Individual free amino acids were also studied (Berg, 1953; Meister, 1965; Schiffman and Engelhard, 1975; Schiffman and Dackies, 1975; Shallenberger and Acree, 1971; Yoshida and Saito, 1969). These studies will be discussed in the sections below.

2.3.1 Free amino acids with savoury properties

Cheese flavour intensity is related to free amino acid concentrations. Molina et al. (1999) determined that the water soluble fraction from bovine cheese (cheese type unknown) with highest free amino acids content (178 mg/ml), also had the strongest intensity of cheese-like taste. Glu, Val, Leu, Gln, Asn, Asp, Thr and Ile were the main amino acids in the fraction. Schiffman and Engelhard (1975) summarized the taste of individual amino acids from Berg, 1953; Meister, 1965; Schiffman and Engelhard, 1975; Schiffman and Dackies, 1975; Shallenberger and Acree, 1971; &Yoshida and Saito, 1969 and found that the majority of free amino acids have flavour but glutamic acid and glutamine were the only amino acids described as “meaty”.

Mayer and Fiechter (2013) analysed the free amino acid content of seven commercially produced long ripening Italian extra-hard parmesan types of cheeses. Those cheeses were found to have concentrations of free amino acids up to 9000mg/100g (Mayer and Fiechter, 2013). As it can be seen in Table 3, L-Glutamic acid was a very high percentage of the total free amino acids.

Salles et al. (1995) also investigated two water-soluble extracts of Comté cheese. The first extract was then fractionated by molecular weight. The high molecular weight peptides from the first extract were reported to be tasteless or bitter. The second extract obtained from the water soluble portion of the cheese was then fractionated by gel filtration. Two fractions from the second extract also contained a large amount of glutamic acid (614.0mg/L and 475.1mg/L) and a small amount of aspartic acid. Both fractions were described as umami in flavour sensation with an aroma of potatoes and vegetable stock. These findings are compatible with glutamic acid being the major contributor to umami flavour in cheese.

Table 3: Free amino acid (FAA) contents in commercial cheese samples of Parmesan-type extra-hard cheese retailed as pre-packed slices (Mayer and Fiechter, 2013)

FAA (mg/100g)	Parmesan-type extra-hard cheese retailed as pre-packed slices (n=7)						
	Virgilio Parmigiano Reggiano	S.Paolo Caseifico Parmigiano R.DOP	Parmigiano Reggiano, 24 months	Zarpellon Grana Padano Selezionata	Despar Grana Padano DOP	Grana Padano	Grana Padano, 14 months
ASP	388	311	377	195	188	218	212
SER	599	665	788	278	272	299	317
GLU	1633	1331	1695	1138	1101	1278	1132
GLY	247	216	235	143	138	209	156
HIS	302	260	298	315	300	322	300
ARG	-	-	393	361	354	-	319
THR	284	297	357	240	232	261	256
ALA	443	394	417	340	338	402	372
PRO	788	902	964	654	630	786	666
TYR	274	195	74	164	149	126	188
VAL	696	578	632	471	448	559	511
MET	236	150	161	146	134	166	156
LYS	1082	927	1124	789	756	904	805
ILE	595	440	456	368	361	474	416
LEU	843	538	519	627	606	664	640
PHE	481	400	429	369	347	393	369
Total	8892	7604	8918	6597	6354	7062	6818

Table 4: The taste qualities for amino acids overview (Schiffman and Engelhard, 1975)

Amino Acid Group	FAA	Schiffman and Dackis	Meister	Shallenberger	Yoshida and Saito	Berg	Solms et al.	Schiffman and Englhard	Composite
I. With aliphatic side chain	Glycine	sweet, pleasant, simple	-	Sweet	sweet	-	sweet	-	Sweet
	Alanine	sweet, not constant over time; possible after taste	sweet	Sweet	sweet umami	-	sweet	-	sweet; possibly complex with bitter after taste
	Valine	flat, unclear, probably slightly bitter, with possible sweet component	flat to bitter	Sweet, bitter	bitter sweet	slightly sweetish, yet bitter	flat	-	flat to bitter; slightly sweet
	Leucine	flat, somewhat unpleasant	flat to bitter	bitter	bitter	flat, faintly bitter	bitter	-	flat to bitter
	Isoleucine	flat, somewhat unpleasant	bitter	bitter	bitter	bitter, weakly astringent	flat	-	flat to bitter
II. With side chains containing hydroxylic group	Serine	sweet	faintly sweet	sweet	sweet, possibly sour, umami	faintly sweet, stale after taste	flat	-	flat to sweet; possibly sour, complex
	Threonine	slightly sweet; soft, moderately fatty	faintly sweet	sweet, bitter	sweet, sour	fairly sweet	flat	-	flat to sweet; possibly bitter, sour, or "fatty'
III. With side chains containing sulfur	Cysteine	sulphurous, obnoxious (crystallized from acid solution	-	-	-	-	sulphurous	-	sulphurous, obnoxious
	Methionine	possibly bitter, somewhat nauseous	flat	tasteless	bitter	flat	sulphurous, meaty, slightly sweet	-	flat to bitter; possibly sulphurous, meaty, or sweet
IV. With side chains containing acidic groups or their amides	Aspartic acid	sour	slightly bitter	-	sour	-	flat	-	flat, sour, slightly bitter
	Asparagine	-	flat to bitter	tasteless	-	insipid	flat	-	flat to bitter
	Glutamic acid	-	meaty	tasteless	sour, umami	unique	unique, glutamate	salty, with bitter and possibly sour components	unique, possibly meaty, salty, bitter, sour, complex
	Glutamine	sweetish, meaty, somewhat unpleasant	flat	-	-	-	-	-	flat, sweet, meaty, somewhat unpleasant
V. With side chains containing basic groups	Arginine	sharp, alkaline, bitter, complex taste	-	-	bitter	-	flat	-	flat to bitter, alkaline complex
	Lysine	-	-	-	-	-	flat	complex, minerally	flat, complex, minerally
	Lysine HCL	bitter, salty, complex	-	-	bitter, sweet	-	-	-	bitter, complex, salty sweet
	Histidine	-	flat to bitter	tasteless	bitter, possibly sweet	tasteless to bitter	flat	weak, bitter, minerally	flat to bitter, minerally
V.I. Containing aromatic rings	Phenylalanine	bitter, complex	faintly bitter	bitter	bitter	faintly bitter	bitter	-	bitter; possibly complex and strangling
	Tryrosine	flat	flat to bitter	bitter	-	bitter, weakly astringent	bitter	-	flat to bitter
	Tryptophan	bitter	flat	tasteless	bitter	flat	bitter	-	flat to bitter
VII. Imino acids	Proline	complex, salty, sour, possibly sweet	-	sweet	sweet, bitter	flat, slightly sweet	flat, slightly sweet	-	sweet; possibly complex with salty or sour components

2.3.1.1 Glutamic acid

Glutamic acid is the most savoury flavoured of all the amino acids. Glutamic acid (HOOC-CH2CH2CH(NH2)COOH) is regarded to be one of most important free amino acids in cheeses (Anderson et al., 2010; Berg, 1953; Meister, 1965; Salles et al., 1995; Salles et al., 2000; Schiffman and Engelhard, 1975; Schiffman and Dackies, 1975; Subranmanian et al., 2011; Shallenberger and Acree, 1971; Yoshida and Saito, 1969). It is an acidic amino acid, carries a hydrophilic acidic group and has a negative charge around pH 7.4. The amino acid is located on the outer surface of the protein, which makes it water soluble.

There are two pathways to release glutamic acids from bovine proteins (Figure 1). The first method is by hydrolysis, which is to use protease and peptidase enzymes to release glutamic acid from the proteins (Adler-Nissen, 1983; Oshita et al., 2000). The second pathway is to use L-glutaminase to hydrolyse to free glutamines at the amide bond producing glutamic acid and ammonia (Gilbert, Price and Greenstein, 1949; Nandakumar, Yoshimune, Wakayama and Moriguchi, 2003; Ohsita et al., 2000).

Glutamic acids that are adjacent to proline were found to be difficult to release by hydrolysis (Moller, Andrew and Cheeseman, 1977; Shih, 1985;). Proline has been shown to be resistant to enzyme hydrolysis and may also inhibit the hydrolysis of neighbouring peptide bonds (Moller et al, 1977). However, Tsao and Otter (1998) found that proline did not affect releasing glutamine from β-casein using aminopeptidase M even though there are 38% of the glutamine residues are adjacent to proline. Glutamine can also convert to pyro-glutamic acid which will be discussed later.

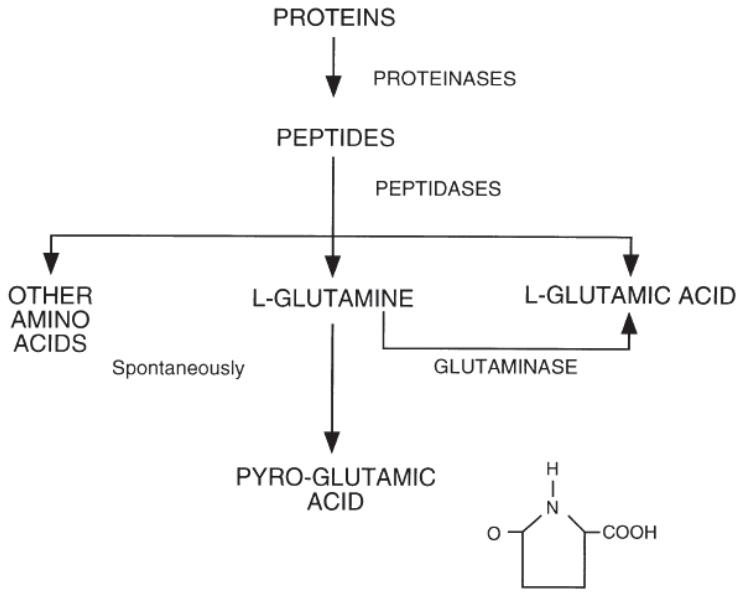


Figure 1: L-Glutamic acid formation during enzymatic hydrolysis of proteins (Oshita et al., 2000)

Research studies also verified the importance of free glutamic acid to flavour development in cheeses (Anderson et al., 2010; Subranmanian et al., 2011). Amino acids, organic acids and changes were monitored during cheddar cheese ripening (Subranmanian et al., 2011). The research confirmed that glutamic acid was very important in Cheddar cheese ripening and flavour development. Twelve Cheddar samples were ripened for 73 days and the samples were tested periodically. Glutamic acid and leucine had the most significant increase in concentration in all 12 samples. Glutamic acid increased from 14 nmol/g to 65 nmol/g whereas leucine increased from 8.1 nmol/g to 66 nmol/g in 73 days. The main amino acids at the end of the cheese ripening were leucine, glutamic acid, asparagine, phenylalanine, valine, ornithine, lysine, glutamine, alanine, and glycine. Anderson et al. (2010) studied taste-active compounds including peptides from water-soluble extract of mature cheddar cheese. Overall, glutamic acid was one of the key drivers for fractions with umami taste. Two taste-active fractions were isolated from each of the two cheeses by gel permeation chromatography (GPC). Glutamic acid was found to be predominating in the two fractions at concentrations of 9.12 mmol/L and 14.35 mmol/L which accounted for 34% and 31% of the total amino acids in those fractions.

2.3.1.2 Pyroglutamic acid

Pyro-glutamic acid is important to consider in the context of cheese flavour because it represents a pool of glutamic acid content that cannot participate in flavour development. Pyroglutamic acid, 5-oxo-L-proline or pyrrolidone carboxylic acid (pGlu) exists either as a free acid or located at the N-terminal of proteins and peptides. It is a by-product of L-glutamine conversion (Figure 1) (Mucchetti et al, 2000). Pyroglutamic acid is converted from glutamine during cheese maturation and this is significant because pyroglutamic acid had no taste and it was difficult to convert pyroglutamic acid back to glutamic acid once formed (Nandakumar et al., 2003). It is controversial how pyroglutamic acid (pGlu) is formed, enzymatic or non-enzymatic.

High pyroglutamic acid content was determined in traditional Italian cheeses. Muchetti et al. (2000) studied the levels of free pyroglutamic acid in traditional Italian cheeses. It was found that pyroglutamic acid was present in high amounts (0.5/100g of cheese) in extensively ripened Italian cheese (Grana Padano and Parmigiano Reggiano) produced with thermophilic lactic acid bacteria as starters and high cooking temperature. The accumulation of pGlu probably was due to its unusual cyclisation of the N-terminal structure that resists proteolysis (Sforz et al., 2009).

Pyroglutamyl peptides were also found in cheeses and wheat hydrolysate. Masotti et al (2010) also found a pyroglutamyl peptide (Pγ3-CN) that originated from the cyclisation of the N-terminal Glu of γ3-CN and increased linearly during ripening of Grana Padano and Parmigiano Reggiano cheese. Low pH value environments such as pH 4.6 were the most efficient for formation of Pγ3-CN in Fontina

cheese (Masotti et al, 2013). Schlichtherle-Cerny and Amadò (2002) identified four pyroglutamyl peptides in wheat hydrolysates: pGlu-Pro-Ser, pGlu-Pro, pGlu-Pro-Glu, and pGlu-Pro-Gln. Those peptides were most likely formed during the pasteurization of the hydrolysates from the corresponding N-terminal glutamine residues.

Interconversions of glutamine, glutamic acid and pyroglutamic acid under physical conditions such as pH and temperature have been studied. Glutamine and glutamic acid can be easily converted to pyroglutamic acid at elevated temperatures and prolonged storage (Shih, 1985). Airauda, Gayte-Sorbier and Armand (1987) determined that glutamine converts to glutamic acid first and then to pyroglutamic acid during heating. They reported that when a glutamine solution was boiled under reflux for 60 minutes with pH ranged 3 to 10, there were no glutamic acids discovered and all the glutamine converted to pyroglutamic acid. Pyroglutamic acids was only converted to glutamic acid when pH of the pyroglutamic acid solution was below 2.5 or above 11 and was boiled under reflux for 60 minutes. Physical conditions that prevent formation of pyroglutamic acid were not considered by Airauda et al (1987).

Oshita et al (2000) determined that an enzyme called glutaminase was able to reduce pyroglutamic acid formation by converting free glutamine to glutamic acid, which left less glutamine to convert to pyroglutamic acid (Oshita et al., 2000). The conversion of glutamine and glutamic acid to pyroglutamic acid is potentially a significant problem in the current project as it is necessary to inactivate enzymes to ensure a food safe and stable ingredient. Typically, enzymes are inactivated thermally.

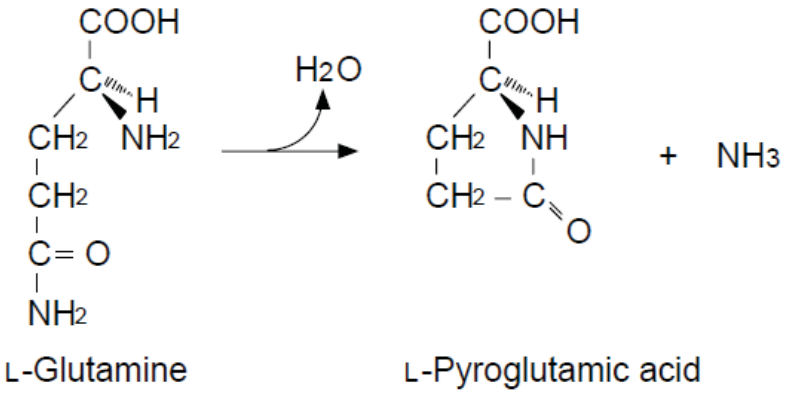


Figure 2: Conversion of glutamine into pyroglutamic acid (Nandakumar et al., 2003)

2.3.2 Savoury peptides from cheeses

Many researchers have focused on flavour functionality of free amino acids in different types of cheeses. Other researchers discovered that there were peptides that do not contain glutamic acids but they still contribute to savoury flavour (Anderson et al., 2010; Salles et al., 1995; Salles et al., 2000;

Subranmanian et al., 2011). Anderson et al (2010) mentioned glutamic acid had a masking effect on salty taste but it was not the only contributor to umami taste.

Umami taste can arise from interactions between compounds or from other compounds and it is frequently found in cheese fractions that do not contain glutamic acid presumably due to other components that were not detected such as nucleotides (Salles et al., 1995). Salles et al (1995) found two gel permeation fractions from water-soluble extract of Comté cheese that had umami taste with cheesy and stock flavour but there was no glutamic acid in the fractions. Salles et al. (2000) further investigated taste compounds in water-soluble extract of goat cheeses. Two types of goat cheeses were investigated, Bouton de culotte and Crottin de Chavignol. Ten fractions were separated from the water soluble extractions of both cheeses. Two fractions from each cheese were described by the panellists as umami. Yet, flavour amino acids, glutamic acid are responsible for the umami taste were at a much lower concentration than their threshold.

Boosting savoury flavour by adding glutamic acid into taste-active fractions was also trialled. Anderson et al (2010) added glutamic acid into all four taste-active fractions collected from water-soluble extractions of mature cheddar cheese and surprisingly no significant difference was observed for umami taste although a range between 0.95 – 14.35 mmol/L of glutamic acid was spread across all four taste-active fractions. Molina et al. (1999) also found that there was no relationship between the intensity of umami taste and the concentration of glutamic acids in 5 different water soluble fractions that were all lower than 1000 Da from cheeses of cows', ewes' and goats' milk.

2.3.2.1 Kokumi Flavour

A new flavour sensation was discovered called "Kokumi". Kokumi flavour peptides can be generated from dairy protein (casein) during the cheese maturation process, vegetable protein (wheat and soy) during the soy sauce fermentation process and fish protein during the fish fermentation process. These peptides have no taste by themselves but they give mouth feel and long lasting savoury taste when they interact with other compounds in foods. This might be the reason that some researchers only focused on free amino acids before as free amino acids have flavour sensation by themselves. Toelstede et al. (2009) and Toelstede and Hofmann (2009) analysed Kokumi compounds in mature Cheddar, Gouda and Blue cheese.

Toelstede et al. (2009) analysed water soluble extract of a 44 week (WSE44) and 4-week old Gouda cheese (WSE4). 44-week old Gouda was found to have much more mouthfulness and long-lasting taste complexity. Eight α-L-glutamyl and ten γ-L-glutamyl dipeptides were selected as candidate kokumi-enhancing molecules. Only γ-L-glutamyl dipeptides were found to give an enhancing kokumi taste to mature cheese. Generation of γ-L-glutamyl dipeptides was also more favourable than α-L-glutamyl

dipeptides during the maturation. The study determined that γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Met, γ-Glu-Gln, γ-Glu-Leu, and γ-Glu-His were key kokumi molecules from those peptides discovered in the cheeses.

Four week and 44-week Gouda cheeses were further studied by Toelstede and Hofmann (2009). The researchers found that α- and γ-glutamyl peptides were minimal in 4-week old Gouda cheese but the high concentrations of the major glutamyl peptides of γ-Glu-Glu and γ-Glu-Met were detected in the areas which were close to the surface of the 44-week old Gouda cheese. α-Glu-Met was not detected in any sample close to the surface but they were detected in the middle of the 44-week old Gouda cheese, which showed that α-glutamyl peptides were not related to the generation of γ-glutamyl peptides. The researchers concluded that the γ-glutamyl peptides might be produced by an enzyme called γ-glutamyl transferase (GGT) and that GGT-catalysed transpeptidation reactions to produce the γ-glutamyl peptides were enhanced in the surface areas of Gouda cheese where water content was low. Toelstede et al. (2009) revealed that bitter and sour/salty as well as umami are also important molecules that required for Kokumi effect. pH, NaCl and amino acids contributed to kokumi-enhancing activity of the γ-L-glutamyl dipeptides.

Toelstede and Hofmann (2009) also studied Kokumi-active glutamyl peptides in blue cheese and how they were generated by Penicillium roquefortii. Blue Shropshire, a blue veined cheese made from pasteurised milk had very high γ-glutamyl peptides concentration with 3590.0 μmol/kg dm. The cheese also had GGT activity of 0.54 U/g. This result was very interesting as milk GGT is heat sensitive so it should be deactivated during milk pasteurisation process. Therefore, the GGT activity was from P.roquefortii (0.37 U/g of protein), which was generated from the mould of the blue cheese. Free amino acids were also incubated with P.roquefortii to verify the theory. P.roquefortii of 1080 (a type of P.roquefortii) was then incubated with the donor amino acid L-glutamine and a mixture of acceptor amino acids L-glutamic acid, L-leucine, L-methionine, and L-histidine each in a concentration of 25mmol/L to generate γ-glutamyl peptides (γ-Glu-His and γ-Glu-Met) successfully. The researchers also tried to improve the yield of γ-glutamyl peptides. Reducing amino acids concentration from 25 to 6.25 mmol/l was the only factor that improved γ-glutamyl peptides yield as γ-Glu-His and γ-Glu-Met was increased from 7.4 and 2.5 mmol/mol to 16.0 and 25 mmol/mol respectively. Temperature, pH, energy source (glucose and sucrose), atmosphere (anaerobic, aerobic, dark and light), substrate adjustments all did not improve the yield. The research also confirmed the finding by Tomita, Yana, Tsuchida, Kumagai and Tochikura (1990) that neural amino acid L-methionine was the most preferred and most effective amino acid acceptor for the γ-glutamyl transferase that helps γ-glutamyl peptides formation.

2.4 Savoury flavour from other protein sources

Fish, wheat and soy protein have also been studied as alternative savoury sources apart from cheeses (Noguchi, Arai, Yamashita, Kato and Fujimaki, 1975; Noguchi, Yamashita, Arai and Fujimaki, 1975; Schlichtherle-Cerny and Amadò, 2002; Rhyu and Kim, 2011). The savoury peptides that were determined from those proteins shared similar properties: Firstly, they were all acidic and low molecular weight peptides. Secondly, glutamic acids in peptides were often present but not essential for savoury flavour.

A fish protein hydrolysate was investigated by Noguchi et al. (1975[a]). The fish proteins were treated with Pronase giving a high content of glutamic acid and there was no bitterness created. The glutamic acid content was increased by three fold from 13.05% to 40.2% w/w. Interestingly, the low molecular acidic fraction was found to be completely non-bitter and have a brothy taste in spite of not containing any free glutamic acid. It was also found that acidic oligopeptide fractions have the ability to mask the bitterness caused by some neutral oligopeptides and amino acids. Noguchi et al (1975[a]) also indicated that peptides with high molar ratios of Glu had a mono sodium glutamic acid (MSG) like flavour and dipeptides with L-Glu and a hydrophilic C-terminal gave umami taste too. Furthermore, Fujimaki, Arai, Yamashita, Kato and Noguchi (1973) also found that Pronase itself had a potent MSG like flavour as well as bitter flavour, and that a low molecular weight acidic peptide fraction contributed significantly to this MSG-like flavour activity. The highly acidic hydrophilic L-glutamyl oligopeptides (an N-terminal Glu residue) also possessed an umami taste (Arai, Yamashita, Noguchi and Fujimaki, 1973). Noguchi et al. (1975[b]) further analysed a different fish protein hydrolysate that at least four dipeptides, Glu-Asp, Glu-Glu, Glu-ser and Thr-Glu and five tripeptides, Asp-Glu-Ser, Glu-Asp-Glu, Glu-Glu-Glu, Glu-Gly-Ser and Ser-Glu-Glu with high molecular ratios of glutamic acid provide the flavour perception of MSG. However, those di- and tri-peptides had a weaker flavour intensity than MSG. The results from the two studies showed that fish protein peptides followed a similar trend to cheese protein peptides.

Schlichtherle-Cerny and Amadò (2002) studied taste-active compounds from wheat hydrolysates from three different enzyme hydrolysis combinations. The first wheat gluten hydrolysate (WGH1) used Flavorzyme 1000L that is a protease and peptidase mix for enzyme hydrolysis only. The second wheat gluten hydrolysate (WGH2) used Flavorzyme and glutaminase-c for enzyme hydrolysis and deamination representing at the same time and the third wheat gluten hydrolysate (WGH3) used hydrochloric acid for acidic deamination first and then used Flavorzyme for enzymatic hydrolysis. WGH1 was described as mainly bitter but only slightly glutamate-like, sour, salty. WGH2 had the highest free glutamic acid content 7.6% with a distinct glutamate-like taste and it was less bitter than WGH1. WGH3 had the lowest free glutamic acid content but it had most glutamate-like flavour with the least bitterness in all three hydrolysates. A solution was formulated based on analytic results of

amino acid, organic acids and salts of WGH3 but the sensory results did not match the flavour profiles of WGH3. Fractionation analysis was then performed on WGH3. Seven fractions from WGH3 were detected to have savoury taste and three of seven fractions were described as predominant savoury taste even though they had the lowest free glutamic acid content in three different enzyme hydrolysis combinations. One fraction was also tasted had significantly stronger saltiness then the others. Six out of seven fractions contained low molecular weight compounds (Mr < 700), which indicated that low molecular weight compounds contributed to the glutamate-like taste of WGH3 not free glutamic acids.

Rhyu and Kim (2011) investigated the compounds that contributed to the taste characteristics of doenjang (a Korean traditional fermented soybean paste) water extract (DWE). DWE reduced bitterness intensity in hydrolysed fish protein solution significantly. 5 fractions were successfully isolated from DWE. The fraction with the highest umami taste contained the largest amount of low molecular weight peptides, those with a Molecular weight between 500 and 1000Da, and the concentration of Glu in peptides was 1.5 to 20 times higher than the other fractions and followed by Asp, Gly, Ser or Lys or Thr. The research suggested that that low molecular weight peptides were produced during fermentation. The sub-fractions, acidic, basic, neutral and aromatic peptides were also collected. The acidic peptide fraction had the highest umami taste, the aromatic peptides had slightly umami and basic and neutral peptide fractions had no taste. The research identified a kokumi peptide γ-glutamyl-valyl-glycine in 6 brands of dark-coloured soy sauce, two brands of light coloured soy sauce and one brand of white soy sauce. The peptide was detected in all soy sauce samples. The dark soy sauce samples had the most kokumi peptides and white coloured soy sauce had the least amount. However, the differences among all the samples were not significant. The protein content was also a factor as the dark soy sauces had the highest amount of protein. Suzuki and Yamada (2007) found that the dipeptide, Val-Gly was a substrate of GGT. Kuroda et al 2013 predicted that Val-Gly was generated by protease activity during the fermentation stage and it was then converted to γ-glu-val-Gly via GGT

2.5 Controversial structure of savoury peptides

Researchers also verified that savoury di- and tri-peptides have umami sensation. Van den Oord and Van wassenaar (1997) summarized 31 di- and tri-peptides that were reported to have an umami taste from Arai et al., 1973, Noguchi et al.,1975[b], Ohyama, Ishibashi, Tamura, Nishizaki and Okai, 1988, and Tamura et al., 1990. Twelve dipeptides and 4 tri-peptides from 31 di and tripeptides and additional three dipeptides were synthesised and subjected for sensory assessments. The results of the study are summarised on Table 5. The additional three dipeptides were not reported for umami taste but they were added by Van den Oord and Van wassenaar (1997) as they represented different polarities. Glu-Trp is an extreme nonpolar addition to glutamic acid, Asp-Val is an intermediate polarity in the Asp-X range and Glu-Val is an extension of Glu-X range. Overall, the selected dipeptides were divided into two groups, which were Asp-X and Glu-X. X groups were either polar/hydrophilic or nonpolar/hydrophilic amino acids. None of 19 peptides had umami flavour in pH 6 and 4 solutions. In all Glu-X peptides the umami effect of the parent L-glutamic acid was lost which disagree with the theory of umami Glu-X peptides existence.

Anderson, Schlichtherle-Cerny and Ardo (2008) also investigated savoury hydrophilic di- and tri-peptides isolated/identified from cheeses by Mojarro-Guerra, Amado, Arrigoni and Solm (1991), Roturier, Le Bars and Gripon (1995), Roudot-Algaron, Kerhoas, Le Bars and Einhorn and Gripon (1994) and Schlichtherle-Cerny, Afflolter and Cerny (2003). Those di and tri peptides were Trp-Gly-Tyr, Glu-Ala, Ala-Glu, Gly-Glu, Thr-Glu, Glu-Asp, Asp-Glu, Glu-Ser, Glu-Glu-Glu and Arg-Lys. Anderson et al (2008) used HILIC-ESI-MS to analyse if these di and tri peptides existed in two types of mature cheddar cheeses. Reference amino acids and di- and tri-peptides were used to identify those compounds in water extracts. All amino acids and di- and tri-peptides references were detected by HILIC-ESI-MS but no peaks were detected for di- and tri-peptides from the hydrophilic cheese extract. This might due to the peptide concentration being below the detection level. The author thought that glutamic acid was one of the main contributors to mature cheddar cheese flavour. All small hydrophilic peptides containing glutamic acid have been reported not to accumulate in cheese (Fernandez, Singh and Fox, 1998). None of the alleged taste active hydrophilic glutamyl peptides were identified from the two mature cheddar cheeses.

Table 5: Evaluated peptides, their reported and experimentally determined taste properties (Van den Oord and Van wassenaar, 1997)

Peptide	Supplier / catalogue no.	Taste as reported (pH)	Threshold concentration (reported) mM	Original reference	Concentration (mM) tasted in present work	Taste perceived in Van den Oord and Van wassenaar, 1997 (pH 6.0)
Asp - Asp	Sigma; A6416	Salty/umami (6.0)	4.79 mM	Tamura	2.67 / 14.60	Not umami, no other taste at either level
Asp - Glu	Sigma; A1916	Salty/umami (6.0)	1.25 mM	Tamura	2.67 / 10.0[a]	Not umami, no other taste at either level
Asp - Gly	Sigma; A8634	Flat (6.0)	200 mg%	Noguchi	2.67	not umami; slightly bitter
Gly - Asp - Gly	Bachem; H1308	Umami ('neutral')	1.5 mM	Ohyama	2.67 / 5.34 / 10.0[a]	Not umami, no other taste at any level
Asp - Leu	Serva; 51315	Bitter (6.0) Umami ('neutral')	300 mg% 2.5 mM	Noguchi Ohyama	2.67	Not umami, no other taste
Asp - Val	Sigma; A6296				2.67	not umami; slightly bitter
Ala - Glu	Sigma; A0378	Umami ('neutral')	1.5 mM	Ohyama	2.67	Not umami, no other taste
Ala - Glu - Ala	Schaaper	Umami ('neutral')	0.8 mM	Ohyama	2.67 / 5.34 / 10.0[a]	Not umami, no other taste at any level
Glu - Ala	Sigma; G3376	Flat (6.0)		Arai	2.67 / 5.34	Not umami, slightly bitter at either level
Glu - Asp	Bachem; G1910	Brothy (6.0) MSG - like ('neutral') Salty/umami (6.0)	 200 mg% 3.14 mM	Arai Noguchi Tamura	2.67 / 10.0[a]	Not umami, no other taste at either level
Glu - Glu	Sigma; G3640	Brothy (6.0) MSG - like (6.0) Salty/umami (6.0)	 150 mg% 2.73 mM	Arai Noguchi Tamura	2.67 / 5.34 / 10.50[a]	Not umami, slightly bitter at any level
Glu - Glu - Glu	Serva; 51352	MSG - like (6.0)	300 mg%	Noguchi	2.67 / 10.0[a]	Not umami, no other taste at either level
Glu - Leu	Schaaper	Bitter (6.0) Umami ('neutral')	 3 mM	Arai Ohyama	5.34 / 10.0[a]	Not umami, no other taste at either level
Glu - Lys	Sigma; G3390	Umami (6.0)	3.12 mM	Tamura	2.67 / 5.34 / 10.0[a]	Not umami, no other taste at any level
Glu - Ser	Bachem; G1980	Weak brothy (6.0) MSG - like (6.0)	 200 mg%	Arai Noguchi	2.67	Not umami, no other taste
Glu - Trp	Sigma; G0505	Umami ('neutral')			2.67	Not umami, no other taste
Glu - Val	Sigma; G3005	Flat (6.0)		Arai	2.67 / 5.34 / 10.0[a]	Not umami, slightly bitter at any level
Val - Glu - Val	Schaaper	Umami ('neutral')	1.5 mM	Ohyama	2.67 / 5.34 / 10.0[a]	Not umami, no other taste at any level
Lys - Gly	Bachem; G2650	Salty/umami (6.0)	1.22 mM	Tamura	2.67 / 5.34 / 10.0[a]	Not umami, slightly bitter at any level

[a] At these concentrations the peptide was tasted also with 0.6% (0.10 M) sodium chloride

2.6 Flavour acceleration in cheeses

2.6.1 Accelerated cheese ripening

Cheese maturation is a long, complex and expensive process that takes large amount of storage space. Table 6 has demonstrated maturation time for six major cheese varieties. This is because proteolysis in cheeses is very slow in particular due to low moisture (Fox et al., 1996).

Table 6: Ripening time for different varieties of Cheese (El Soda and Awad, 2011)

Cheese types	Cheese variety Ripening time (months)
Cheddar cheese	6–12
Swiss cheese varieties	6–12
Blue cheese varieties	3–4
Parmigiano	24–28
Provolone	10–12
Gouda	1–2
Ras	3–4

An extensive review by Fox et al. (1996) highlighted that there are 6 methods for acceleration cheese ripening: (1) elevated ripening temperatures, (2) exogenous enzymes, (3) chemically or physically modified cells, (4) genetically modified starters, (5) Adjunct cultures (6) cheese slurries. Researchers also reviewed advantages and limitations of each method with examples.

Table 7: Methods for accelerating cheese ripening and their advantages and limitations (Fox et al., 1996)

Maturation Method	Example	Advantages	Limitations/Problems
Elevated temperature	-	· Effective · No legal barriers · Technically simple · No cost, perhaps saving	· Non-specific · Increased risk of microbial spoilage · Applicable to relatively few varieties, for example Cheddar
Exogenous enzymes	· Rennet	· Natural additive	· Cheap not effective
	· Plasmin	· Indigenous milk enzyme	· Expensive
	· Other proteinases/ peptidases	· Low cost; specific action; choice of flavour options	· Limited choice of useful enzymes; possible legal barriers; difficult to incorporate uniformly; risk of over-ripening; limited commercial use to date
Chemically or physically modified cells	· Attenuated starters	· Easily incorporated; natural enzyme profile	· May be expensive
	· Other types of bacterial cells	· Easily incorporated; range of enzyme options?	· Perhaps legal problems in some cases
Genetically modified starters	-	· Easily incorporated; · Desirable enzyme profiles	· Possible legal barriers; · Key enzymes not yet identified
Adjunct cultures	-	· Natural microflora; · Appear to be effective; · Flavour options; · Commercially available	· Careful selection required
Cheese slurries	· High moisture cheese	· Very rapid flavour development; commercially used	· High risk of microbial spoilage; suitable only as a food ingredient
	· Addition of free amino acids to cheese curd	· Choice of flavour	· Maybe too expensive; limited work to date

2.6.2 Enzyme modified cheese

As can been seen from the previous section, accelerated cheese ripening has its benefits and challenges. Low-cost enzyme modified cheese was developed to fill the demand for prepared consumer foods, which required enhancing of an existing cheese taste or to conferring a specific cheese character on a food product (Moskowitz and Noelck, 1987). In general, EMCs are 5 to 25 times stronger than the same variety of natural cheeses (Moskowitz and Noelck, 1987). Enzyme modified cheeses are manufactured from cheese substrate of various ages, exogenous enzymes, other dairy components such as butter, casein, skim milk powder and whey and also flavour potentiator such as monosodium glutamate (Moskowitz and Noelck, 1987; Kilcawley, Wikinson and Fox, 1998; Wilkinson et al, 2011). There are two ways to manufacture EMCs (Kilcawley et al, 1998 and Wilkinson et al, 2011). The first method is to hydrolyse fat and protein of cheese substrate simultaneously (Figure 3) and the second method is to create several flavour components (protein and fat) separately and combine them at the end of the process (Figure 4).

Figure 3: Enzyme modified cheese - one step manufacturing approach (Wilkinson et al, 2011)

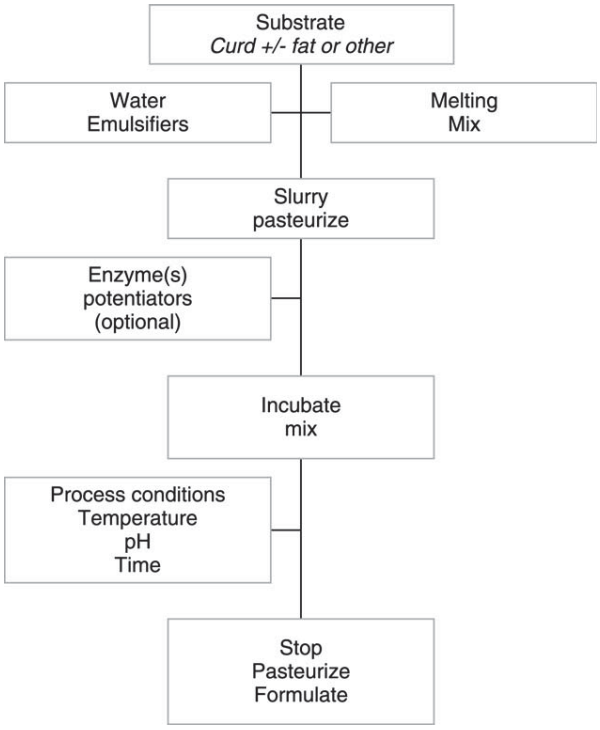
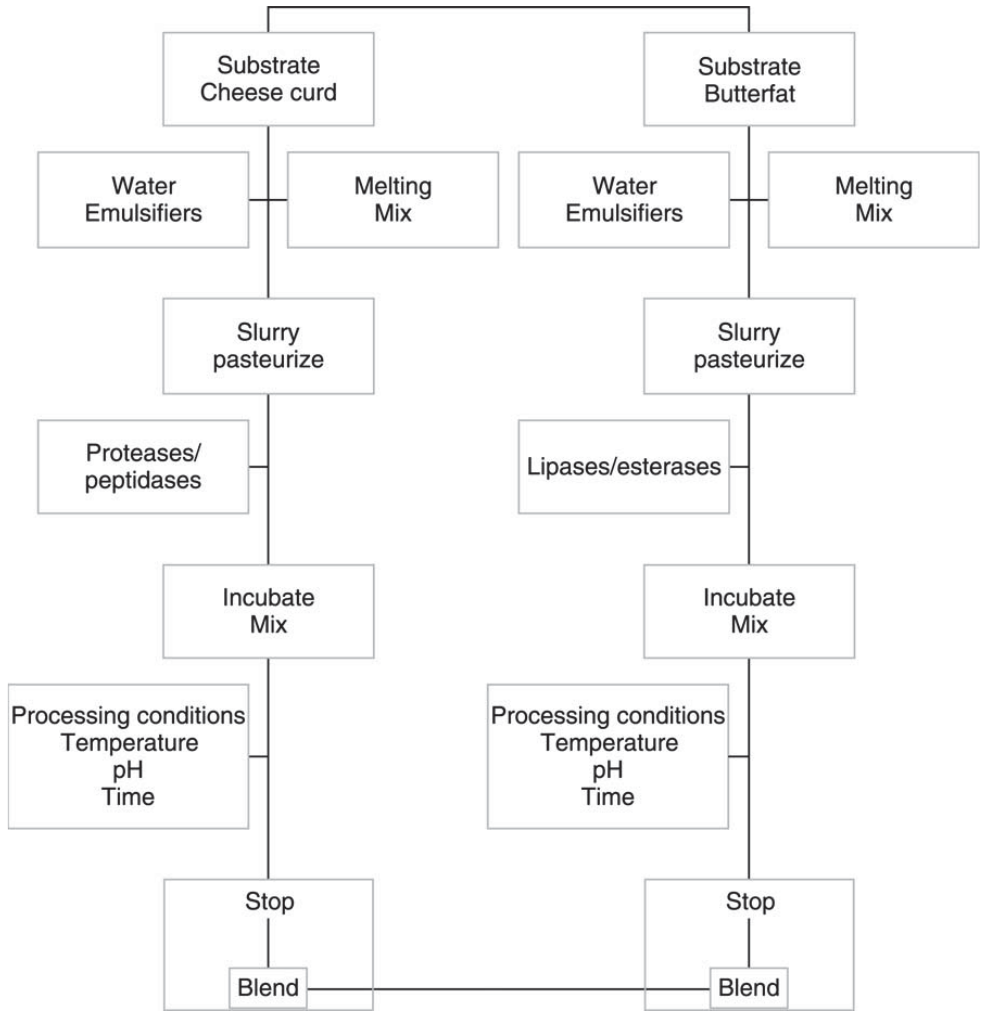


Figure 4: Enzyme modified cheese – component manufacturing approach (Wilkinson et al, 2011)



Kilcawley, Wikinson and Fox (2000) studied fifteen commercial Cheddar EMC products and four natural Cheddar cheeses aged 6 to 12 month from a local supermarket. From the results of Table 8 and 9, the proteolysis of EMCs was more extensive than the natural cheddar cheese resulting in a large amount of small peptides and free amino acids. It was also determined that there were greater batch variations in EMCs from water soluble nitrogen (WSN) and phosphotungstic acid – nitrogen (PTA-N) levels (data not shown). The predominant FAAs in the EMCs were glutamic acid, leucine and lysine. EMC 3A had the highest free glutamic acid of 90% of total FAA, which is very different from the other cheeses and suggests that this cheese had added MSG.

Table 8: Protein, levels of pH4.6 soluble N (WSN) or 5% phosphotungstic acid (PTA-N), molecular weight distribution profile of pH 4.6 water soluble peptides in commercial Cheddar enzyme modified cheese (Kilcawley et al., 2000)

Code	Batch	Protein %	PTA-N %	WSN %	Percentage of total profile %						
					> 20 kDa	20 > 10 kDa	10 > 5 kDa	5 > 2 kDa	2 > 1 kDa	1 > 0.5 kDa	< 0.5 kDa
EMC - 1A	Mean	34.6	65.5	84.6	0.3	0.5	0.4	0.7	2.4	7.4	88.3
EMC - 1B	Mean	33.7	64.5	77.3	0.1	0.4	0.2	0.4	2	4.3	89.4
EMC - 1C	Mean	22.6	42.3	59.5	0.3	0.6	0.9	1.9	2.4	6.7	87.3
EMC - 1D	Mean	33.3	5.1	34.8	0.3	0.6	1.3	8.6	22.8	20.9	45.6
EMC - 2A	Mean	43.9	7.3	25.1	4.2	2.7	3.3	15.1	12.7	11.6	50.3
EMC - 2B	Mean	40.8	6.5	47.4	1	1.7	3.4	17.4	16.4	12.1	48
EMC - 2C	Mean	43.1	6.2	23.9	0.9	1.1	3	20.9	15.4	11.7	47
EMC - 2D	Mean	41.4	14.3	41.2	0.7	1.7	4.4	17.9	15.4	11.4	48.5
EMC - 3A	Mean	18.9	47	57.5	0.7	0.2	0.8	1.4	2.5	5.9	88.2
EMC - 4A	Mean	31.2	21.9	52.1	0.4	0.8	2.2	13.4	17.8	14.9	50.5
EMC - 5A	Mean	37.8	34.6	59.7	0.3	0.4	0.3	1.5	8.1	15.4	74.2
EMC - 6A	Single	43.1	12.2	32.6	1.6	2.3	3.6	13.9	12	12.6	54
EMC - 7A	Mean	31.5	19.5	50.1	4.6	4.7	2.7	7	9.5	13.3	58.5
EMC - 7B	Mean	13.5	30.7	69.4	1.6	3.1	0.3	5.6	8.2	11.3	69.9
EMC - 8A	Single	39.1	5.3	26.8	1.6	4.6	7.7	16	13.9	11	42.1
Natural Cheddar Cheese 1	Single	40.3	1.4	13.1	3	6.4	10.8	25.8	13.2	13.7	27.1
Natural Cheddar Cheese 2	Single	41.1	6.6	26.5	2.4	3.8	7.6	21.5	14.6	14	36.2
Natural Cheddar Cheese 3	Single	39.1	7.5	28.4	1.9	3.3	6.3	20.3	14.6	12.5	41.1
Natural Cheddar Cheese 4	Single	41.7	6.8	20.6	1.7	3.7	6.2	22.3	14.1	11.7	40.5

Table 9: Total free amino acid content and individual free amino acids expressed as percentage of total in commercial Cheddar enzyme modified cheeses (Kilcawley et al., 2000)

FAA Average (%)	ASP	THR	SER	GLU	PRO	GLY	ALA	CYS	VAL	MET	ILE	LEU	TYR	PHY	HIS	LYS	ARG	TRP	Total FAA mg/g Mean (SD)
EMC - 1A	2.4	4.3	4.9	10.2	6.7	1.3	3.0	1.3	7.6	3.5	4.7	11.4	3.2	6.0	4.3	10.9	5.6	6.7	118 (28.5)
EMC - 1B	2.8	4.3	5.7	12.6	6.2	1.4	3.4	1.2	8.0	3.9	4.9	12.9	2.1	7.0	4.8	13.3	5.8	0.0	125 (9.3)
EMC - 1C	3.3	5.1	5.1	13.2	9.8	1.4	3.4	0.5	6.7	3.7	4.4	13.3	7.6	6.3	3.2	9.2	3.7	0.0	72 (13.1)
EMC - 1D	1.2	13.8	3.6	12.0	0.6	1.2	2.7	7.9	4.1	1.2	0.7	18.6	2.1	7.2	12.6	4.6	6.4	0.0	5
EMC - 2A	1.4	2.7	3.4	19.9	11.3	2.0	3.7	2.4	9.4	3.7	3.7	18.6	3.6	7.9	0.5	6.1	0.0	0.0	25
EMC - 2B	3.7	3.6	3.1	20.8	7.9	2.6	4.2	1.7	9.5	3.3	5.2	17.1	3.9	7.6	0.7	5.5	0.0	0.0	62
EMC - 2C	2.3	3.5	4.5	20.4	9.6	2.5	3.2	3.3	9.6	2.2	4.4	17.6	0.0	7.5	0.0	8.5	1.2	0.0	29
EMC - 2D	3.7	2.4	3.4	15.7	9.8	2.2	5.7	2.3	10.2	3.2	5.3	16.7	5.5	7.2	0.6	5.4	1.2	0.0	43
EMC - 3A	0.5	0.4	0.5	90.7	2.0	0.1	0.3	0.7	0.6	0.1	0.4	1.0	0.1	0.2	0.2	1.2	1.0	0.0	134 (56.3)
EMC - 4A	2.1	3.3	3.5	18.4	5.1	1.6	4.0	1.8	10.1	4.1	5.6	14.6	2.2	5.5	4.2	11.7	0.0	0.0	44
EMC - 5A	2.0	3.2	3.4	9.8	2.5	0.4	2.2	2.8	6.0	3.8	4.4	14.0	5.8	9.0	6.6	15.5	5.6	3.6	53
EMC - 6A	0.4	5.0	6.3	5.7	0.0	3.5	6.0	2.9	13.2	4.8	8.2	20.3	1.7	9.2	0.0	12.7	0.0	0.0	44
EMC - 7A	1.2	1.5	2.7	18.3	1.3	0.7	2.4	3.5	4.8	3.3	2.6	18.3	7.1	6.5	4.0	22.2	0.0	0.0	35
EMC - 7BC	4.9	3.7	5.1	12.5	4.0	2.3	5.5	3.8	6.5	2.4	4.4	10.5	5.0	5.7	3.4	11.0	5.2	4.0	29 (8.1)
EMC - 8AC	5.9	3.3	3.3	18.5	7.9	2.2	5.6	0.8	9.5	3.5	5.4	11.9	1.7	6.1	2.8	11.0	1.1	0.0	37
Natural Cheddar Cheese 1	4.4	3.3	9.0	17.5	0.0	1.0	3.1	6.0	0.0	1.1	0.0	21.1	0.0	17.0	5.2	11.4	0.0	0.0	3
Natural Cheddar Cheese 2	2.1	2.3	7.2	20.7	2.8	2.1	2.6	7.4	7.0	2.8	1.7	16.1	3.9	10.8	2.3	6.6	1.7	0.0	21
Natural Cheddar Cheese 3	2.6	2.1	8.1	23.9	2.8	2.3	2.6	1.0	9.9	3.3	1.4	17.1	4.0	8.3	2.3	7.4	0.8	0.0	33
Natural Cheddar Cheese 4	2.9	1.8	6.8	22.2	2.3	1.6	2.6	0.8	12.4	4.0	2.0	15.8	4.0	8.5	1.5	7.1	3.6	0.0	32

2.7 Summary

Savoury amino acids and peptides were reviewed in this chapter. The literature review started from the proteolysis of the cheese maturation process that identified predominant casein proteins and their structures in cheeses, protease enzymes from rennet and milk and proteases and peptidase from lactic bacteria. The review further investigated potential savoury free amino acids and peptides and their structures in various cheeses. From free amino acids, free glutamic acid was described as umami by many researchers. However, there were mixed views around glutamic acid contribution in savoury peptides. This was because some peptides without glutamic acid were also found to be savoury and some peptides with Glutamic acid are not savoury. Moreover, a new type of savoury flavour was described as Kokumi, the peptides were tasteless themselves but they provided long lasting and mouthfulness savoury sensation when they synergised with other effects such as glutamic acid, pH and salt. Other non-dairy proteins such as fish, soy fish were also reviewed to understand if there were any similarities between different proteins. Finally, accelerated maturation methods in cheese manufacturing and enzyme modified cheeses manufacturing process were examined. Most of researchers focused on savoury flavours in the fundamental of understanding of cheese maturation. There were no published studies concentrating on generating savoury flavour hydrolysate from casein proteins.

The aim of this research is to develop a potent savoury casein hydrolysate that can be used in premium savoury food applications. The key implications from the literature on this project will involve:

- Minimisation of glutamic acid conversion to pyroglutamic acid during enzyme inactivation
- The importance of selection of enzymes
- Optimisation of hydrolysis conditions by proteases and peptidases
- Optimisation of hydrolysis conditions by physical and chemical conditions such as pH, temperature and substrate concentration

3 General approach

3.1 Choice of substrate

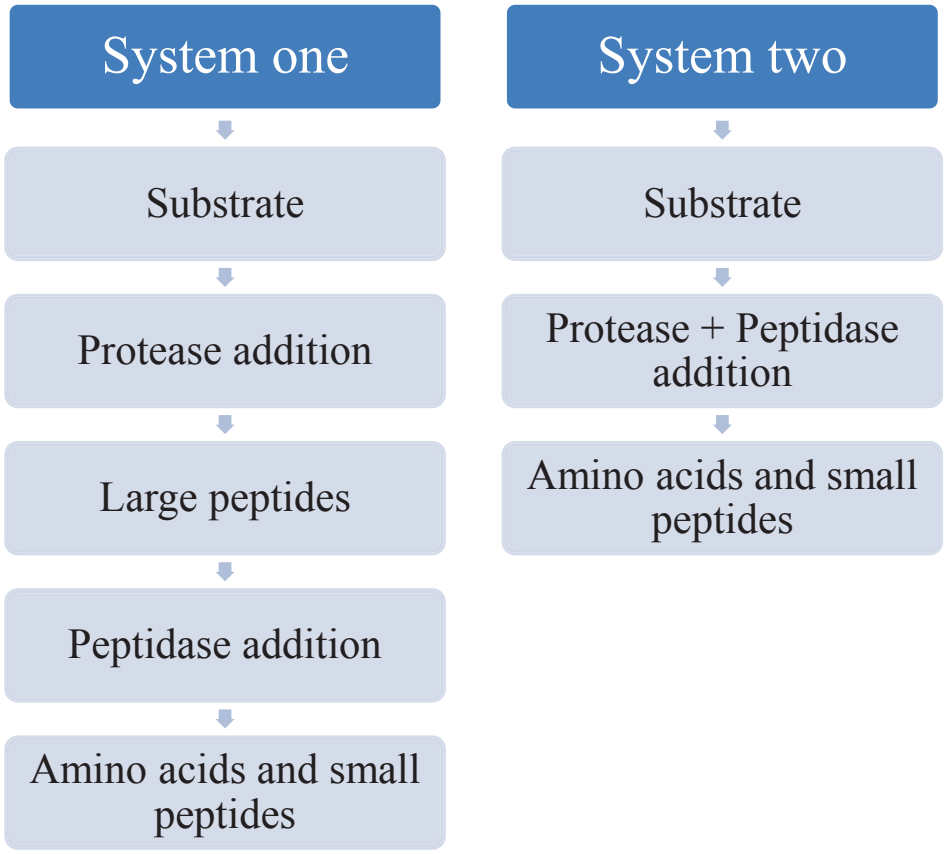
From the literature review, bovine casein protein is a main ingredient of cheese manufacturing. There are two casein products that are manufactured at Tatua and they are lactic sodium caseinate and sodium caseinate. Sodium caseinate is made from neutralising casein curd with sodium hydroxide. Sodium caseinate is easier to spray dry and more soluble than casein. Lactic sodium caseinate was selected as the protein substrate of the enzymes over sodium caseinate for the experiments. This was because the lactic casein manufacturing acidification process is very similar to cheese manufacturing. The casein and whey separation process is initiated by acid produced from lactic bacteria during cheese manufacturing, and lactic sodium caseinate follows a very similar process. However, a mineral acid is used for whey and casein seperation during sodium caseinate manufacturing. As a result, lactic sodium caseinate was selected over sodium caseinate.

In order to generate free amino acids especially free glutamic acid from casein proteins, two different types of enzyme activity are needed. The first type is protease activity and the second type is peptidase activity. Among those commercial food grade enzyme preparations, some of them have broad enzyme activities which mean can that they have functions of both a protease and a peptidase. Other commercially available enzyme preparations are either peptidase or protease. Thus some enzyme combinations might only need one enzyme preparation that has both protease and peptidase activities. The other combinations will need at least one protease and at least one peptidase enzyme preparation.

3.2 Enzyme systems

Two different enzyme systems were trialled to determine the best combinations for generating free glutamic acid. System one was a combination of two commercial enzyme preparations that consisted of an enzyme preparation with a protease as the primary activity and a second enzyme preparation with mainly peptidase function. This provided a systematic approach that peptides were produced by proteases first and followed by second peptide hydrolysis to produce free amino acids. System two had only one enzyme preparation with both protease and peptidase functions. Both systems followed the same cheese proteolysis pathway and it was of interest to determine which system was the most efficient in generating free glutamic acid.

Figure 5: System one and system two process flow



3.3 Manufacturing constraints

3.3.1 Microbiology consideration

Four main food safety related microorganisms were considered for the experimental design. The food safety information was collected from New Zealand Food Safety Authority website (Table 10). These bacteria can potentially cause human illness if they are not treated properly. Growth conditions such as pH and temperature of Escherichia coli, Bacillus cereus, Salmonella and Listeria were determined in Table 11. The optimum pH ranges of the most enzymes used for the experiments were around neutral, which was within the growth pH range of those bacteria. As an outcome, temperature was the key microbiology control point for the experiments and involved two steps.

1) The protein substrate slurry is needed to be heat treated before enzyme addition. This ensures that all microorganisms are inactivated in the slurry so it does not seed any microbial growth during enzyme hydrolysis.

2) Temperature of the hydrolysis condition is also a key element to control those food safety related bacteria growth. The temperature of the hydrolysis should be above 50°C to avoid their microbial temperature growth range according to food safety information in Table 11.

Table 10: Information of illness causing food safety bacteria

Bacteria	Food safety information
Escherichia coli (Anon, 2001)	A pathogenic variant of an organism that is generally regarded as harmless. The organism invades the gut and then produces a toxin. The results of infection can range from being asymptomatic to kidney disease and death. It also can grow in the presence or absence of oxygen.
Bacillus cereus (Anon, 2015)	Bacillus cereus is a spore-forming bacterium. It can form spores that are resistant to heating and dehydration and can therefore survive cooking and dry storage. It also produces toxins and causes vomiting or diarrhoea and, in some cases, both. This depends on the kinds of toxin it produces.
Salmonella (**Anon, 2013)**	Salmonella spp. is bacteria that cause salmonellosis, a common form of foodborne illness in humans. Outcomes from exposure to Salmonella spp. can range from mild symptoms to severe disease and can be fatal.
Listeria (Anon, 2001)	Two forms of diseases: Invasive (people with weakened immune systems): Include 'flu'-like symptoms (e.g. fever, headache), diarrhoea, vomiting, meningitis, septicaemia, spontaneous abortion. Non-invasive (high cell number): Diarrhoea, fever, muscle pain, headache, and less frequently with abdominal cramps and vomiting. Attack rate reported to be 74%.

Table 11: Temperature and pH growth conditions of the determined food safety bacteria

Growth Information	Growth Temperature (°C)	Inactive Temperature (°C)	Growth pH	Inactive pH
Escherichia coli (Anon, 2001)	7 to 46 (optimum 37)	> 46	4.4 to 9.0	< 4.4 or > 9.0
Bacillus cereus (Anon, 2015)	Cell: 4 to 55 (optimum 30 to 40) Toxin: 10 – 40 (optimum 20 – 25)	> 55	4.5 to 9.5 (optimum 6 to 7)	< 4.5
Salmonella (Anon, 2013)	5.2 to 46.2 (optimum 35 - 43)	> 46.2	3.8 – 9.5 (optimum 7 – 7.5)	< 3.8 or > 9.5
Listeria (Anon, 2001)	-1.5 to 45 (optimum 37)	> 70	4.4 to 9.4 (Optimum 7.0)	< 4.4 or > 9.4

3.3.2 Plant restrictions

One of the main targets of this project was to significantly reduce hydrolysis time from the 58 hours of the existing process environment to 24 hours. The current process is lengthy and vulnerable to microbiology contamination. The long hydrolysis time of the current process is due to a low pH hydrolysis environment. The pH was intentionally lowered to control microbiology growth. As a result, the activity of the enzymes was decreased because the enzyme preparations have lower activities in a low pH range and thus take longer to hydrolyse casein proteins. Selecting suitable optimum pH and temperature for enzyme preparations will help to reduce hydrolysis time, and operational cost but will need to be balanced by both microbiology contamination and microbial growth rate risk.

4 Enzyme substrate preparation

4.1 Introduction

Tatua lactic sodium caseinate T1300 was selected to make the protein substrate solution. This can help the project to save potential cost and it is also more convenient for production planning. The goal of this stage was to prepare the enzyme substrate in bulk and pack into small packs.

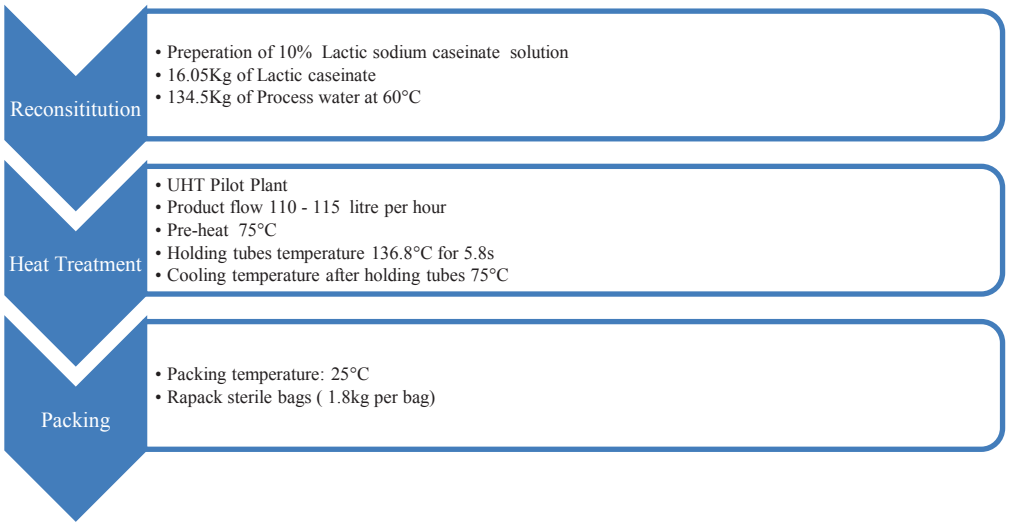
4.2 Materials and methods

The process flow for substrate preparation is shown in Figure 5. Ten percent total solids (TS) concentration was carefully chosen to be the slurry concentration. There was a concern that higher TS might be too viscous and cause processing difficulties. Optimisation of the slurry concentration however was out of scope for this project. The slurry was then heat treated (high temperature short time) through ultra-high temperature (UHT) process plant to sterilise the substrate.

Process water (134.5kg) was added into a temperature controlled stainless steel tank with agitation. The process water was then heated to 60°C. 16.05kg of lactic sodium caseinate was added slowly into the tank and the slurry was mixed for 1 hour after reconstitution.

A SPX UHT pilot plant was setup up in indirect downstream mode. The plant was sterilised at 127°C for 30 minutes using water first and it was then adjusted to pre-heat at 75°C, holding tubes at 138°C and cooling at 75°C. The UHT plant was ready for processing after all the temperatures had stabilised with water. The lactic sodium caseinate slurry was processed at 100 – 110 litres per hour through the UHT plant and packed at around 25°C.

Figure 6: Process flow of substrate reconstitution, heat treatment and packing



4.3 Final format of enzyme substrate

Fifty-four of 1.8kg bags were packed and they were stored in a chiller at 4°C to preserve maximum freshness and quality. The final TS of slurry packed were 10.8% and pH was 6.44. This was a very convenient way to make substrate slurry as they have a long shelf life and all bacteria were deactivated. Moreover, it saved a large amount of preparation time during the trials. Each slurry bag was used for one enzyme combination trial. It saved time by eliminating repetitive lactic sodium caseinate reconstitution and heat treatment steps before enzyme addition.

5 Selection of protease for the two enzyme preparation system

5.1 Introduction

The first stage of the two enzyme preparation system (system one) hydrolysis was designed to determine the most efficient enzyme preparation with mainly protease function from the selected preparations. Based on product information supplied by manufacturers, all the enzyme preparations dosages was set at 1% of dry matter of lactic casein protein. This dosage enabled all the enzyme preparations to be directly compared for product efficiency and cost benefit could be compared easily. The selected enzyme preparation from the stage one was then used to combine with enzyme preparations with peptidase activity producing final products, which was the second stage of hydrolysis.

5.2 Materials and methods

A detailed process flow was drawn in chart 6 for the first stage of system one. The overall process and process conditions for each stage as shown. An enzyme preparation table was also constructed to make sure all critical hydrolysis conditions for each enzyme preparation were followed. All the materials used for experiments were also included in material section. The effective of the proteases was determined by the measurement of DH (degree of hydrolysis) after 8 hours.

5.2.1 Experiment materials and testing methods

5.2.1.1 Experiment materials

Anchor NS1170 ETHANOL (ETHYL ALCOHOL) - Fonterra
Sodium hydroxide (NaOH) 11.9% - Orica
Lactic sodium caseinate slurry bags 10% total solids, UHT treated - Tatua
Stainless steel vessel with lid 2 litre
Eurostar Digital Overhead Stirrer with a blade attachment - IKA
Water bath - Grant
Water bath heating unit – Grant T100
pH meter - Mettler Seven Compact
pH probe - Scott
Brix meter - Atago PAL-1 (0 – 53°Brix)
Osmolality meter - The Advanced Osmometer model 3250
Nutritional indirect UHT plant - Tatua
Spray drier – GEA Niro Minor

5.2.1.2 Testing methods

The test methods used are shown in Table 12.

Table 12: Test parameters and methods of spray dried powder

Test parameters	Method
Degree of hydrolysis	Tatua internal – LCHPM2/4.14/2
Protein content (TN x 6.47)	Tatua internal – LCHPM/3.30/20
Free amino acid profile	New Zealand AgResearch method based on AOAC988.15 and 994.12
Molecular weight profile	Tatua internal - LCHPM2/13.3/2

Table 13: System one – Stage one Enzyme preparation hydrolysis conditions and dosage of proteases

System One Enzyme Preparations: Stage 1	Enzyme Type	Starting pH	Hydrolysis Temperature (°C)	Hydrolysis Time (h)	Dosage (%)
Neutrase 0.8L	Protease	7.52	53	8	1
Protamex	Protease	7.50	53	8	1
Alcalase	Protease	7.53	65	6	1
Protin SD-AY10	Protease	7.50	65	6	1
MaxiproNPU	Protease	7.53	53	6	1
Maxipro BAP	Protease	8.75	53	6	1
Promod 144MDP	Protease	7.50	64	6	1

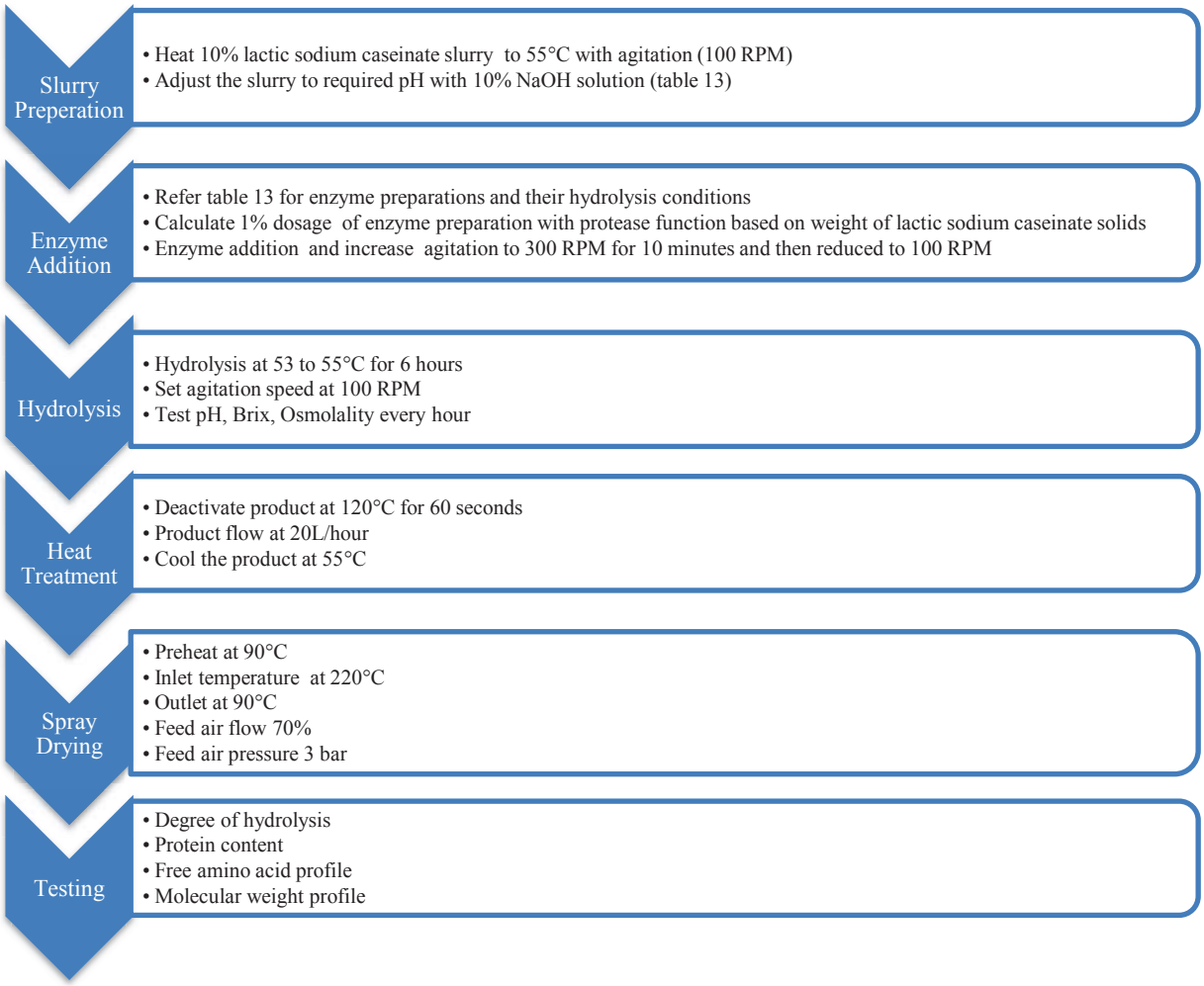
5.2.2 Experiment methodology

A 2 litre stainless steel vessel was sanitised using 95% ethanol and 1750g of lactic sodium caseinate were added into the pot afterwards. A stainless steel stir blade attachment was placed in the pot with a lid positioned on the top of the pot. The whole vessel was then put into a 53°C Grant water bath. The blade attachment was then attached to an IKA power stir head. The agitator speed was set at 100 RPM. The slurry pH was then adjusted from 6.39 to 7.52 using 14.4 g of 11.9% NaOH when the slurry temperature reached 53°C. 1.9g of Neutrase 0.8L was added into the vessel at 300 RPM agitation speed and reduced back to 100 RPM after 10 minutes when the enzyme preparation was mixed homogenously in the slurry. The slurry was tested for Brix, pH and Osmolality every hour for 8 hours. The slurry enzyme was deactivated using a nutritional UHT pilot plant at 120°C at the end of 8th hour. The slurry was then spray dried using Niro Minor spray drier.

The hydrolysis time was adjusted to 6 hours after the first two experiments (Protamex and Neutrase 0.8L) due to pH and Brix osmolality reaching a plateau very early at around 4 hours. This indicated that the majority of hydrolysis had finished. Only Neutrase 0.8L and Protamex were hydrolysed for 8 hours and the rest enzyme preparations were reduced down to 6 hours. It was unnecessary to keep tracking hydrolysis progress when it was almost finished.

The trials for the other enzyme preparations with protease function followed the same process flow and testing regime. The individual enzyme preparation conditions were recorded in Table 13. The final products were tested for degree of hydrolysis, protein content, free amino acid profile and molecular weight profile (Table 12).

Figure 7: Process flow of the stage one of system one



5.3 Results and discussion

Three main trends were observed during stage one hydrolysis. As shown in figure 7, the hydrolysis reaction was very intense in the start for all the enzyme preparations. pH, Osmolality and Brix were stabilised around the 3rd to 4th hour. Firstly, all enzyme preparations had a sharp decrease of pH during the first hour of hydrolysis and which stabilised afterwards. This was due to the production of H^+ during hydrolysis reaction. Secondly, Osmolality also rapidly increased in the first hour and then increased slowly afterwards. Osmolality is a highly effective means of determining the total number of particles in solution. Breaking down of protein enzymatically releases peptides and amino acids, which increases the total number of particles in the slurry. As a result, osmolality increases. Thirdly, Brix decreased in the first hour then gradually increased afterwards. The decrease in Brix was due to initial insoluble peptides produced from the hydrolysis process causing the slurry colour to change from opaque to cream white. Although Brix values started to increase slightly, they were all still under the starting value of 12.8°Brix. This showed that the all the slurries contained large amounts of insoluble peptides. From the three trends, the start of hydrolysis was very important as the majority of enzymatic reaction happened there. The constant agitation was the key as it was required to stop sedimentation of insoluble peptides to the bottom of the hydrolysis silo.

Figure 8: System one –a) pH, b) Brix and c) Osmolality of stage one hydrolysis

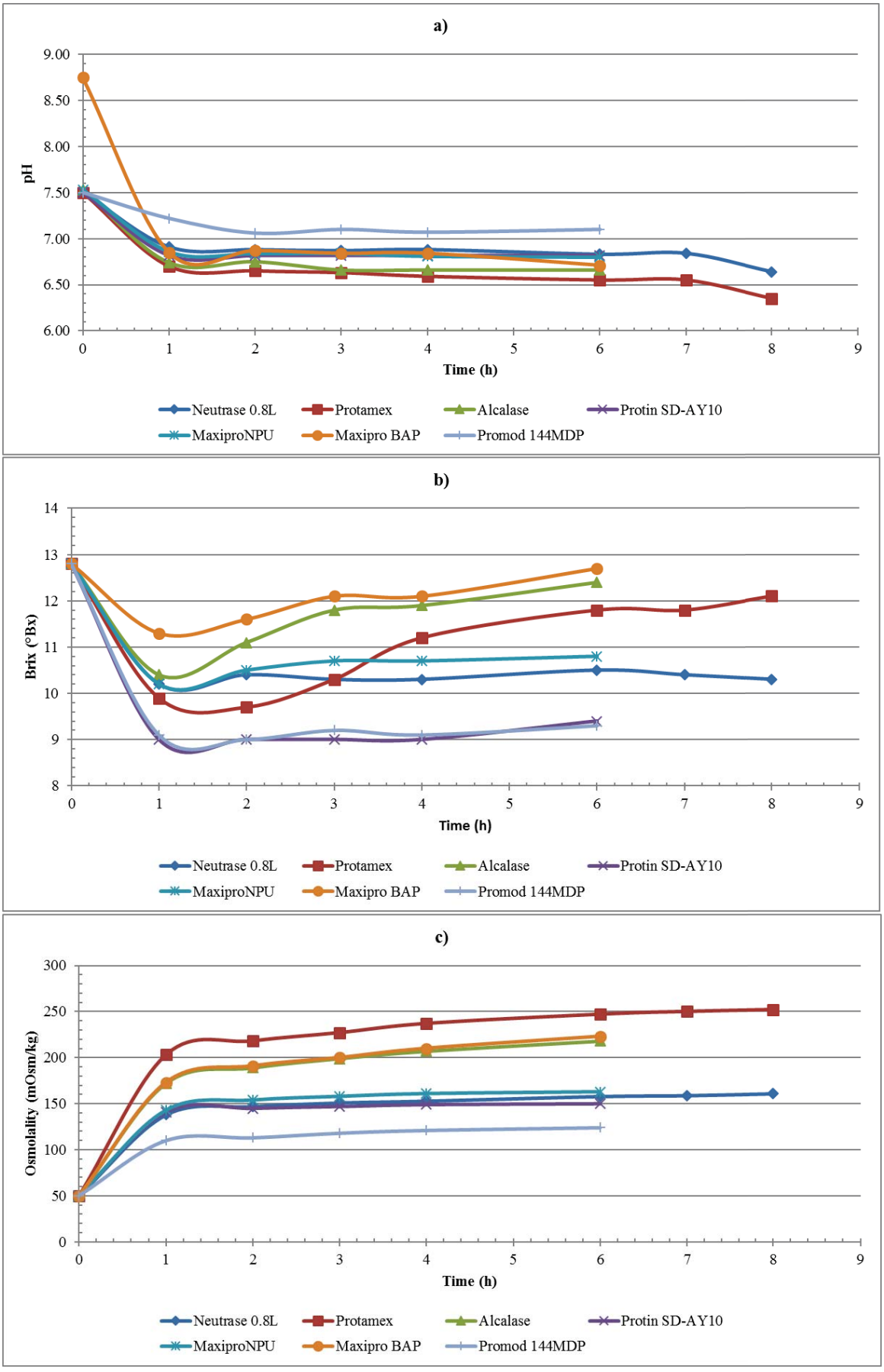


Table 14: Stage one hydrolysate powder results of all enzyme preparations with protease function

Enzyme preparations /Parameters	DH (%)	Protein - N x 6.38 (%)	FAA (mg/g Protein)
Neutrase 0.8L (P4021)	6.9	89.6	0.5
Protamex (P4022)	12.8	87.0	4.8
Alcalase (P4023)	12.1	88.7	13.1
Protin SD-AY10 (P4024)	5.6	90.3	4.1
MaxiproNPU (P4032)	7.6	90.3	0.6
Maxipro BAP (P4033)	12.2	88.3	9.9
Promod 144MDP (P4034)	2.5	90.0	0.5

Table 14, shows the degree of hydrolysis (DH), protein content (measurement in the dried powder) and free amino acid profile (FAA) data for all the enzyme hydrolysis trials. FAA profiles were converted to mg/g of protein. The conversion better reflected hydrolysis performance and it was also more accurate to compare results. From the data of Table 14, Protamex was the most efficient enzyme preparation in terms of functionality. It had the highest degree of hydrolysis value of 12.8%, which means that 12.8% of the total peptide bonds were broken. Alcalase and Maxipro BAP were not far behind with DH value of 12.1 and 12.2% respectively. Promod 144 MDP had the lowest DH value of 2.5%. The interesting finding was that Protamex had the highest DH value and a lower FAA content of 4.8 mg/g of protein compared to Alcalase (13.1 mg/g of protein) and Maxipro BAP (9.9 mg/g of protein). The most likely reason for this was that Protamex produced more small peptides than Alcalase and Maxipro BAP whereas Alcalase and Maxipro BAP produced more free amino acids. The molecular weight profile, figure 8 demonstrated that Protamex was effective in producing peptides below 1000Da whereas Alcalase and Maxipro BAP produced more peptides between 1000 and 5000Da. The molecular weight range supported the theory that Protamex produced more di and tripeptides than Alcalase and Maxipro BAP. Protamex produced 15.2% peptides between 200 and 500 Da whereas Alcalase and Maxipro BAP produced 12.5% and 12.6% respectively. Although Protamex had more intact casein protein (7%) than Alcalase (5.1%) and Maxipro BAP (3.1%), it still produced more short chain peptides than the rest.

Figure 9: Molecular weight profile of Protamex, Alcalase and Maxipro BAP powder hydrolysates

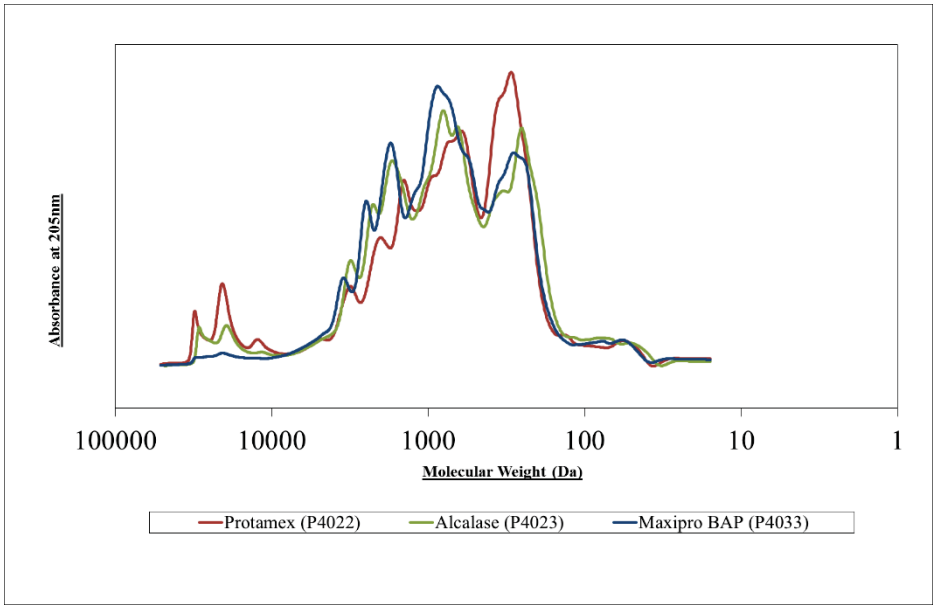


Table 15: Molecular weight profile range results of Protamex, Alcalase and Maxipro BAP powder hydrolysates

Molecular Weight Range (Da)	Protamex (P4022) %	Alcalase (P4023) %	Maxipro BAP (P4033) %
>5000	7.0	5.1	3.1
5000-1000	15.0	19.7	19.4
1000-500	11.9	11.9	13.8
500-200	15.2	12.5	12.6
<200	3.7	3.8	4.0

5.4 Selection of protease considerations

In order to select the most suitable enzyme preparation with protease function, all the enzyme preparations were evaluated against enzyme function, enzyme cost, process chemistry, process microbiology and process complexity (Table 16). Protamex, Alcalase and Neutrase 0.8L all had the highest score with 4 points. All the enzyme preparations had good process complexity and microbiology scores. A simple pH adjustment step was required to adjust the pH to the pre-enzyme addition pH for all the enzyme preparations. The hydrolysis temperatures of all the enzyme preparations were all above 53°C which was over the growth temperatures of Escherichia coli, Bacillus cereus, Salmonella and Listeria. From all the enzyme preparations, Protamex, Alcalase and Neutrase 0.8L passed the process chemistry criteria with higher DH values than the rest of the enzyme preparations. Protamex was the only enzyme preparation that passed enzyme function. Firstly, it was better at smaller peptides

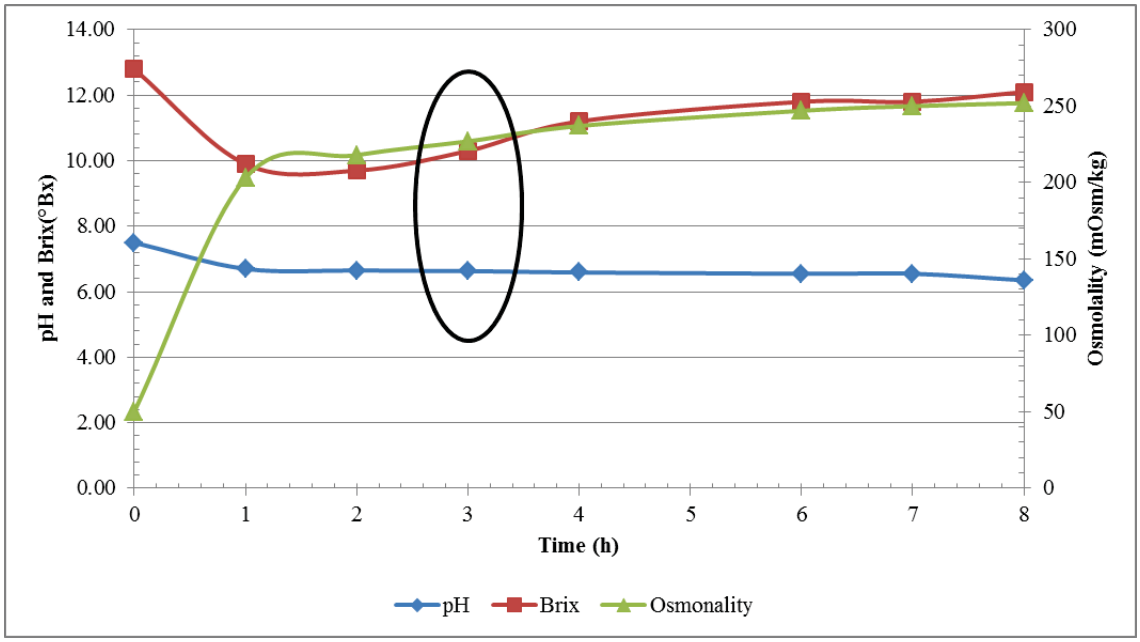
generation (<1000Da). It provided a very good peptide source for the next stage of proteolysis with peptidase. Secondly, it is consisted of two enzymes, derived from Bacillus licheniformis and Bacillus amyloliquefaciens. The Bacillus amyloliquefaciens enzyme has de-bittering function according to its enzyme manufacturer, which could be very beneficial for casein hydrolysis. Alcalase and Neutrase 0.8L contain Bacillus licheniformis derived enzyme only. However, cost of Protamex was more than Alcalase and Neutrase 0.8L. After careful consideration, Protamex was selected as the enzyme preparation to pair with selected peptidases due to its ability to generate low molecular weight peptidases, combination of two different enzymes and its de-bittering function. Although Protamex was more expensive than the other two enzyme preparations, enzyme functionality was considered more important than cost at this stage of processing.

Table 16: Enzyme preparations with protease functionality score board

Enzyme Preparations /Parameters	Enzyme Function (debittering, small peptides)	Enzyme Cost	Process Chemistry (DH)	Process Microbiology	Process Complexity	Score
Neutrase 0.8L (P4021)	-	+	-	+	+	3
Protamex (P4022)	+	-	+	+	+	**4**
Alcalase (P4023)	-	+	+	+	+	**4**
Protin SD-AY10 (P4024)	-	-	-	+	+	2
MaxiproNPU (P4032)	-	+	-	+	+	3
Maxipro BAP (P4033)	-	+	+	+	+	**4**
Promod 144MDP (P4034)	-	+	-	+	+	3

The aim of the protease and peptidase hydrolysis time was 24 hours. It was very important to utilise 24-hour hydrolysis time more efficiently so it was key to add peptidases when protease hydrolysis just reached plateau. The shorter protease hydrolysis time was better as it provided peptidases more time to break down peptides producing smaller peptides and free amino acids. The optimum time point was determined at the end of 3rd hour from Protamex in-process hydrolysis plot (Figure 9). The osmolality reached 227mOsm/kg in the first 3 hours but it only increased by 25mOsm/kg next 5 hours. The stabilisation of pH also showed less amount of H+ produced from hydrolysis. Both Osmolality and pH indicated that the majority of hydrolysis reaction is complete after 3 hours. However, peptidases addition at the 3rd hour of first stage hydrolysis does not mean that protease hydrolysis ends there. Protease hydrolysis reactions will continue but its activity depend on temperature and pH of slurry of selected peptidases.

Figure 10: Protamex in-process hydrolysis trend and its optimum reaction point



Therefore, in summary the first stage of the two enzyme system was selected as Protamex, for 3 hours. Picking one protease greatly decreases the number of experiments required to test system one combinations and makes the combination feasible within the time constraints of the project.

6 Comparison of two enzyme and single enzyme preparation systems

6.1 Introduction

The two different hydrolysis systems were designed to maximise free glutamic acid generation. In system one peptides were initially created by proteases first before they were broken down to smaller peptides and free amino acids in a second enzyme reaction by peptidases. System two was designed with single enzyme preparation containing both protease and peptidase functions. The hydrolysis process of system two was not as ordered as system one; system two hydrolysis was more random with proteases and peptidase breaking down lactic casein protein at the same time. The aim of this section is to compare both systems and determine the best enzyme combination based on enzyme cost, process chemistry, process microbiology, process complexity and free glutamic acid generation.

6.2 Experiment materials and methods

Two detailed process flow diagrams were designed for system one and two. The overall process and process conditions were carefully considered for each stage of both systems. Optimum hydrolysis temperature, pH, time and dosage rate were determined for all enzyme preparations for system one and two. System one had four different combinations and system two had nine different enzyme preparations.

6.2.1 Experiment materials and testing methods

6.2.1.1 Experiment materials

Anchor NS1170 ETHANOL (ETHYL ALCOHOL) - Fonterra
11.9% sodium hydroxide (NaOH) - Orica
10% TS lactic sodium caseinate slurry bags - Tatua
2 litre stainless steel vessel with lid
Eurostar Digital Overhead Stirrer with a blade attachment - IKA
Water bath - Grant
Water bath heating unit (T100) - Grant
pH meter - Mettler SevenCompact
pH probe - Scott
Brix meter (0 – 53°Brix) - Atago PAL-1
Osmolality meter - The Advanced Osmometer model 3250
Nutritional indirect UHT plant - Tatua
Niro Minor Spray drier - GEA

6.2.1.2 Testing methods

The testing methods of degree of hydrolysis, protein content, free amino acid profile and molecular weight profile were the same methods as Table 12.

Test parameters	Method
Degree of hydrolysis	Tatua internal – LCHPM2/4.14/2
Protein content (TN x 6.47)	Tatua internal – LCHPM/3.30/20
Free amino acid profile	New Zealand AgResearch method based on AOAC988.15 and 994.12
Molecular weight profile	Tatua internal - LCHPM2/13.3/2

6.2.2 Experiment methodology

6.2.2.1 System one methodology

A 2 litre stainless steel vessel was sanitised using 95% ethanol and placed on a weighing balance. Lactic sodium caseinate (1750g) was added into the pot. A stainless steel stirring blade attachment was placed in the pot. A stainless steel lid was positioned on the top of pot and the blade attachment went through the hole in the centre of the lid. The whole vessel was then put into a Grant water bath that was set to a constant 53°C. The top of blade attachment was then attached to an IKA power stir head. The agitator speed was set at 100 RPM. The slurry pH was adjusted from 6.40 to 7.60 using 15.2 g of 11.9% NaOH when the slurry temperature reached 53°C. Protamex (1.9g) was added into the vessel when the agitation speed was set at 300 RPM. The speed was reduced back to 100 RPM when the enzyme preparation was mixed with the slurry homogenously after 10 minutes. During the first 3 hours, the slurry was tested for Brix, pH and Osmolality every hour. Promod215P (1.9g) was added as the second enzyme preparation at the end of 3rd hour. Before the second enzyme preparation addition, the agitation speed was increased to 300 RPM. After 10 minutes mixing at 300 RPM, to ensure that the Promod215P homogeneously mixed throughout the slurry the agitation speed was reduced back to 100 RPM.

All the designed in-process tests were continued at the 4th, 6th, 22nd, 23rd and 24th hour of the hydrolysis after mixing. The slurry pH at the end of the hydrolysis was adjusted from the final pH of 6.47 to 6.80 using 9.0g of 11.9% NaOH for product specification reasons. The enzyme preparations were then deactivated using a nutritional UHT pilot plant at 120°C for 60 seconds after the pH adjustment. The deactivated slurry was then spray dried using the Niro Minor spray drier.

All the other three enzyme preparation combinations followed the same process flow (Figure 10) and testing regime (Table 12). The other enzymes were added at the same dosage but temperature and initial pH were changed based on the manufacturer's information and are detailed in Table 17. The final products were tested for degree of hydrolysis, protein content, free amino acid profile and molecular weight profile.

Figure 11: Process flow of system one hydrolysis

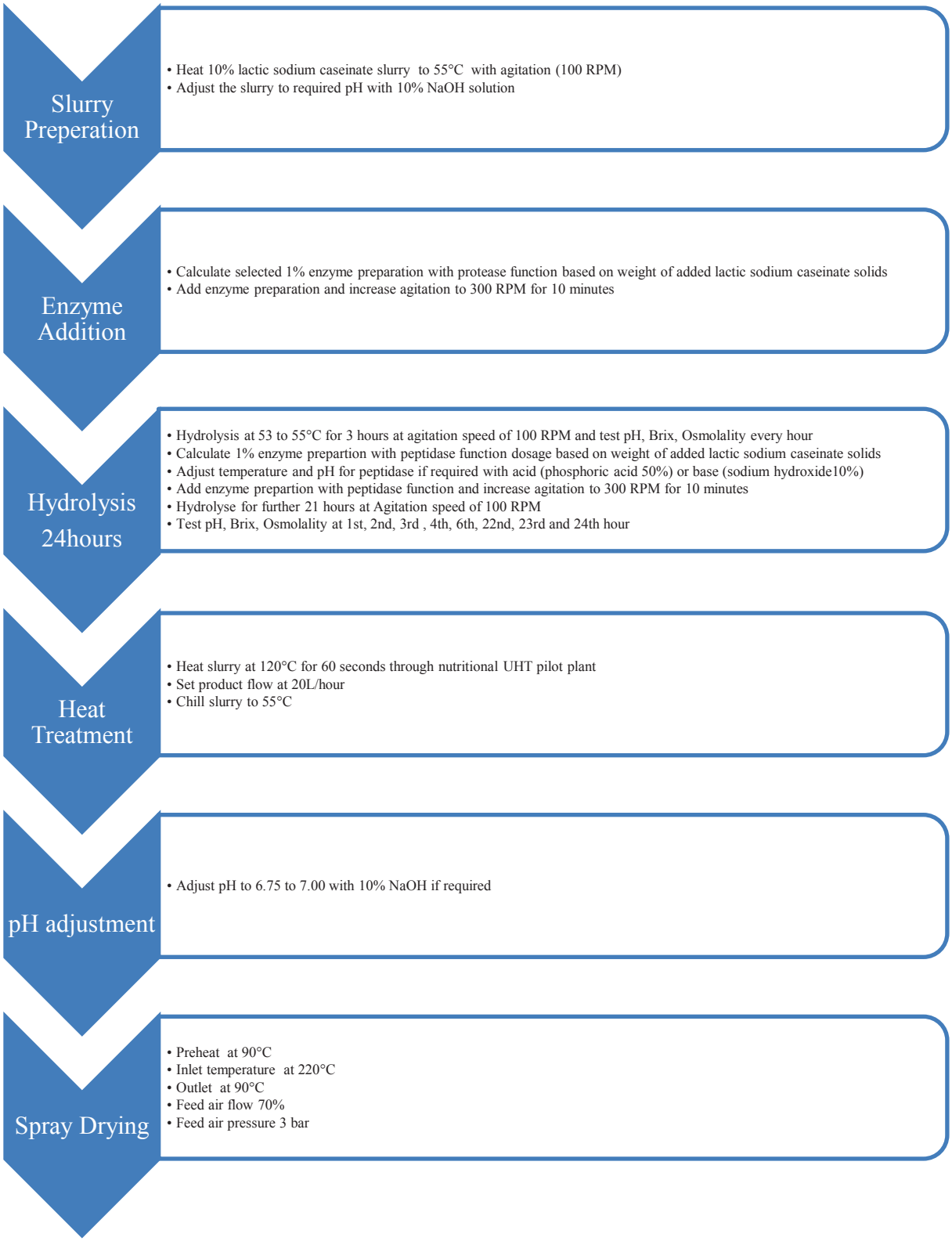


Table 17: System one – Enzyme preparations for hydrolysis step one and two

System One Enzyme Preparations: Step 1	Enzyme Type	Starting pH	Hydrolysis Temperature (°C)	Dosage (%)
Protamex	Protease	7.5	54	1
System One Enzyme Preparations: Step 2	**Enzyme Type**	**Starting pH**	**Hydrolysis Temperature (°C)**	**Dosage (%)**
Promod215P	Peptidase	6.70	54	1
Flavorpro937P	Peptidase	6.67	54	1
PDN N48/3	Peptidase	6.00*	54	1
Peptidase R-K	Peptidase	6.57	45	1

***Adjusted with Phosphoric acid 50%

6.2.2.2 System two methodology

A 2 litre stainless steel vessel was sanitised using 95% ethanol and placed on a weighing balance. Lactic sodium caseinate (1750g) was added into the pot. A stainless stir blade attachment was placed in the pot. A stainless steel lid was positioned on the top of pot and the blade attachment went through the hole in the centre of the lid. The whole vessel was then put into a Grant water bath that was set to constant 53°C. The top of blade attachment was then attached to an IKA power stir head. The agitator speed was set at 100RPM. The slurry pH was adjusted from 6.39 to 7.52 using 14.4 g of 11.9% NaOH when the slurry temperature reached 53°C. 3.8g of Promod 845MDP was added into the vessel at 300 RPM of agitation speed and reduced back to 100 RPM when the enzyme preparation was mixed with the slurry homogenously after 10 minutes. The slurry was tested for Brix, pH and Osmolality at 1st, 2nd, 3rd, 4th, 6th, 22nd, 23rd and 24th hour. The enzyme preparation was deactivated using a nutritional UHT pilot plant at 120°C at the end of 8th hour. The deactivated slurry was then spray dried using Niro Minor spray drier.

All other enzyme preparation combinations followed the same process flow (Figure 11) and testing regime (Table 12). The individual enzyme preparation conditions used are described in Table 18. The final products were tested for degree of hydrolysis, protein content, free amino acid profile and molecular weight profile.

Figure 12: System two hydrolysis process flow

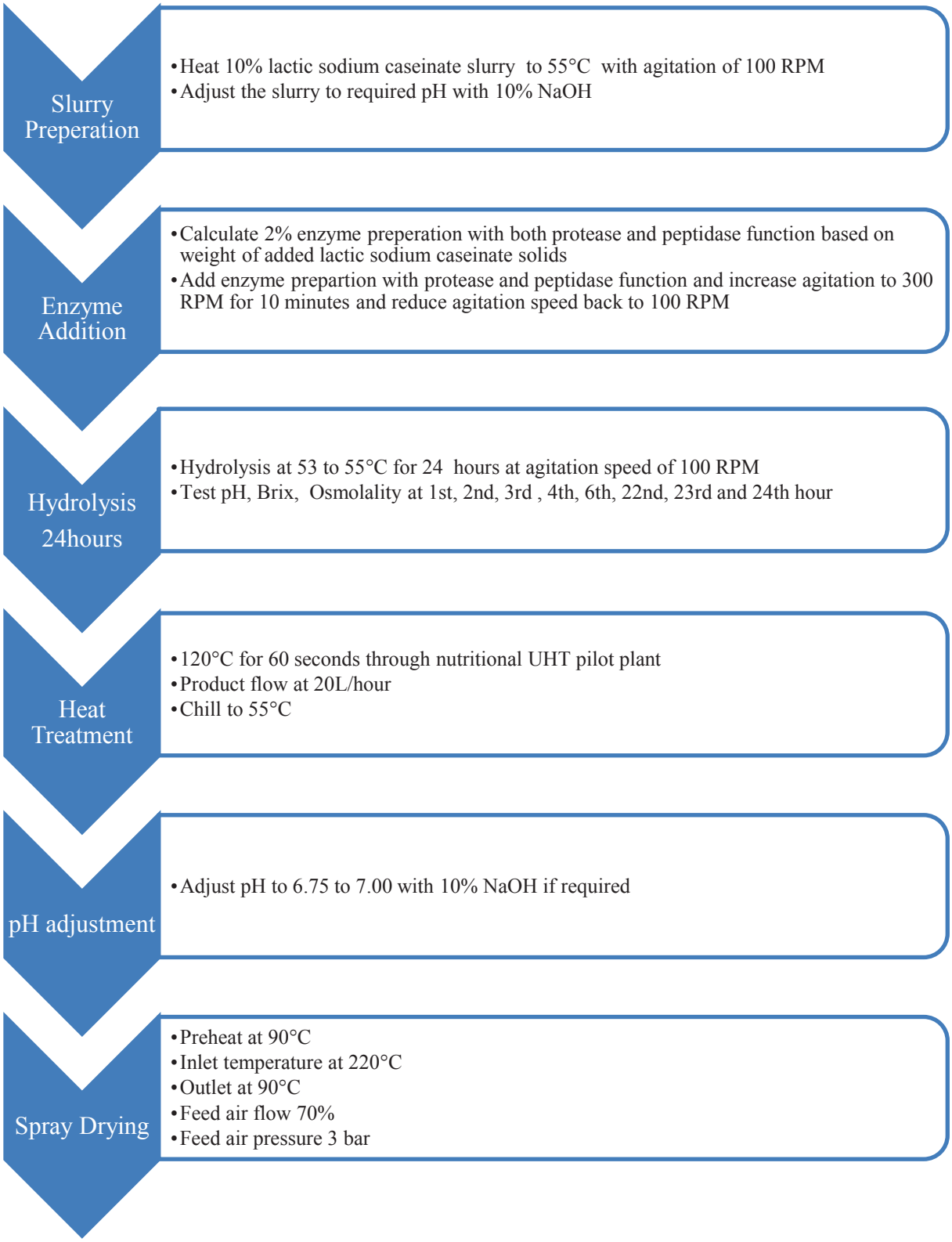


Table 18: System two – Enzyme preparations dosage and hydrolysis conditions

System One Enzymes	Enzyme Type	Starting pH	Hydrolysis Temperature (°C)	Dosage (%)
Promod 845MDP	Protease and Peptidase	7.50	53	2
Promod 903 MDP	Protease and Peptidase	7.54	53	2
Protease A 2SDK	Protease and Peptidase	7.50	53	2
Protease M SDK	Protease and Peptidase	7.00	53	2
Protease AX	Protease and Peptidase	9.05	53	2
Flavorzyme 1000L	Protease and Peptidase	7.50	53	2
Maxipro FPC	Protease and Peptidase	7.50	53	2
Flavopro 852MDF UMAMI	Protease and Peptidase	7.60	53	2
Flavorpro F795MDF	Protease and Peptidase	7.50	53	2

6.3 Results and discussion

6.3.1 System one - in-process results

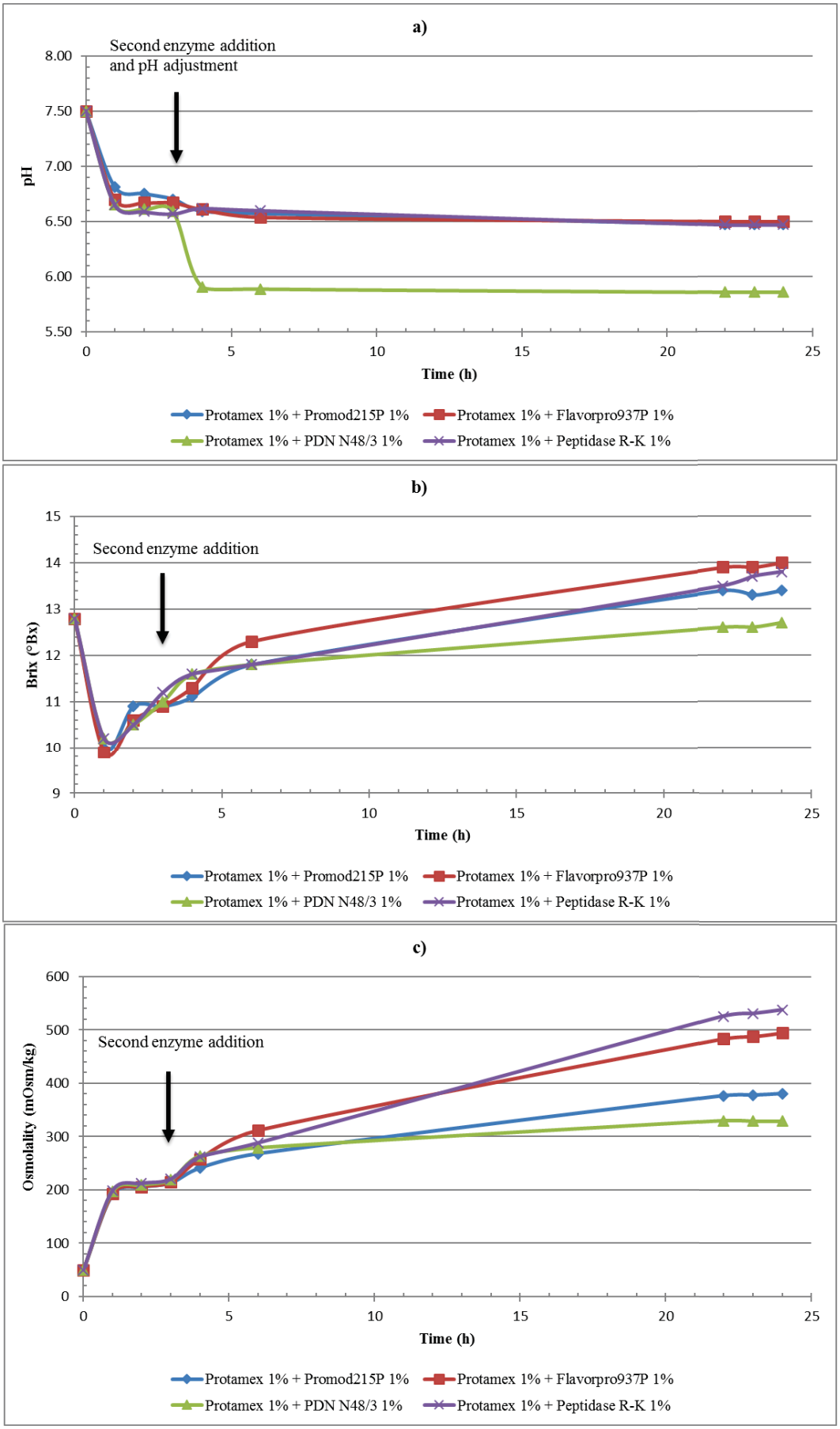
Promod215P, Flavorpro937P, PDN N48/3 and Peptidase R-K enzyme preparations with peptidase function were selected to be in combination with Protamex (Table 17). These four enzyme preparations were the only commercial enzyme preparations with peptidase function available at the time of this research. The dosage rate of the four enzyme preparations was 1% of dry matter of lactic casein in the slurry. At the end of the 3rd hour of Protamex hydrolysis, the slurry was adjusted to each of the enzyme preparation's optimum pH and temperature conditions. From the four enzyme preparations, PDN N48/3 and Peptidase R-K were the only preparations that required pH and temperature adjustment. Before PDN N48/3 addition, the pH of the slurry was adjusted to 6.00 with 50% phosphoric acid and the hydrolysis temperature was kept the same at 54°C. The slurry temperature of Peptidase R-K was adjusted to 45°C and pH of the slurry was unchanged.

Overall, pH, Brix and Osmolality of all the four enzyme combinations with peptidase function followed the similar trend (Figure 12). Slurry pH decreased sharply during the 1st hour of hydrolysis and then had a relatively small drop over the rest of 23 hours. Brix values also had a sharp decline in the first hour to below its original value due to the formation of insoluble peptides but it went back to the same as the starting value of 12.8°Brix or higher after 24 hours of hydrolysis. This was due to peptidases

continuing to break down peptides to smaller peptides and free amino acids that made them more soluble. The appearance of the Protamex + Flavorpro937P, and Protamex + Peptidase R solutions changed from translucent to white milky colour after first three hours of hydrolysis and then changed to back to being a translucent solution at the end of the 24th hour. Protamex + Promod 215P, and Protamex + PDN N48/3 stayed a white milky colour till the end of the hydrolysis. This might be due to their lower activity or their specificity compared to the other combinations and therefore insoluble peptide remaining. The solubility of a peptide depends on the isoelectric point of the peptide relative to the environment. The isoelectric point of a peptide is in turn a function of the peptide's amino acid sequence. The specifity of an enzyme will dictate what peptide sequences are generated. If an insoluble peptide, due to its amino acid sequence and/or conformation, is not accessible to further hydrolysis by an enzyme then that peptide will remain insoluble.

The osmolality parameter gives a good indication of enzyme hydrolysis performance. During the experiments, osmolality equilibria was reached about 3 hours after protease was added and then it gradually increased only after peptidase addition. From the in-process results of all four combinations, Protamex and Peptidase R-K combination had the highest hydrolysis efficiency as it had the highest osmolality of 538 mOsm/kg whereas Protamex and PDN N48/3 combination had the lowest value (329 mOsm/kg). All the enzyme preparations were inactivated by UHT heat treatment and spray dried into powders with designed conditions (Figure 11). All system one and system two final powder results were compared together to select the best enzyme combination in section 6.3.3.

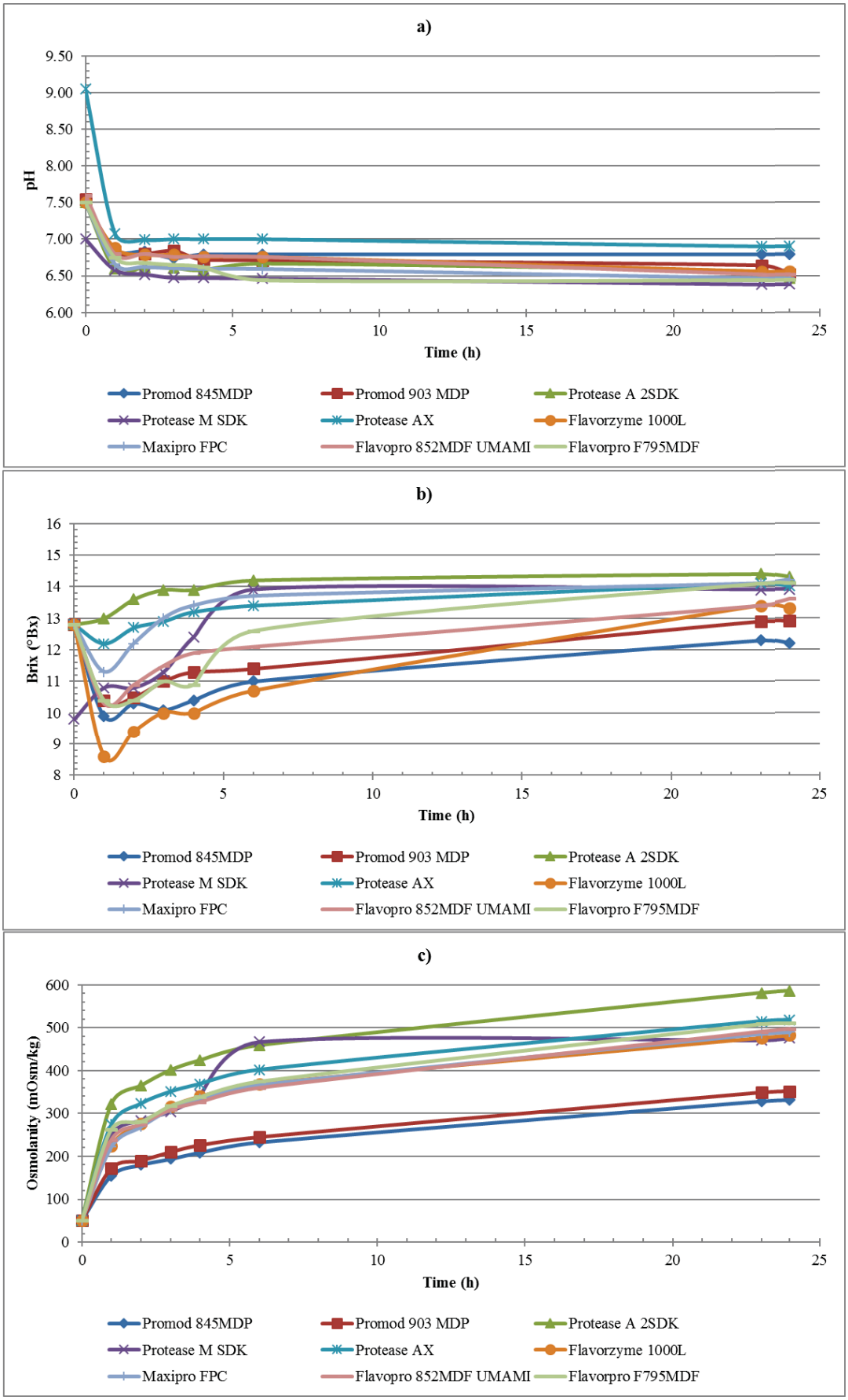
Figure 13: System one – In-process graph of a) pH, b) Brix and c) Osmolality



6.3.2 System two - In-process results

Nine different enzyme preparations with both protease and peptidase properties were trialled according to the experimental plan. From in-process results of all nine enzyme preparations in Figure 13, pH, Brix and Osmolality trends were very similar. pH of all enzyme preparations decreased with time, Osmolality increased throughout the hydrolysis process and Brix decreased initially and then increased gradually afterwards. The trend of Osmolality also appeared smooth and did not have a plateau around the 2nd and 3rd hour that occurred in system 1 in-process trends (Figure 12). With enzyme preparations having a combination of proteases and peptidases, the initial hydrolysis speed was faster as peptides generated from proteases were quickly broken down by peptidases to smaller peptides and free amino acids. The slurry with Protease A 2SDK had the highest Osmolality with 586mOsm/kg whereas the slurries of Promod 845MDP and Promod 903MDP had two lowest Osmolality, 332 and 352mOsm/kg respectively. Osmolality results of the rest of enzyme preparations were between 476 and 518 mOsm/kg. All the enzyme preparations from the slurries were inactivated after 24 hours of hydrolysis and spray dried. Both system one and system two powder products were tested and are compared in section 6.3.3.

Figure 14: System two – In-process trend of a) pH, b) Brix and c) Osmolality



6.3.3 Summary of system one and two

All the sprayed dried final powders from system one and system two were tested for Ash, DH, protein and FAA profile (Table 19 and Figure 14). FAA results were converted to milligram per gram of protein based on protein content of each product. For comparison the properties of the existing commercial product are included as the control.

6.3.3.1 System one

From the final product results of System one, Protamex + Peptidase R-K (P4084) was the most efficient enzyme combination. Protamex + Peptidase R-K (P4084) had the highest DH of 36.2% and Protamex + Flavorpro937P (P4082) had the second highest DH of 35.8%. The DH results of the other two combinations were significantly less. The DH of Protamex + Promod215P (P4081) was 25.4% whereas Protamex + PDN N48/3 (P4083) had the lowest DH of 18%.

The degree of hydrolysis results had direct relationship with FAA content. Protamex + Peptidase R-K (P4084) had the highest FAA of 358.0 mg/g of protein and Protamex + PDN N48/3 (P4083) had the lowest FAA of 47.4 mg/g of protein.

The level of ash in the final products was affected by pH adjustment. The more sodium hydroxide or phosphoric acid required for pH adjustment, the higher the ash results. Protamex + PDN N48/3 (P4083) had the highest ash of 5.9% due to the second pH adjustment. The other three combinations had similar ash results.

6.3.3.2 System two

Protease A 2SDK (P4002) had the highest DH and FAA results in system two, 43.5% and 446.8 mg/g of protein respectively. DH and FAA results of Protease AX (P4004) and Flavorzyme 1000L (P4010) were second and third highest in system two. Protease AX (P4004) had DH of 36.1% and FAA of 362.4 mg/g of protein whereas Flavorzyme 1000L (P4010) had DH of 35.2% and FAA of 374.8 mg/g of protein. Promod 903 MDP (P4001) had the lowest DH (24%) and FAA (193.8 mg/g of protein) result in system two. All the ash results were comparable among all the enzyme preparations; they were all between 4.5 to 4.8%.

Although all the enzyme combinations had the same dosage of 2% of total lactic caseinate solids, DH results were different. Protease A 2SDK (P4002) had the highest DH result of 43.5% that was higher than the control (40.3%). Five products had DH result more than 35%, almost all those five products had similar FAA content as the control (358.0 mg/g of protein) except P4082 that had 313.3 mg/of protein. From the general trend in Table 19, the higher DH values generated more total FAAs. P4002

had the highest DH value with FAA content of 446.8mg/g of protein. In contrast, P4080 had the lowest DH value of 18% and FFAs were only 47.4mg/g of protein. FAA results of N4232, P4001, and P4081 were also in the lower range, they were below 200mg/g of protein. However, the control's average DH result was 40.3% that is about 5% higher than those batches. The average FFAs of the control was similar to P4004, P4010 and P4084.

6.3.3.3 System one and system two comparison

Ash contents of all the products were significantly less than the control. The ash test measures the overall mineral content in dairy products. As a dairy ingredient, it is important to keep mineral content as low as possible. There are two main reasons: The first reason was to maintain dairy wholesomeness of the product without adding too much minerals during manufacturing process. The second reason to minimise mineral content in dairy ingredients is that it can benefit downstream processes, some final food products that use hydrolysates may have strict mineral requirement. The highest ash content from the trials (Protamex + PDN N48/3 (P4083) was 5.9% which compared favourably to 9.3% of the control. The high ash content of the control was caused by its complicated manufacturing process, which required multiple pH adjustments. Both system one and two had more simple processes than the control. Most of the final products were only pH adjusted at the start and end of the process except P4083. The pH of P4083 was adjusted before the second enzyme preparation addition.

Table 19: Final powder results of the control, system one and system two

Enzymes/Parameters	Ash (%)	DH (%)	Protein (%)	FAA (mg/g Protein)
Control (average)	9.3	40.3	87.1	358.0
System One (two enzyme preparations)				
Protamex + Promod215P (P4081)	5.3	25.4	85.3	141.2
Protamex + Flavorpro937P (P4082)	4.7	35.8	83.5	313.7
Protamex + PDN N48/3 (P4083)	5.9	18.0	84.0	47.4
Protamex + Peptidase R-K (P4084)	4.8	36.2	82.5	352.8
System Two (single enzyme preparations)				
Promod 903 MDP (P4001)	4.6	24.0	84.9	193.8
Protease A 2SDK (P4002)	4.8	43.5	83.1	446.8
Protease M SDK (P4003)	4.5	34.0	83.8	330.0
Protease AX (P4004)	4.8	36.1	83.6	362.4
Flavorzyme 1000L (P4010)	4.5	35.2	84.4	374.8
Maxipro FPC (P4011)	4.7	33.7	83.9	328.6
Flavopro 852MDF UMAMI (P4014)	4.8	33.4	83.0	311.3
Flavorpro F795MDF (P4015)	4.8	35.3	82.4	312.5

The detailed FAA profiles of all thirteen final products and the control were compared in Figure 14. All FAA were followed similar trend. L-Glutamic acid (L-Glu), L-Glutamine (L-Gln) and L-Proline (L-Pro) were key free amino acids to be analysed. As result, L-Glu, L-Gln and L-Pro were taken out the Figure 14 and plotted with sum of L-Glu and L-Gln in Figure 15. It can be seen that the results of L-Glu, L-Gln, L-Proline and L-Glu + L-Gln were very different from the control. None of the enzyme combinations reached the same L-Glu level as control. However, Flavorzyme 1000L (P4010) from the system one and Protamex + Peptidase R-K (P4084) from system two have great potential to match the L-Gln content of the control if all their L-Gln are converted to L-Glu. Those four major parameters are compared in Figure 15. A more detailed analysis is required beyond straight data comparison by considering process parameters such as degree of hydrolysis and total amino acid content for all 13 combinations. This would benefit further process improvement for potential enzyme combinations.

Figure 15: Free amino acid profile of all final products from control, system one and system two

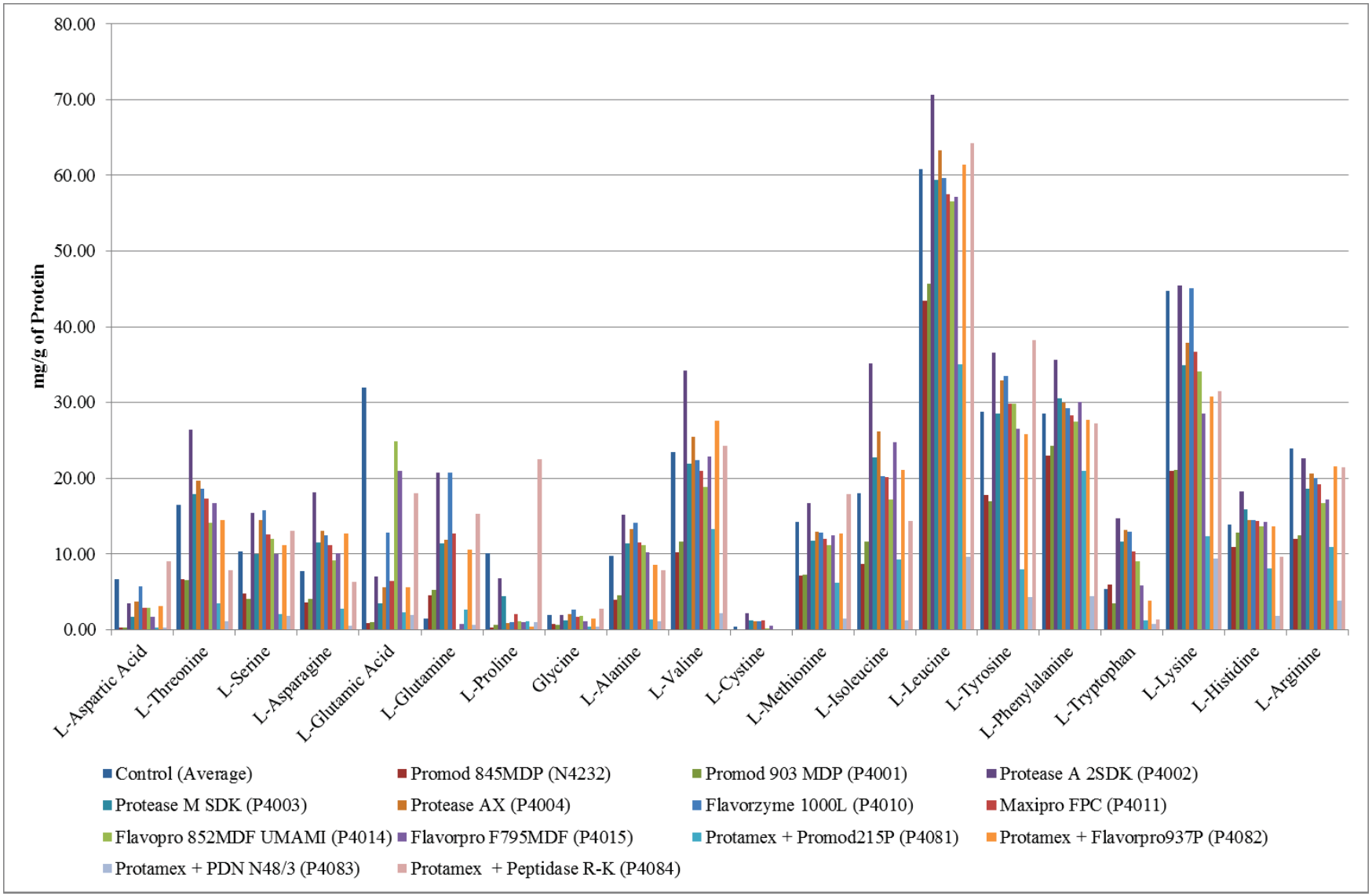
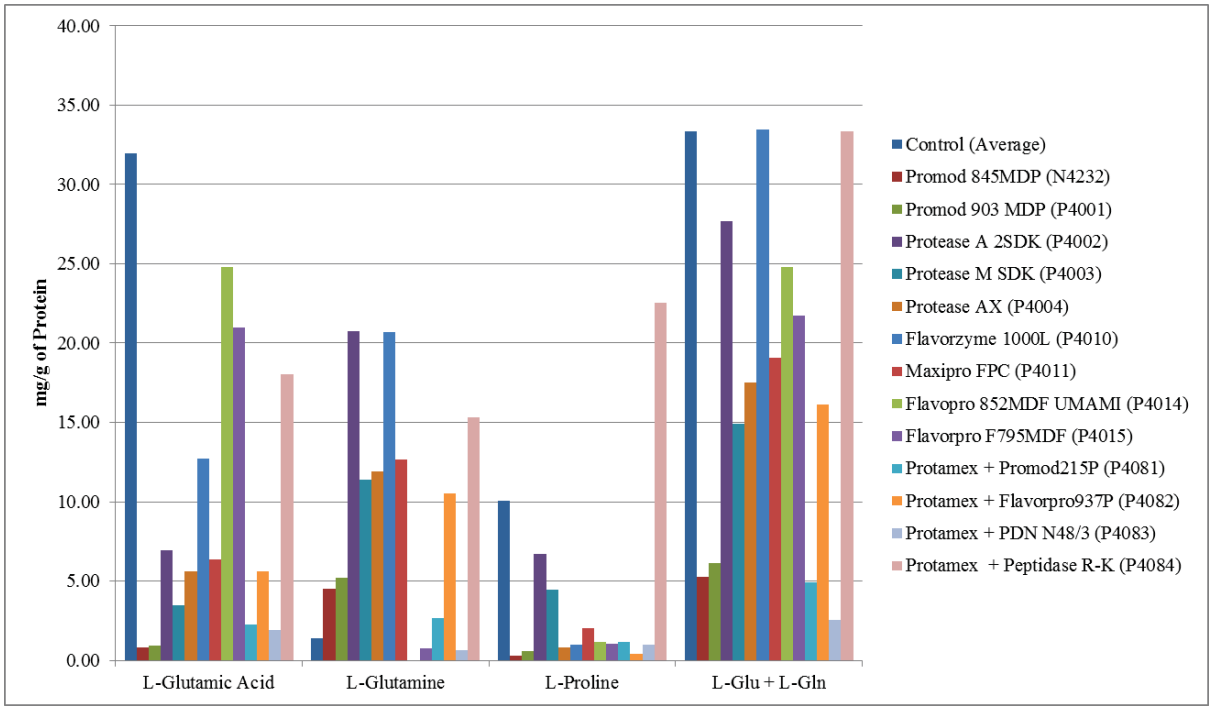
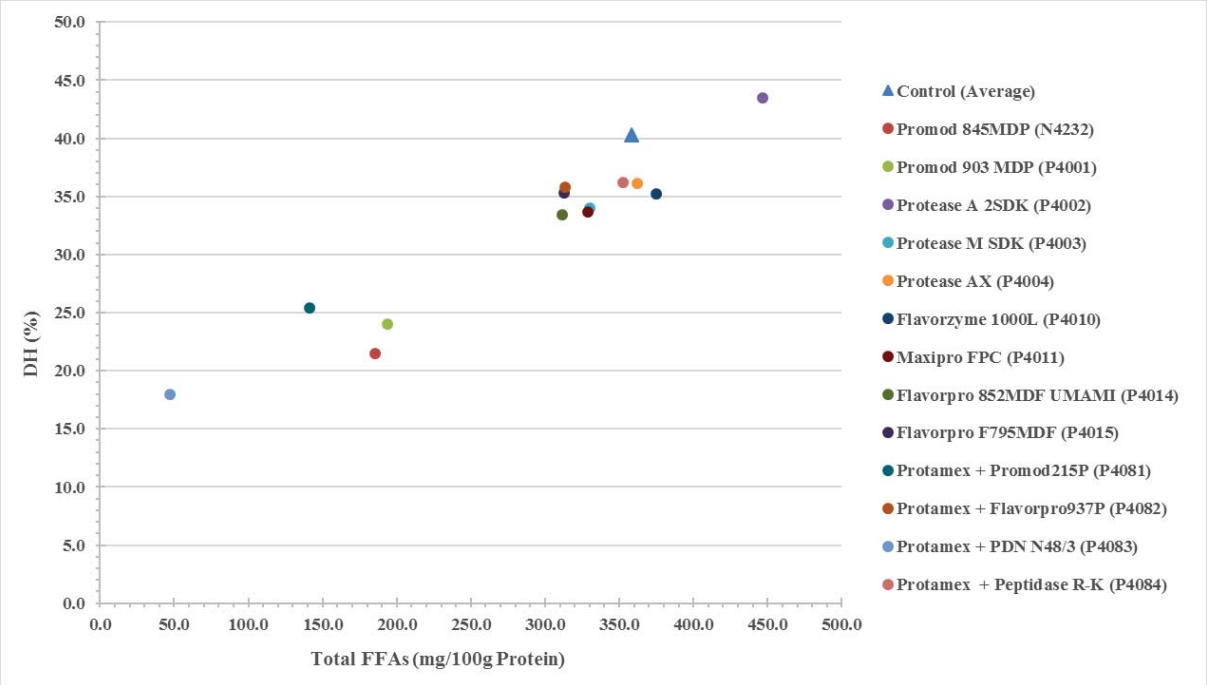


Figure 16: Selected free amino acids of all final products from control, system one and system two



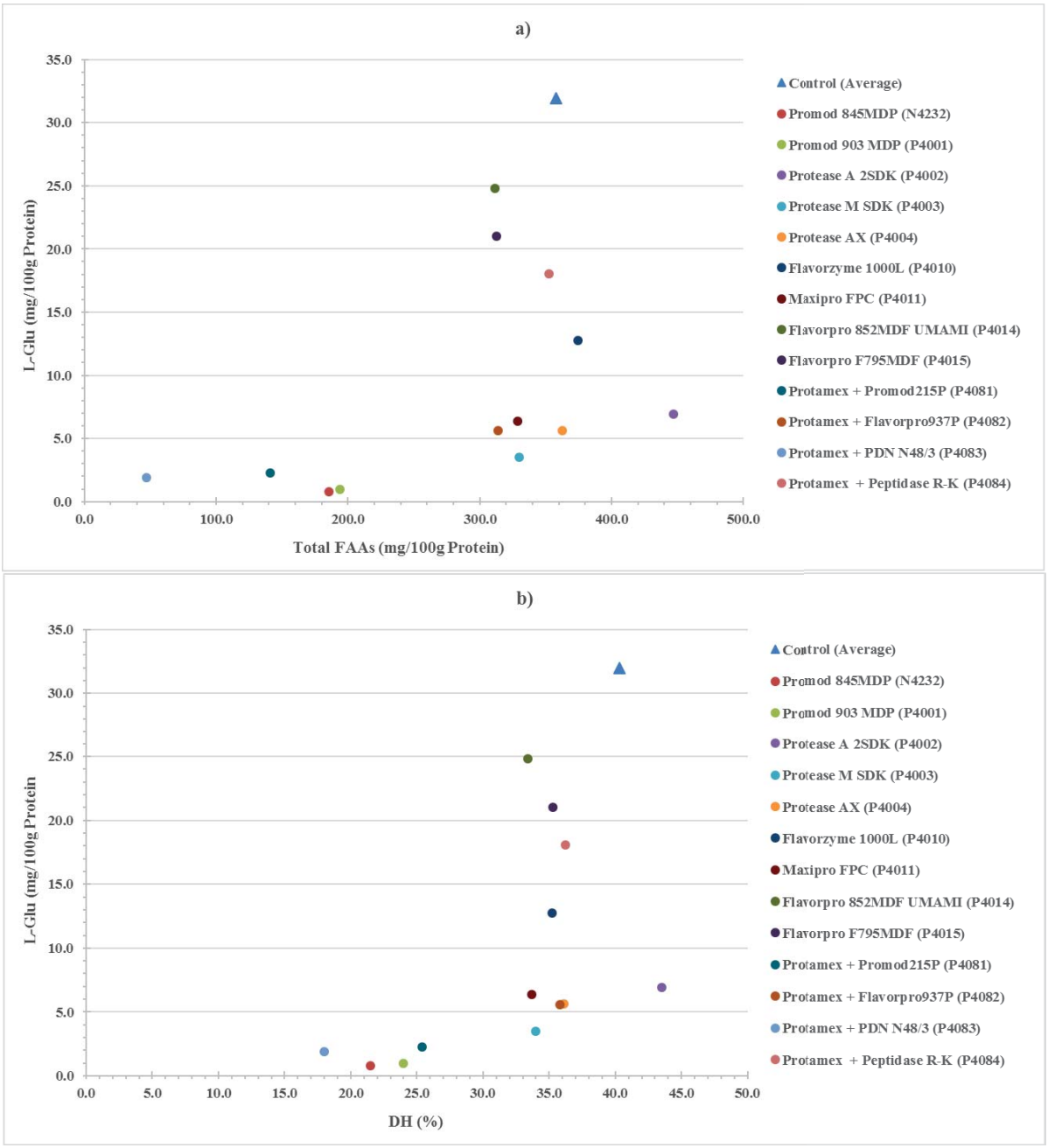
The degree of hydrolysis values of all 13 enzyme combinations were plotted against their total free amino acid in Figure 16. The relationship of the DH and total FFA was that the higher DH gave more free amino acids. Protease A 2SDK (P4002) had the highest DH value of 43.5% and it also had the highest free amino acid of 446.8 mg/100g of protein. The lowest DH belonged to Protamex + PDN N48/3 (P4083) that corresponded with lowest free amino acid value (47.4 mg/100g of protein). The direct relationship between DH and free amino acids raises a question if the higher free amino acids means more L-Glu.

Figure 17: Degree of hydrolysis VS Total free amino acids



The L-Glutamic acid content was plotted against total free amino acids to determine if there was a direct relationship between the two parameters in Figure 17. The observation was that the highest free amino acid content did not generate the highest free L-Glu. Nine out of thirteen combinations of L-Glu were below 10mg /100g of protein although their total free amino acid contents were between 47.4 to 446.8 mg/100g of protein. Protease A 2SDK (P4002) had the highest free amino acid content and it only ranked fifth on L-Glu content (6.66mg/g of protein) in all 13 combinations. Flavorpro 852 MDF UMAMI had the highest L-Glu value of 24.81mg/100g of protein and the total free amino acids was only 331.28 mg/100g of protein in all the enzyme combinations except the control. The control still had the highest L-Glu of 31.95mg/100g of protein with the total free amino acids of 357.98 mg/100g of protein. Moreover, the degree of hydrolysis also did not affect the release of L-Glu. As it can be seen in Figure 17, the pattern of L-Glu vs degree of hydrolysis was very similar to L-Glu vs total free amino acids. It makes sense that a higher the DH gives a higher total free amino acid value but L-Glu is not necessarily higher.

Figure 18: a) L-Glu VS Total free amino acids and b) L-Glu VS Degree of hydrolysis



The L-Glutamine results were plotted against total free amino acid and degree of hydrolysis (Figure 19). A trend was found that amount of L-Gln increased with the quantity of total free amino acid content except three combinations. The control, Flavorpro 852MDF UMAMI (P4014) and Flavorpro F795MDF (P4015) were three outliners. The interesting note was that total free amino acids of the three outliners were between 300 to 350 mg/100g of protein but their L-Gln were below 2 mg/100g of protein. Flavorpro 852MDF UMAMI (P4014) had 0 mg/100g of protein. Protease A 2SDK (P4002),

Flavorzyme 1000L (P4010) and Protamex + Peptidase R-K (P4084) had the top three highest L-Gln release and they were 20.7, 20.8 and 15.3 mg/100g of protein against 446.8, 374.8 and 352.8 mg/100g of protein of total free amino acids. L-Gln against degree of hydrolysis were followed the same trend (Figure 19).

Figure 19: a) L-Gln VS Total free amino acids and b) L-Gln VS Degree of hydrolysis

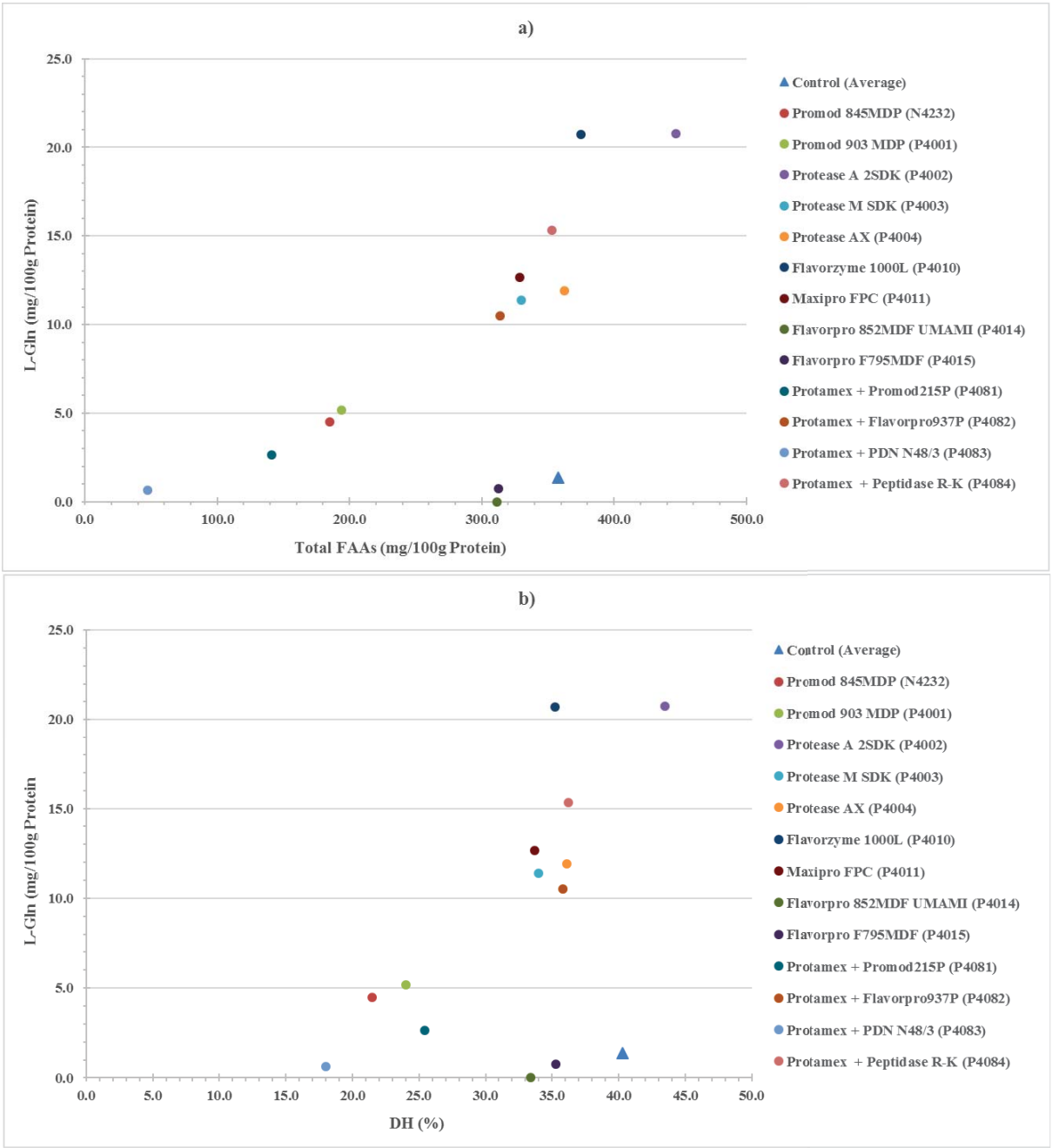
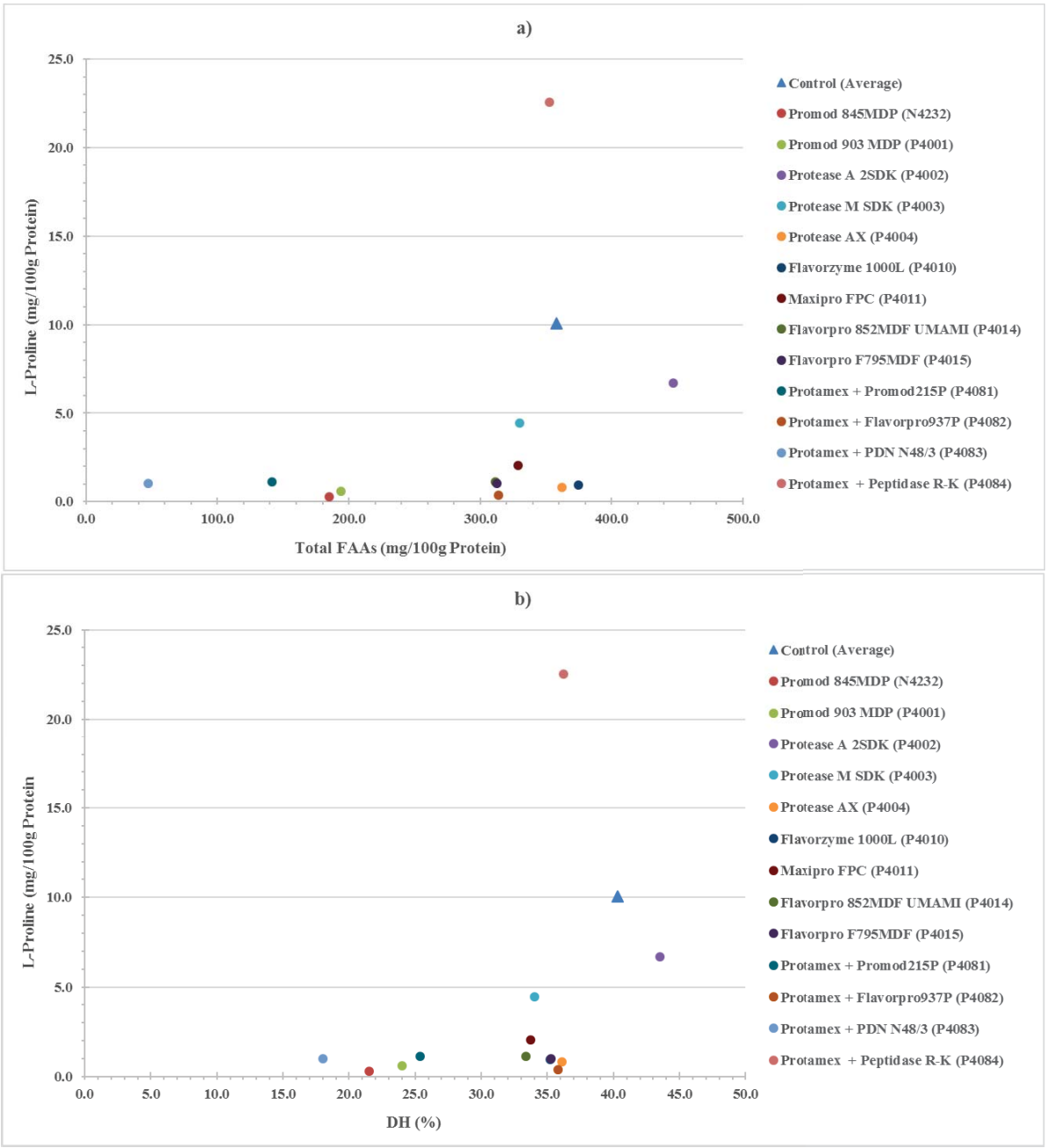


Figure 20: a) L-Proline VS Total free amino acids and b) L-Proline VS Degree of hydrolysis



There does not appear to be any pattern to the formation of Free L-Proline release (refer Figure 19). Both the free amino acid content and degree of hydrolysis had no effect on amount of L-Proline freed. There were nine combinations with an L-Proline content below 5.0mg/100g of protein but their degree of hydrolysis range was between 15 to 40%. The combination that released the most L-proline in 24 hours was Protamex + Peptidase R-K (P4084) with 22.6 mg/100g of protein. Protease A 2SDK (P4002) had the second highest L-Proline content of 6.7 mg/100g of protein.

6.3.3.4 Hypotheses of System one and System two experiments

There were three main hypotheses that were considered when the experiments were designed. The first hypothesis was that those thirteen enzyme preparations/ enzyme preparation combinations were sufficient to produce the same amount or more L-Glu compared to the control (31.95mg/g of protein). However, from the results in the Figure 17, none of the thirteen enzyme preparations/enzyme preparation combinations had produced more L-Glu than the control. Flavorpro 852MDF UMAMI (P4014) and Flavorpro F795MDF (P4015) had the top two L-Glu contents, 24.8 and 21.0 mg/g of protein respectively. They were only 77.6% and 65.7% of L-Glu of control. Promod 845MDP (N4232) and Promod 903 MDP (P4001) had the lowest L-Glu values, 0.80 and 0.95 mg/g-protein respectively. This was consistent with their low DH and total FAAs results, which indicated that certain DH values need to be reached to generate more FAAs including L-Glu. An interesting finding was that Protease A 2SDK (P4002) only had a L-Glu level of 6.94 mg/g of protein. Protease A 2SDK (P4002) had the highest DH and total FAAs among all the final products (Table 19). The result showed that high DH and FAAs did not necessarily mean high L-Glu generation and it is the key to finding an L-Glu specific enzyme preparation and an optimum DH value.

The second hypothesis was that if there were strong glutaminase side any activities in the thirteen enzyme preparations/ enzyme preparation combinations, it would convert most of L-Gln to L-Glu during hydrolysis. A side enzyme activity means that it is not main activity of an enzyme preparation but both side and main enzyme activities are from a single microbial source. There was only one product (P4014) that had 0 mg/g of protein L-Gln although another product Flavorpro F795MDF (P4015) had a very low L-Gln value of 0.75mg/g. These two enzyme preparations were dry blended with glutaminase according to the enzyme suppliers which explains this result - presumably the peptidases in these preparations were not very effective at releasing free Glu and/or Gln. As a result, they did not possess glutaminase side activities on their own and they were considered a second enzyme preparation.

The control had high L-Glu of 31.95 mg/g of protein content with a low L-Gln value (1.38 mg/g of protein). However, there was no declared glutaminase enzyme in the enzyme preparations used in the control. There might be three causes for this. Firstly, the long hydrolysis time of 55 hours and L-Glu specific enzyme preparations allowed high L-Glu release. Secondly, there might be weak glutaminase side activities slowly converting L-Gln to L-Glu over this 55-hour period. Protease A 2SDK (P4002), Flavorzyme 1000L (P4010) and Protease AX (P4004) achieved similar DH and total FAAs as the control within a short period of time (24Hours) but they had lower L-Glu level. Large amounts of L-Gln were still remaining; Protease A 2SDK (P4002) had 20.76 mg/g of protein, Flavorzyme 1000L (P4010) had 20.71 mg/g of protein and P4084 had 15.32 mg/g of protein. The L-Gln results of three products were all more than their L-Glu. This might be due to there not being enough time for any weak glutaminase side activities to convert L-Gln to L-Glu.

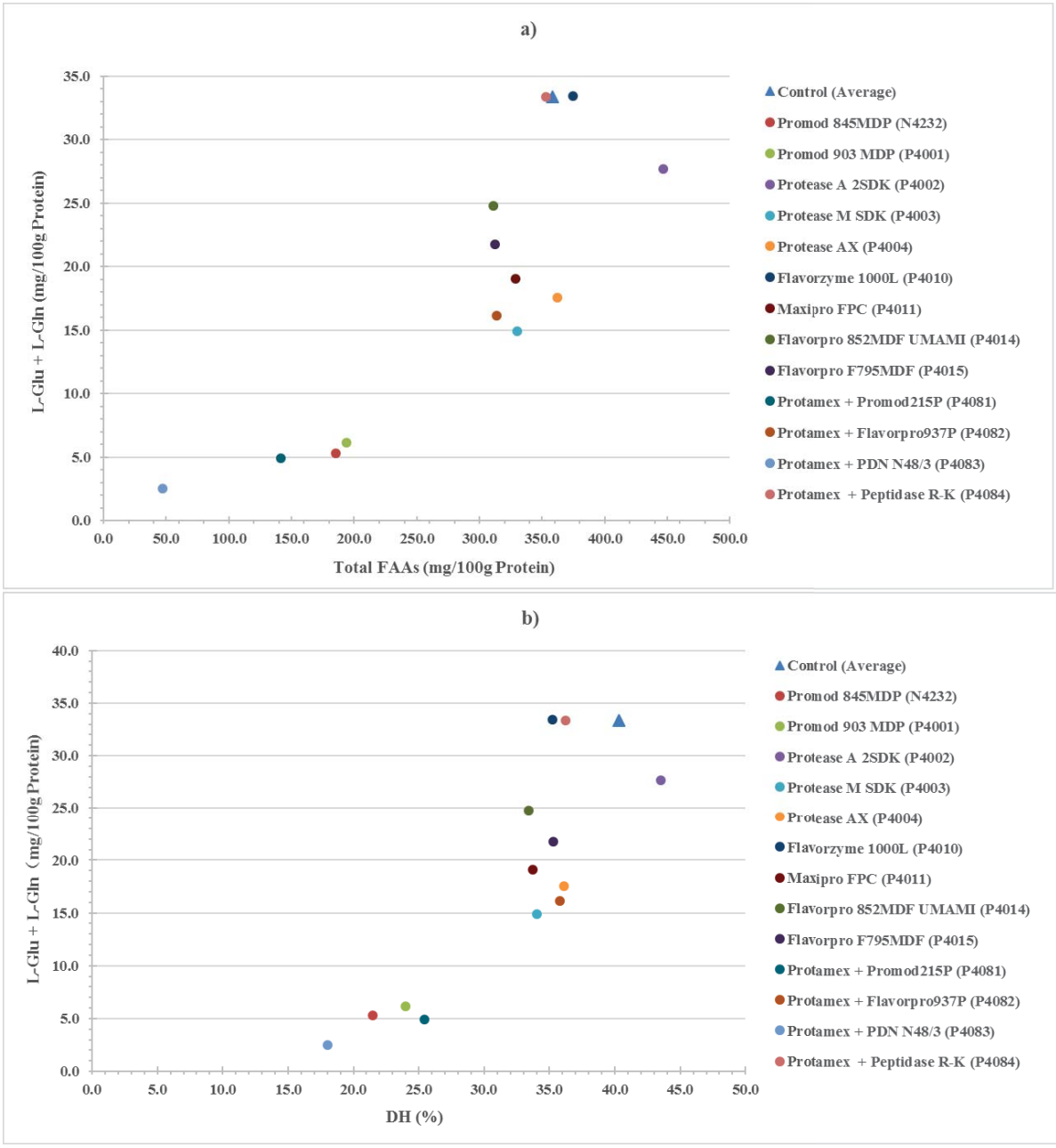
The third hypothesis was that glutamic acids that are adjacent to proline were found, according to the literature (Moller, Andrew and Cheeseman, 1977; Shih, 1985), to be difficult to release by hydrolysis so releasing more L-Pro from the casein protein would create more L-Glu and L-Gln. From Figure 19, P4084 had the highest amount of L-Pro (22.6mg/g of protein) compared to the second place P4001 (6.70mg/g of protein) and the control (10.08mg/g of protein). L-Glu content of P4084 (18.05 mg/g of protein) was the highest after Flavorpro 852MDF UMAMI (P4014) and Flavorpro F795MDF (P4015) but they were not selected for comparison because they were pre-blended with a glutaminase preparation by their manufacturer. L-Gln result of P4084 (15.32mg/g of protein) was still behind of P4002 (20.76 mg/g of protein) and P4010 (20.71mg/g of protein). Protamex + Peptidase R-K (P4084) enzyme preparation combination was definitely proline specific which freed up proline. This might help to release more Glu and Gln. However, according to Table 20, Glu and Gln that are adjacent to proline are minimal so its impact on final free glutamic acid content is small. Release of L-proline will not be a key way to increase free glutamic acid content in the casein hydrolysate project.

Table 20: Number of Glutamic acids and Glutamine in four types of casein protein sequences (Farrell et al, 2004)

Casein Proteins	**αs1-casein**	**αs2-casein**	**β-casein**	**κ-casein**
Reference Proteins	CN B-8P	A-11P	A2-5P	A-1P
Ratio	40%	10%	45%	5%
Glu(s) in protein sequence	25	24	19	12
Glu(s) are adjacent to Pro(s)	2	0	2	3
Gln(s) in protein sequence	14	16	20	14
Gln(s) are adjacent to Pro(s)	2	2	6	2

To summarise all three points, it was determined that it was very difficult to generate glutamic acid and convert glutamine to glutamic acid in a shorter time frame (24 hours) compared to the control (56 hours) except Flavorpro 852MDF UMAMI and Flavorpro F795MDF. The two enzyme preparations that converted Gln to Glu in that time frame however they were not as effective in releasing free Glu and Gln as some of the other combinations. P4084 was a good enzyme preparation combination for L-Pro release but it could not generate enough L-Glu due to the limited number of L-Glu was adjacent to L-Pro (Table 20). As a result, an enzyme preparation with glutaminase as its main function was needed to convert L-Gln to L-Glu during hydrolysis.

Figure 21: a) Sum of L-Glu and L-Gln VS Total free amino acids and b) Sum of L-Glu and L-Gln VS Degree of hydrolysis



In order to determine if there was a potential that L-Glu content was more or equal than the control after L-Gln to L-Glu conversion, a sum of L-Gln and L-Glu was determined to predict a final L-Glu content in the products. As it can be seen in Figure 20, there were two products, P4010 and P4084, that had good potential where the sum of Gln and Glu was almost the same as the control. This was important as it indicated that there was a potential that final L-Glu results might be equal or more than the control if a glutaminase was used during hydrolysis.

6.4 Selection of enzyme for optimisation

There were 13 different enzyme combinations from system one and system two. It was very important to select the most suitable enzyme preparation to work with glutaminase. All thirteen final products were compared in five categories and all the enzyme preparations were evaluated against enzyme function, enzyme cost, process chemistry (DH), process microbiology (Temperature and pH), process complexity (process steps) and L-Glu and L-Gln generation. Each categories was rated pass (+) or fail (-) and an enzyme combination with most of passes was the best option to combine with glutaminase.

From all the enzyme combinations, Flavorzyme 1000L was scored the highest with 5 points whereas two other enzyme combinations, Protease M SDK (P4003) and Maxipro FPC (P4011) came second with 4 points. L-Glu and L-Gln generation was certainly the most important for all the criteria, only Flavorzyme 1000L (P4010) and Protamex + Peptidase R-K (P4084) were scored positive from all thirteen products. As can be seen from Table 21, Flavorpro 852MDF UMAMI (P4014) and Protamex + Peptidase R-K (P4084) had almost identical sum of L-Glu and L-Gln. However, Protamex + Peptidase R-K (P4084) scored poorly in enzyme cost and process microbiology. Hydrolysis temperature of Protamex + Peptidase R-K (P4084) was around 45°C, which was susceptible to microbial growth. The growth temperature ranges of E. coli, B. cereus, salmonella and listeria are all around 45°C. On the other side, the hydrolysis temperature of Flavorpro 852MDF UMAMI (P4014) was around 50°C, which is within the recommended the growth temperature range of the food safety microbial. The enzyme preparation cost difference between (P4084) and Flavorpro 852MDF UMAMI (P4014) was significant, the enzyme preparation (Peptidase R-K) used in Protamex + Peptidase R-K (P4084) was about 6 times more than Flavorzyme 1000L that was used in Flavorpro 852MDF UMAMI (P4014). As a result, Flavorzyme 1000L was selected as the candidate to combine with glutaminase.

Table 21: Final products rating table of system one and system two

Enzymes/Parameters	Enzyme Cost	Process Chemistry	Process Microbiology	Process Complexity	L-Glu and L-Gln Generation	Score
Promod 845MDP (N4232)	+	-	+	+	-	3
Promod 903 MDP (P4001)	+	-	+	+	-	3
Protease A 2SDK (P4002)	-	+	+	+	-	3
Protease M SDK (P4003)	+	+	+	+	-	4
Protease AX (P4004)	-	+	+	+	-	3
Flavorzyme 1000L (P4010)	+	+	+	+	+	**5**
Maxipro FPC (P4011)	+	+	+	+	-	4
Flavopro 852MDF UMAMI (P4014)	-	+	+	+	-	3
Flavorpro F795MDF (P4015)	-	+	+	+	-	3
Protamex + Promod215P (P4081)	+	-	+	+	-	3
Protamex + Flavorpro937P (P4082)	-	+	+	+	-	3
Protamex + PDN N48/3 (P4083)	+	-	+	-	-	2
Protamex + Peptidase R-K (P4084)	-	+	-	+	+	3

7 Enhancing glutamic acid levels with glutaminase

7.1 Introduction

From all thirteen enzyme combinations, Flavorzyme 1000L was determined to have the potential of producing high glutamine content and suitable hydrolysis conditions for microbiology safety. A food grade enzyme preparation with glutaminase as its main activity was needed to pair with Flavorzyme 1000L. Glutaminase SD-C100S was the only commercial food enzyme preparation available at the time of selection. Glutaminase SD-C100s was selected to pair with Flavorzyme 1000L to generate more L-Glu content during the hydrolysis process.

7.2 Experiment materials and methodology

7.2.1 Experiment materials and testing method

7.2.1.1 Experiment materials

Anchor NS1170 ETHANOL (ETHYL ALCOHOL) - Fonterra
11.9% sodium hydroxide (NaOH) - Orica
10% lactic sodium caseinate slurry bags - Tatua
2 litre stainless steel vessel with lid
Eurostar Digital Overhead Stirrer with a blade attachment - IKA
Water bath - Grant
Water bath heating unit (T100) - Grant
pH meter - Mettler SevenCompact
pH probe - Scott
Brix meter (0 – 53°Brix) - Atago PAL-1
Osmolality meter - The Advanced Osmometer model 3250
Nutritional indirect UHT plant - Tatua
Niro Minor Spray drier - GEA

7.2.1.2 Testing methods

The testing methods of degree of hydrolysis, protein content, free amino acid profile and molecular weight profile were the same methods as Table 12.

Test parameters	Method
Degree of hydrolysis	Tatua internal – LCHPM2/4.14/2
Protein content (TN x 6.47)	Tatua internal – LCHPM/3.30/20
Free amino acid profile	New Zealand AgResearch method based on AOAC988.15 and 994.12
Molecular weight profile	Tatua internal - LCHPM2/13.3/2

7.2.2 Experiment methodology

Two experiments were conducted with two different glutaminase dosages (0.25% and 0.50%). This was used to determine the optimum glutaminase dosage so enzyme cost could be potentially reduced. The hydrolysis conditions (Table 22) and substrate material (10% total solids lactic casein slurry) was exactly the same as P4010 when only Flavorzyme 1000L was used. During the experiment, Flavorzyme 1000L was dosed first and Glutaminase SD-C100S was added three hours into the hydrolysis. There were two reasons for the time delay. Firstly, three hours after Flavorzyme 1000L was added, pH decreased from 7.5 to 6.8 which was within Glutaminase SD-C100S optimum pH range of 6 to 7. Secondly, Glutaminase SD-C100S is not temperature stable when hydrolysis temperature is over 50°C when there are no L-Glns to covert. Delaying Glutaminase SD-C100S addition would help to wait till some L-Glns are generated so the enzyme deactivation risk is reduced. Also since the glutaminase is likely to lose activity quickly, it makes sense to wait until most of the substrate Gln has been produced before addition. As a result, efficiency of L-Gln to LGlu conversion is increased.

A detailed method is described as following: A stainless steel 2 litre vessel was sanitised using 95% ethanol and 1750g of lactic sodium caseinate slurry were added into the pot. A stainless stir blade attachment was placed in the pot and a lid was positioned on the top of pot. The whole vessel was then put into a Grant water bath that was set to constant at 53°C. The blade attachment was then attached to an IKA power stir head. The agitator speed was set at 100 RPM and the slurry heated to 53°C. The slurry pH was adjusted from 6.40 to 7.60 using 15.2 g of 11.9% NaOH when the slurry temperature reached 53°C. 3.8g of Flavorzyme 1000L was added into the vessel after agitation speed was increased to 300 RPM. The speed was reduced back to 100 RPM when the enzyme preparation was mixed with the slurry homogenously after 10 minutes. From this point of time, the hydrolysis process started. The slurry was tested for Brix, pH and Osmolality every hour for the first 3 hours. 0.48g of Glutaminase C100SD (0.25%) was added as the second enzyme preparation at the end of 3rd hour. During the second enzyme addition, the agitation speed was increased to 300 RPM for 10 minutes temporarily and then

reduced back to 100 RPM. All the designed in-process tests (Brix, pH and Osmolality) were continued at 4th, 6th, 22nd, 23rd and 24th hour of the hydrolysis. At the end of 24-hour hydrolysis, the slurry pH was adjusted from 6.38 to 7.59 using 14.8g of 11.8% NaOH. The slurry enzyme was then deactivated using a nutritional UHT pilot plant at 120°C after the pH adjustment. The slurry was then spray dried using Niro Minor spray drier.

Figure 22: Process flow of Flavorzyme 1000L and Glutaminase SD-C100S combinations

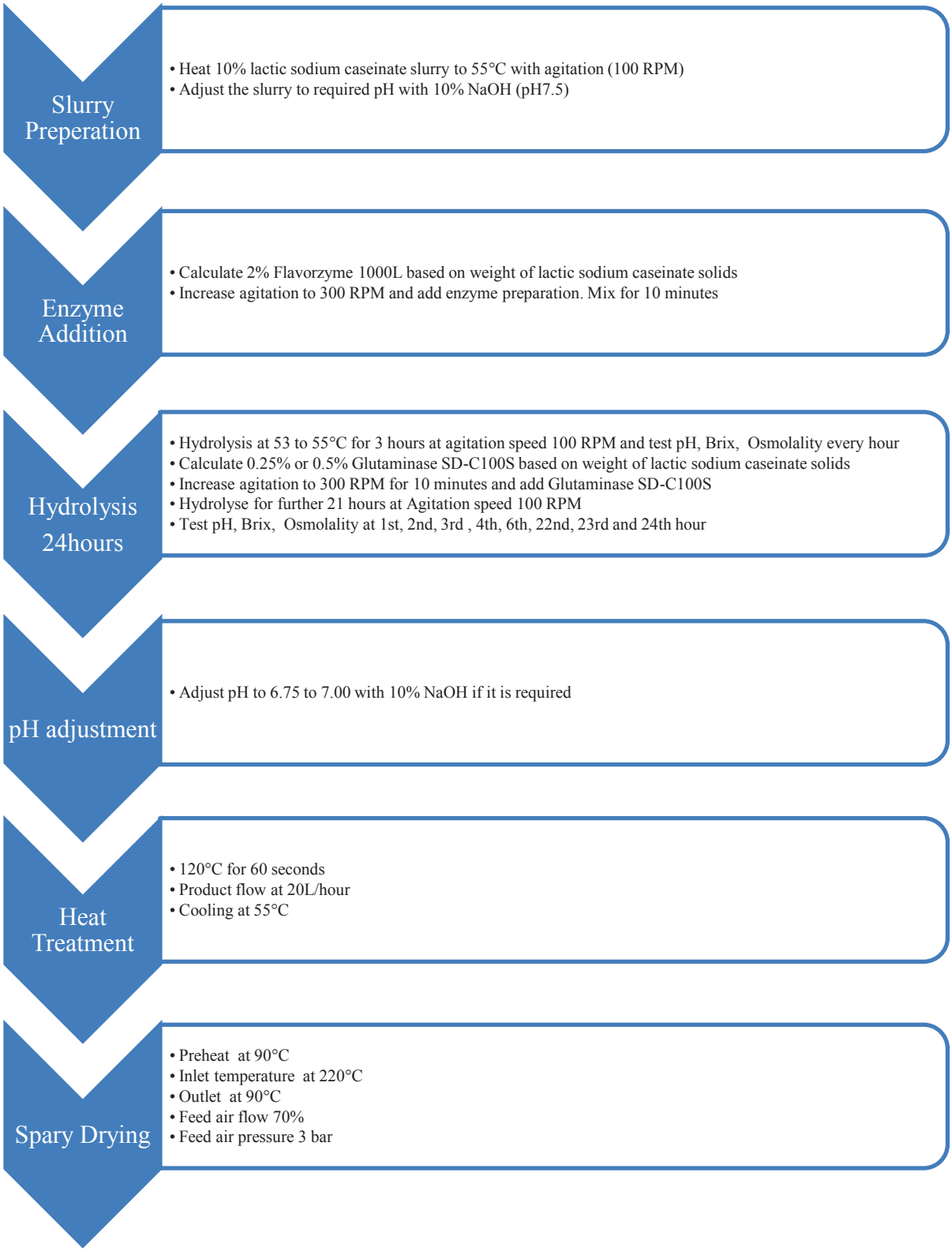


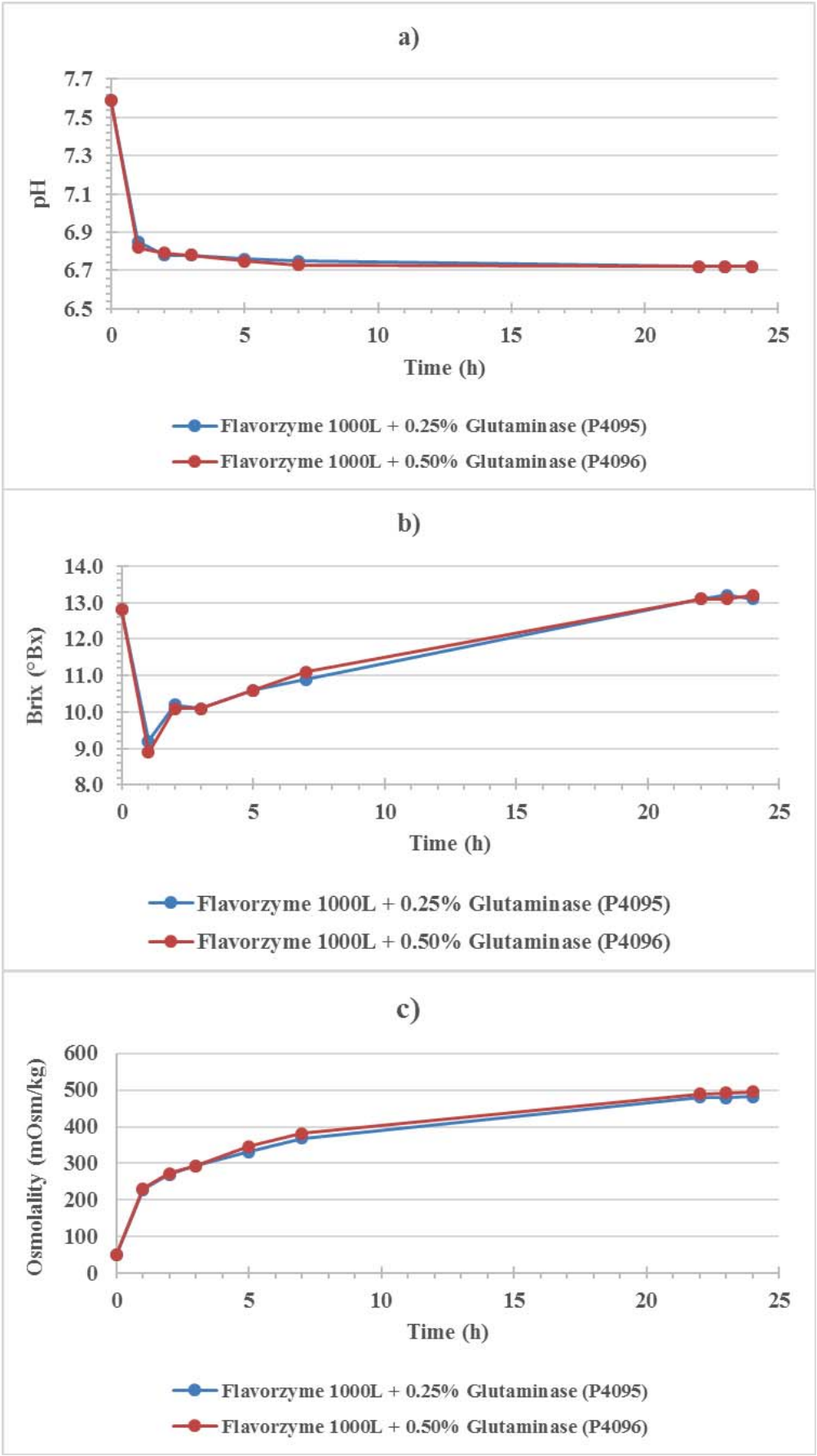
Table 22: Hydrolysis conditions of Flavorzyme 1000L and Glutaminase SD-C100S combinations

Enzymes	Enzyme Type	Starting pH	Hydrolysis Temperature (°C)	Dosage (%)
Flavorzyme 1000L	Protease and Peptidase	7.50	53	2
Glutaminase SD-C100S	Glutaminase	6.78	53	0.25 - 0.5

7.3 Results and discussion

From the experiment results, in-process graph of P4095 and P4096 were almost identical, Brix decreased sharply initially and increased above to the starting Brix (12.8) after 24 hours during hydrolysis. Osmolality increased sharply in the first four hours and plateaued over the last 3 hours. The slurry pH had the biggest change in the first hour and then stabilised after 4 hours.

Figure 23: In-process results of Flavorzyme and Glutaminase combinations: a) pH, b) Brix and c) Osmolality

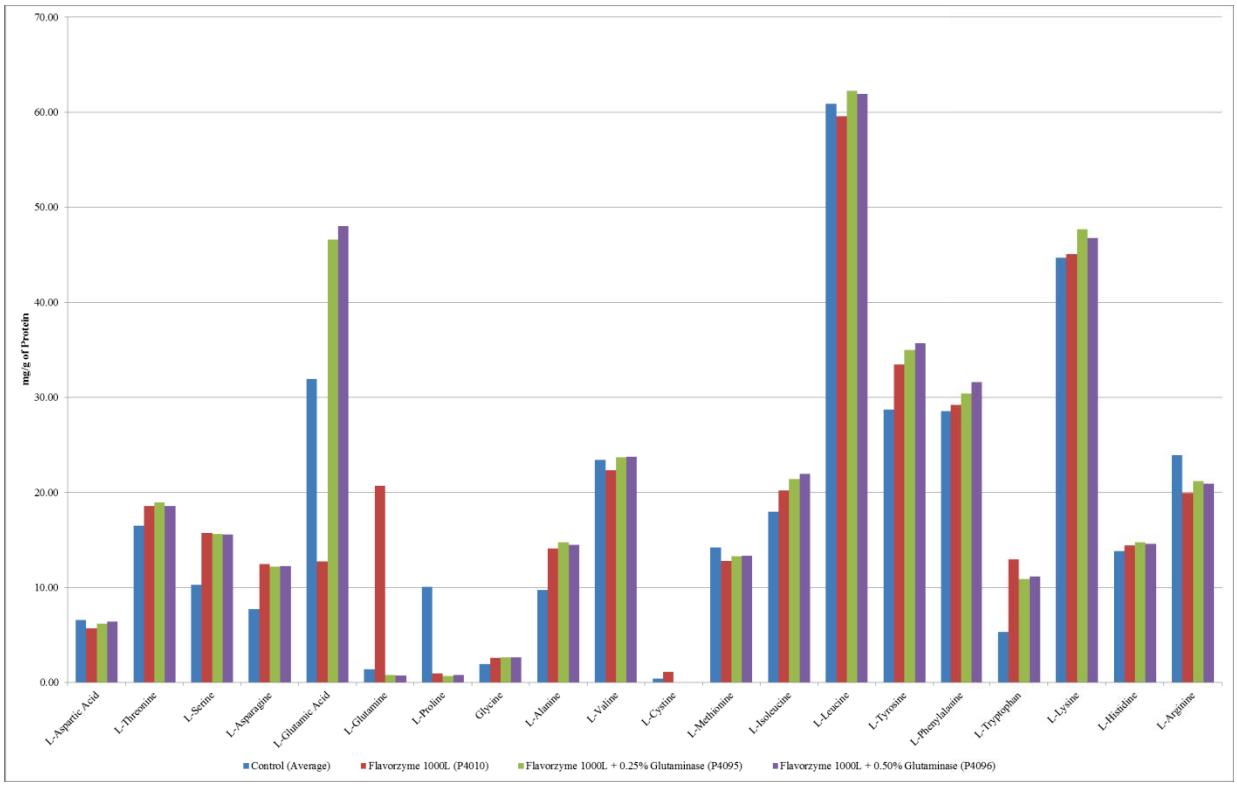


Final powder results of P4095 and P4096 were compared to P4010 that used Flavorzyme 1000L only (Table 23). P4010 had the highest ash result of 4.5% whereas P4095 was 4.2% and P4096 was 4.3%. Total FAA content of P4095 and P4096 were slightly higher than P4010 because they were more hydrolysed than P4010. P4095 (36.2%) and P4096 (36.7%) had higher DH value than P4010 (35.2%). Overall, the results of ash, DH, and protein were very close among three batches. FAA results between P4005 and P4006 were almost identical.

Table 23: Final powder product results of Flavorzyme 1000L and Flavorzyme 1000L and Glutaminase SD-C100S combinations

Enzymes/Parameters	Ash (%)	DH (%)	Protein (%)	FAA (mg/g of Protein)
Flavorzyme 1000L (P4010)	4.5	35.2	84.4	375
Flavorzyme 1000L + 0.25% Glutaminase (P4095)	4.2	36.2	82.7	399
Flavorzyme 1000L + 0.50% Glutaminase (P4096)	4.3	36.7	82.6	401

Figure 24: Free amino acid profile of Control, Flavorzyme 1000L and Flavorzyme 1000L and Glutaminase SD-C100S combinations



FAA profiles of P4095 and P4096 were plotted comparing against the control and P4010 (Chart 18). All free amino acids from all four samples were following the similar trend except L-Glu, L-Gln and proline. Those four free amino acids were selected from the FAA profile chart and plotted on chart 19

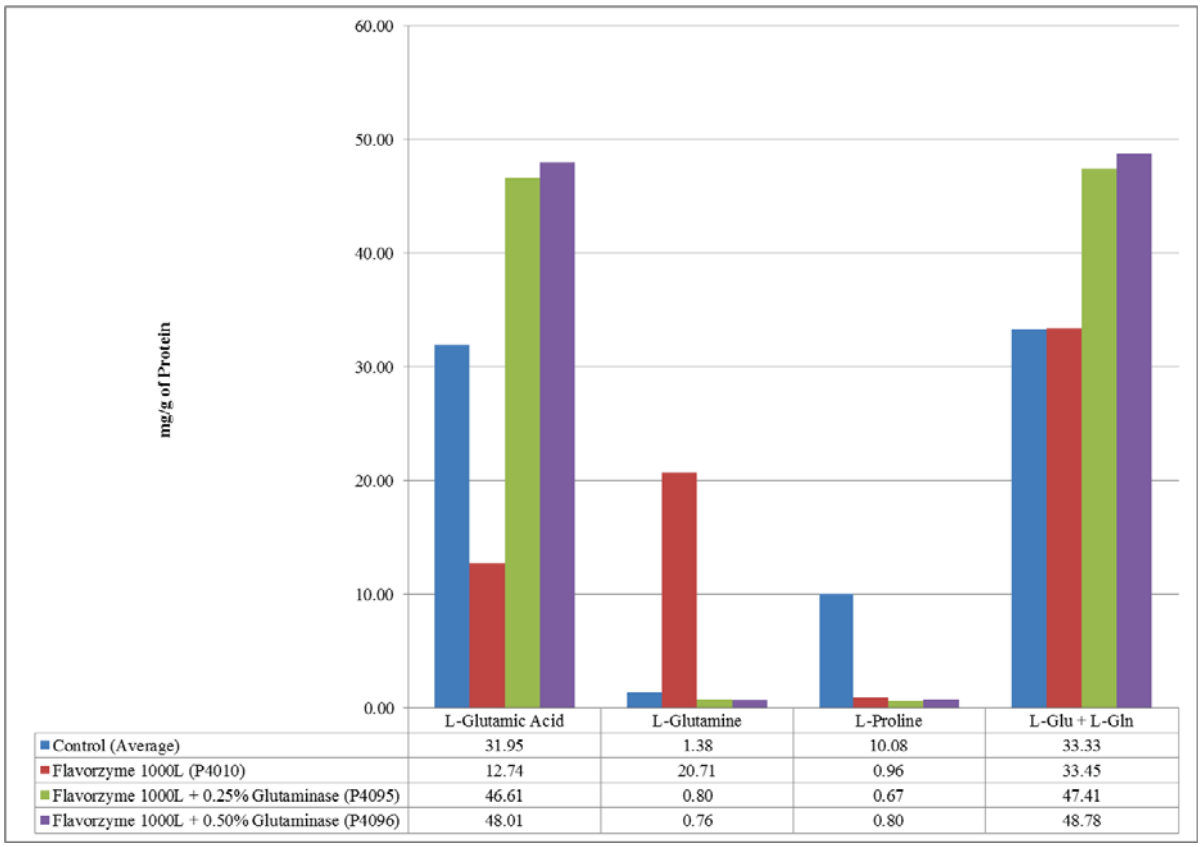
including sum of L-Glu and L-Gln for more detailed analysis. Three main findings were discovered as following:

First of all, the L-Gln content in P4095 and P4096 were significantly reduced by glutaminase. P4010 (without glutaminase) had 20.71mg/g of protein, L-Gln of P4095 and P4096 were significantly reduced to less than 1 mg/g of protein after glutaminase addition. This indicated that glutaminase exercised its function on L-Gln and converted all most all the L-Glns that were available.

Secondly, the final L-Glu content of P4095 and P4096 were much more than the control. The main purpose of adding glutaminase was to convert as many L-Gln as possible to L-Glu. After 21 hours of L-Gln and L-Glu conversion, L-Glu content of P4095 (46.61mg/g of protein) and P4096 (48.01 mg/g) were almost 4 times more than P4010 (12.74mg/g of protein). The average L-Glu content of P4095 and P4096 was 47.31mg/g of protein, which was 15.36 mg/g more than the control (31.95 mg/g). The Flavorzyme 1000L and Glutaminase SD-C100S combination generated significantly more L-Glu compared to the control (48%).

Thirdly, final L-Glu content of P4095 and P4096 were much higher than the calculated L-Glu potential (Figure 19). The calculated L-Glu potential was the sum of L-Glu and L-Gln of P4010. There were almost 14mg/g of protein more L-Glu than the calculated value of 33.45mg/g of protein. From the literature review, some L-Glns were converted to L-pGlu if they were not able to convert to L-Glu in time so some potential L-Glns were lost due this reaction (Oshita et al, 2000). This is significant because pyroglutamic acid had no taste itself and it was difficult to convert pyroglutamic acid back to glutamic acid once formed (Nandakumar et al., 2003). It was determined that an enzyme called glutaminase was able to reduce pyroglutamic acid formation by converting free glutamines to glutamic acids, which left less glutamines available to convert to pyroglutamic acid (Oshita et al, 2000). In summary, the most appropriate explanation for the extra L-Glu was that glutaminase might stop L-pGlu formation by converting extra L-Gln to L-Glu. However, this was only an assumption as L-pGlu was not tested in P4010, P4095 and P4096 as the L-pGlu test was not available commercially at the moment.

Figure 25: Selected free amino acids comparison of control, Flavorzyme 1000L and Flavorzyme 1000L and Glutaminase SD-C100S combinations

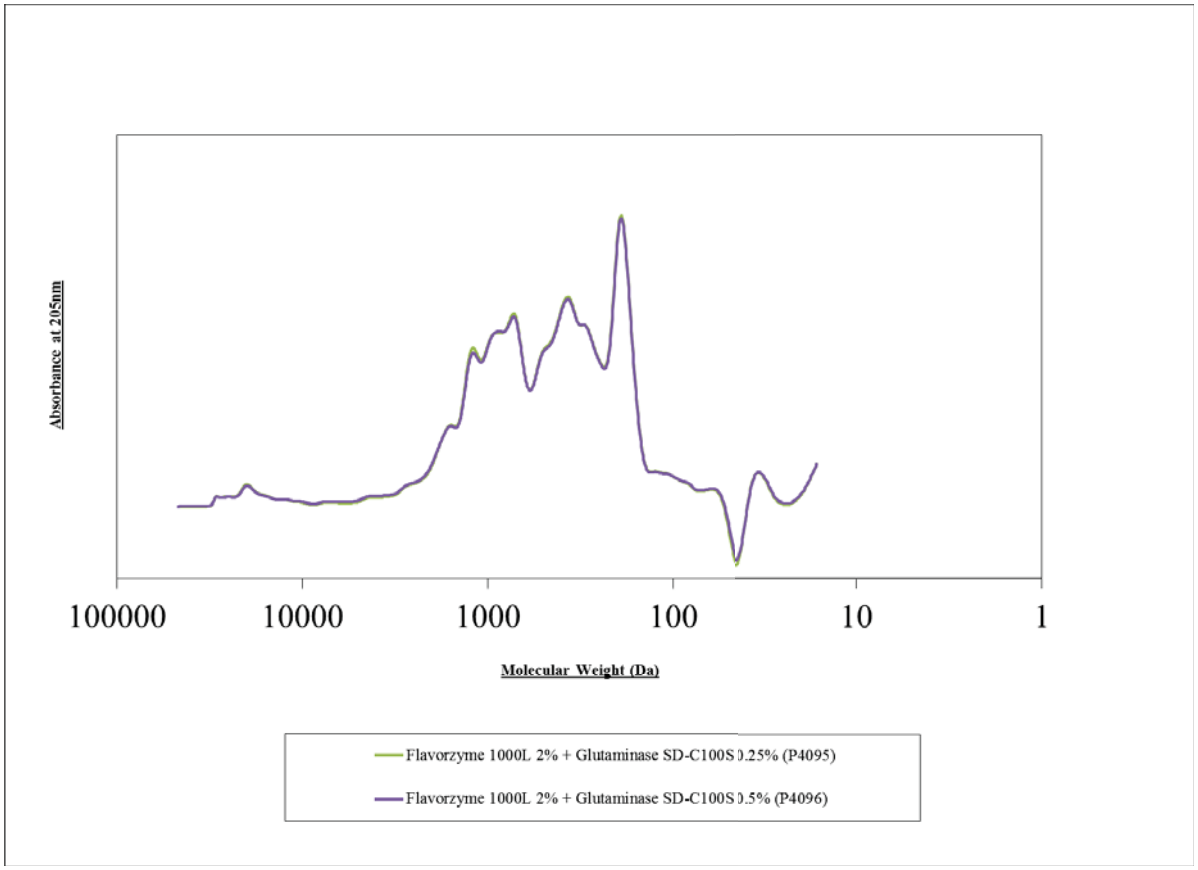


	L-Glutamic Acid	L-Glutamine	L-Proline	L-Glu + L-Gln
Control (Average)	31.95	1.38	10.08	33.33
Flavorzyme 1000L (P4010)	12.74	20.71	0.96	33.45
Flavorzyme 1000L + 0.25% Glutaminase (P4095)	46.61	0.80	0.67	47.41
Flavorzyme 1000L + 0.50% Glutaminase (P4096)	48.01	0.76	0.80	48.78

7.4 Conclusion

From all the data above, it was found that Flavorzyme 1000L and Glutaminase C100SD combinations produced a very high free Glutamic acid level. The actual amount of L-Glu generated were about 48% more than the control based on L-Glu per gram of protein. Almost all the L-Glns were converted for both levels of glutaminase addition experiments and FAA profile and DH were also very close. The Glutaminase SD-C100S combinations of 0.25% and 0.50% generated almost the same amount of L-Glu which showed that 0.25% dosage was sufficient. The MWP (Figure 20) further proved that the experiment showed excellent repeatability as both curves were matching well. The final results showed that both products were almost identical.

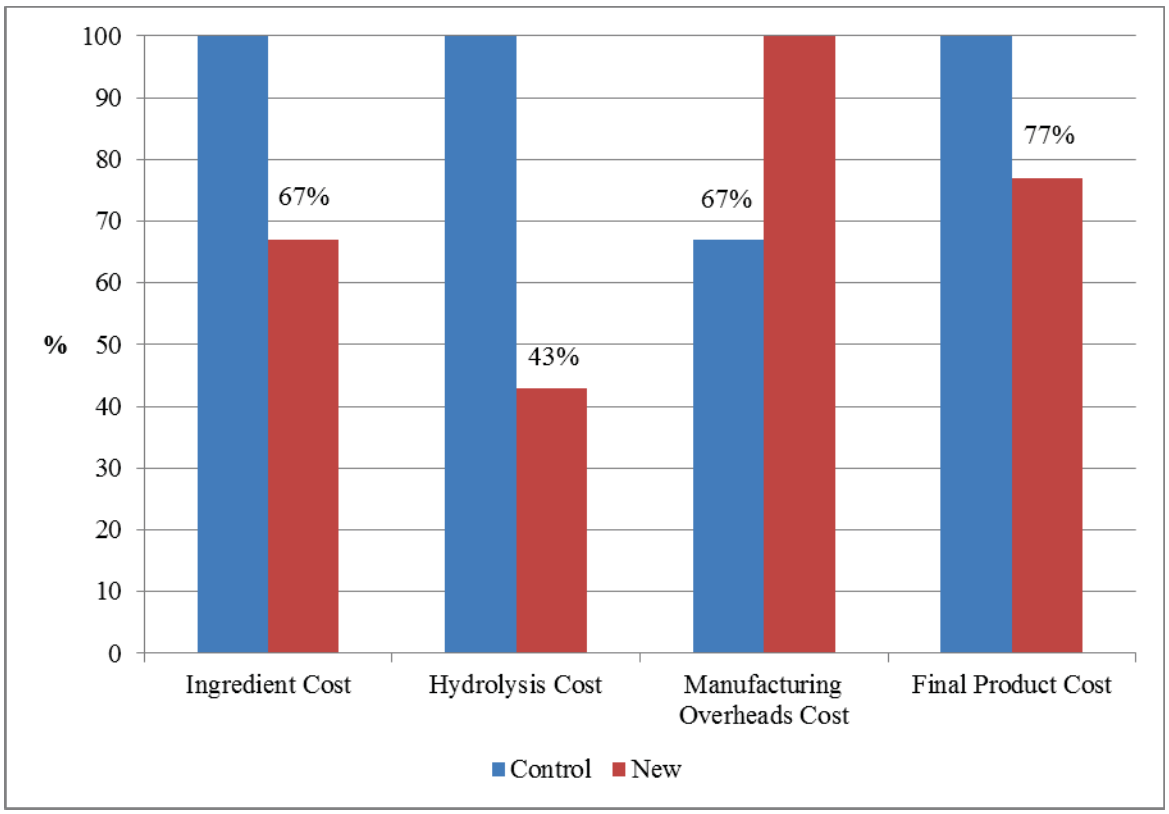
Figure 26: Molecular weight profile of Flavorzyme 1000L and Glutaminase SD-C100S combinations



8 Product improvement and sensory

The new formulation, Flavorzyme 1000L 2% and Glutaminase C100SD 0.25%, and its relevant manufacturing information were used for cost evaluation. The commercial costing model of the new formulation was exactly the same as the control, which made them more comparable. As it can be seen from Figure 21, the new formulation achieved 33% ingredient cost reduction and 59% hydrolysis time reduction. However, the manufacturing overhead cost went up almost a third, this was due to total solids in the new formulation being about 5% lower than the control of 15%. This makes less final product based on volume of hydrolysis silo for every batch of manufacturing. Most importantly, the final product cost still achieved 22.5% cost reduction compared to the control. The final product also passed Tatua's internal informal sensory evaluation and all 5 participants agreed that the new formulation was more savoury than the control.

Figure 27: Process improvement of enzyme cost, hydrolysis time and final product cost



9 Overall conclusion and recommendations

In summary, the project was successful to produce a new formulation of savoury casein hydrolysate. The glutamic acid content of the new combination had a 48% increase compared to the control. The hydrolysis time of the new formulation was 59% shorter and ingredient cost was 33% lower than the control. As a result, the final product cost had a reduction of 23% based on the same commercial cost module as the previous formulation. There were no major differences between system one and system two for the final powder glutamic acid results without adding glutaminase. Both systems could not generate the same amount of glutamic acid as the control. This might due to weak side glutaminase activity which could not convert large amounts of free glutamine to free glutamic acid within 24 hours. In the end, glutaminase was used in the formulation option containing Flavorzyme 1000L to convert free glutamine and this combination was successful.

There are two recommendations for further research. Firstly, the glutamic acid content of the new formulation was significantly higher than the calculated theoretical value of Glu plus Gln before the addition of glutaminase that was only equal to the control. This might be due to converting free glutamine to free glutamic acid stopped or reduced free glutamine converting to pyroglutamic acid directly (Nandakumar et al, 2003). This recommends developing a pyroglutamic acid test method to prove the pathway so the whole pathway can be evaluated. There was no commercial testing lab for testing pyroglutamic acid at the time of experiments. Secondly, further experiments are recommended to increase the total lactic sodium caseinate solids in an enzyme substrate slurry so manufacturing overheads cost can be reduced to further improve final ingredient cost.

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Appendix

Appendix A – Selection of protease for the two enzyme preparation system

Appendix B – Comparison of two enzyme and single enzyme preparation system

Appendix C - Enhancing glutamic acid levels with glutaminase

Appendix D – Test parameter methodologies

Appendix A – Selection of protease for the two enzyme preparation system

System One - stage one: In-process data - liquid

System One: Stage One pH Values							
Time (h)	**Neutrase 0.8L**	**Protamex**	**Alcalase**	**Protin SD-AY10**	**MaxiproNPU**	**Maxipro BAP**	**Promod 144MDP**
0	7.50	7.50	7.53	7.50	7.53	8.75	7.50
1	6.91	6.70	6.74	6.82	6.86	6.85	7.22
2	6.88	6.65	6.75	6.82	6.84	6.87	7.06
3	6.87	6.63	6.66	6.82	6.84	6.84	7.10
4	6.88	6.59	6.66	6.82	6.81	6.84	7.07
6	6.83	6.55	6.66	6.82	6.80	6.71	7.10
7	6.84	6.55	-	-	-	-	-
8	6.64	6.35	-	-	-	-	-

System One: Stage One Brix Values (°Brix)							
Time (h)	**Neutrase 0.8L**	**Protamex**	**Alcalase**	**Protin SD-AY10**	**MaxiproNPU**	**Maxipro BAP**	**Promod 144MDP**
0	12.8	12.8	12.8	12.8	12.8	12.8	12.8
1	10.2	9.9	10.4	9.0	10.2	11.3	9.1
2	10.4	9.7	11.1	9.0	10.5	11.6	9.0
3	10.3	10.3	11.8	9.0	10.7	12.1	9.2
4	10.3	11.2	11.9	9.0	10.7	12.1	9.1
6	10.5	11.8	12.4	9.4	10.8	12.7	9.3
7	10.4	11.8	-	-	-	-	-
8	10.3	12.1	-	-	-	-	-

System One: Stage One Osmolality Values (mOsm/kg)							
Time (h)	**Neutrase 0.8L**	**Protamex**	**Alcalase**	**Protin SD-AY10**	**MaxiproNPU**	**Maxipro BAP**	**Promod 144MDP**
0	50	50	50	50	50	50	50
1	138	203	172	141	143	173	110
2	147	218	189	145	154	191	113
3	151	227	199	147	158	200	118
4	153	237	207	149	161	210	121
6	158	247	218	150	163	223	124
7	159	250	-	-	-	-	-
8	161	252	-	-	-	-	-

System one - stage one: Final powder

Enzymes/Parameters	Ash (%)	DH (%)	Protein (%)	Moisture (%)	FFA (mg/g Protein)
Neutrase 0.8L (P4021)	4.3	6.9	89.6	4.2	0.5
Protamex (P4022)	5.1	12.8	87.0	4.6	4.8
Alcalase (P4023)	4.2	12.1	88.7	4.6	13.1
Protin SD-AY10 (P4024)	3.9	5.6	90.3	3.5	4.1
MaxiproNPU (P4032)	4.1	7.6	90.3	3.5	0.6
Maxipro BAP (P4033)	4.5	12.2	88.3	4.2	9.9
Promod 144MDP (P4034)	4.0	2.5	90.0	4.1	0.5

Appendix B – Comparison of two enzyme and single enzyme preparation system

System One - stage two: In-process data - liquid

System One: Stage Two pH Values				
Time (h)	**Protamex 1% + Promod215P 1%**	**Protamex 1% + Flavorpro937P 1%**	**Protamex 1% + PDN N48/3 1%**	**Protamex 1% + Peptidase R-K 1%**
0	7.50	7.50	7.50	7.50
1	6.81	6.7	6.65	6.65
2	6.75	6.67	6.61	6.59
3	6.70	6.67	6.59	6.57
4	6.59	6.61	5.91	6.62
6	6.57	6.54	5.89	6.6
22	6.47	6.5	5.86	6.47
23	6.47	6.5	5.86	6.47
24	6.47	6.5	5.86	6.47

System One: Stage Two Brix Values (°Brix)				
Time (h)	**Protamex 1% + Promod215P 1%**	**Protamex 1% + Flavorpro937P 1%**	**Protamex 1% + PDN N48/3 1%**	**Protamex 1% + Peptidase R-K 1%**
0	12.8	12.8	12.8	12.8
1	10	9.9	10.2	10.2
2	10.9	10.6	10.5	10.5
3	10.9	10.9	11.0	11.2
4	11.1	11.3	11.6	11.6
6	11.8	12.3	11.8	11.8
22	13.4	13.9	12.6	13.5
23	13.3	13.9	12.6	13.7
24	13.4	14.0	12.7	13.8

System One: Stage Two Osmolality Values (mOsm/kg)				
Time (h)	**Protamex 1% + Promod215P 1%**	**Protamex 1% + Flavorpro937P 1%**	**Protamex 1% + PDN N48/3 1%**	**Protamex 1% + Peptidase R-K 1%**
0	50	50	50	50
1	191	193	197	199
2	205	206	209	212
3	213	215	219	220
4	241	258	264	262
6	268	312	279	288
22	377	484	330	526
23	378	488	329	531
24	381	495	329	538

System one - stage two: Final powder

Enzymes/Parameters	Ash (%)	DH (%)	Protein (%)	Moisture (%)	FFA (mg/g Protein)
Protamex + Promod215P (P4081)	5.3	25.4	85.3	2.8	141.2
Protamex + Flavorpro937P (P4082)	4.7	35.8	83.5	2.6	313.7
Protamex + PDN N48/3 (P4083)	5.9	18.0	84.0	3.6	47.4
Protamex + Peptidase R-K (P4084)	4.8	36.2	82.5	3.8	352.8

System Two: In-process Data - Slurry

System Two: Stage One pH Values									
Time (h)	**Promod 845 MDP**	**Promod 903 MDP**	**Protease A 2SDK**	**Protease M SDK**	**Protease AX**	**Flavorzyme 1000L**	**Maxipro FPC**	**Flavopro 852MDF UMAMI**	**Flavorpro F795MDF**
0	7.50	7.54	7.50	7.00	9.05	7.50	7.50	7.60	7.50
1	6.86	6.83	6.60	6.58	7.08	6.88	6.66	6.80	6.75
2	6.83	6.80	6.62	6.52	6.99	6.79	6.62	6.80	6.68
3	6.74	6.84	6.61	6.47	7.00	6.79	6.60	6.76	6.65
4	6.79	6.72	6.58	6.47	7.00	6.76	6.59	6.77	6.62
6	6.79	6.71	6.67	6.46	7.00	6.76	6.59	6.76	6.44
23	6.79	6.64	6.56	6.38	6.90	6.56	6.46	6.52	6.44
24	6.79	6.52	6.46	6.38	6.90	6.56	6.46	6.52	6.44

System Two: Stage One Brix Values (°Brix)									
Time (h)	**Promod 845MDP**	**Promod 903 MDP**	**Protease A 2SDK**	**Protease M SDK**	**Protease AX**	**Flavorzyme 1000L**	**Maxipro FPC**	**Flavopro 852MDF UMAMI**	**Flavorpro F795MDF**
0	12.8	12.8	12.8	9.8	12.8	12.8	12.8	12.8	12.8
1	9.9	10.4	13.0	10.8	12.2	8.6	11.3	10.3	10.4
2	10.3	10.5	13.6	10.8	12.7	9.4	12.2	10.9	10.4
3	10.1	11.0	13.9	11.3	12.9	10.0	13.0	11.5	11.0
4	10.4	11.3	13.9	12.4	13.2	10.0	13.4	11.9	10.9
6	11.0	11.4	14.2	13.9	13.4	10.7	13.7	12.1	12.6
23	12.3	12.9	14.4	13.9	14.1	13.4	14.1	13.4	14.1
24	12.2	12.9	14.3	13.9	14.0	13.3	14.2	13.6	14.1

System Two: Stage One Osmolality Values (mOsm/kg)									
Time (h)	**Promod 845MDP**	**Promod 903 MDP**	**Protease A 2SDK**	**Protease M SDK**	**Protease AX**	**Flavorzyme 1000L**	**Maxipro FPC**	**Flavopro 852MDF UMAMI**	**Flavorpro F795MDF**
0	50	50	50	50	50	50	50	50	50
1	155	172	322	249	274	224	222	239	263
2	180	190	365	284	323	276	269	275	281
3	194	210	401	305	351	316	310	309	318
4	209	226	424	344	369	340	334	327	339
6	233	245	459	467	402	369	367	360	375
23	329	349	581	471	515	477	486	491	509
24	332	352	586	476	518	483	490	498	511

System Two: In-process Data – Final powder

Enzymes/Parameters	Ash (%)	DH (%)	Protein (%)	Moisture (%)	FFA (mg/g Protein)
Promod 845MDP (N4232)	4.0	21.5	85.8	4.0	185.3
Promod 903 MDP (P4001)	4.6	24.0	84.9	4.2	193.8
Protease A 2SDK (P4002)	4.8	43.5	83.1	3.6	446.8
Protease M SDK (P4003)	4.5	34.0	83.8	4.0	330.0
Protease AX (P4004)	4.8	36.1	83.6	3.3	362.4
Flavorzyme 1000L (P4010)	4.5	35.2	84.4	3.6	374.8
Maxipro FPC (P4011)	4.7	33.7	83.9	3.4	328.6
Flavopro 852MDF UMAMI (P4014)	4.8	33.4	83.0	4.0	311.3
Flavorpro F795MDF (P4015)	4.8	35.3	82.4	3.8	312.5

Free amino acid profile of all system one and system two enzyme combinations

Sample ID (mg/g of Protein)	Control (Average)	Promod 845MDP (N4232)	Promod 903 MDP (P4001)	Protease A 2SDK (P4002)	Protease M SDK (P4003)	Protease AX (P4004)	Flavorzyme 1000L (P4010)	Maxipro FPC (P4011)	Flavorpro 852MDF UMAMI (P4014)	Flavorpro F795MDF (P4015)	Protamex + Promod215P (P4081)	Protamex + Flavorpro937P (P4082)	Protamex + PDN N48/3 (P4083)	Protamex + Peptidase R-K (P4084)
L-Aspartic Acid	6.59	0.30	0.27	3.48	1.68	3.62	5.69	2.91	2.90	1.66	0.21	3.11	0.25	9.05
L-Threonine	16.52	6.69	6.57	26.38	17.86	19.61	18.58	17.29	14.07	16.72	3.48	14.41	1.06	7.84
L-Serine	10.26	4.79	4.05	15.38	10.01	14.44	15.71	12.53	11.96	9.90	2.05	11.09	1.85	12.99
L-Asparagine	7.72	3.61	4.09	18.16	11.44	13.06	12.46	11.18	9.08	10.05	2.74	12.69	0.51	6.30
L-Glutamic Acid	31.95	0.80	0.95	6.94	3.50	5.63	12.74	6.39	24.81	21.00	2.26	5.60	1.89	18.05
L-Glutamine	1.38	4.49	5.18	20.76	11.40	11.90	20.71	12.67	0.00	0.75	2.64	10.50	0.64	15.32
L-Proline	10.08	0.29	0.59	6.70	4.45	0.81	0.96	2.04	1.13	1.02	1.14	0.38	1.01	22.55
Glycine	1.95	0.70	0.57	1.88	1.24	2.08	2.62	1.73	1.76	1.08	0.40	1.40	0.43	2.74
L-Alanine	9.71	3.92	4.49	15.14	11.42	13.30	14.12	11.48	11.10	10.13	1.31	8.55	1.10	7.82
L-Valine	23.41	10.15	11.57	34.19	21.89	25.43	22.35	20.92	18.87	22.85	13.26	27.60	2.12	24.29
L-Cystine	0.39	0.00	0.00	2.15	1.18	1.08	1.14	1.14	0.16	0.53	0.00	0.00	0.00	0.00
L-Methionine	14.19	7.09	7.29	16.67	11.74	12.91	12.80	11.92	11.11	12.39	6.23	12.69	1.45	17.87
L-Isoleucine	17.96	8.67	11.55	35.16	22.70	26.18	20.23	20.11	17.18	24.76	9.21	21.02	1.20	14.36
L-Leucine	60.87	43.43	45.65	70.60	59.39	63.34	59.60	57.54	56.57	57.21	34.98	61.44	9.57	64.23
L-Tyrosine	28.73	17.73	16.97	36.62	28.53	32.91	33.47	29.85	29.78	26.57	7.92	25.76	4.26	38.19
L-Phenylalanine	28.56	22.93	24.23	35.67	30.51	30.00	29.23	28.24	27.45	30.12	20.95	27.66	4.40	27.25
L-Tryptophan	5.30	5.98	3.47	14.72	11.62	13.12	12.93	10.35	9.02	5.85	1.23	3.80	0.67	1.36
L-Lysine	44.69	20.94	21.07	45.40	34.95	37.88	45.07	36.75	34.05	28.48	12.30	30.80	9.33	31.53
L-Histidine	13.81	10.87	12.83	18.19	15.84	14.49	14.42	14.37	13.60	14.22	8.01	13.64	1.85	9.56
L-Arginine	23.92	11.93	12.44	22.64	18.59	20.59	19.95	19.19	16.67	17.22	10.91	21.57	3.76	21.47
Total mg/g	357.98	185.31	193.84	446.82	329.95	362.38	374.76	328.58	311.28	312.52	141.24	313.74	47.36	352.78
L-Glu + L-Gln	33.33	5.29	6.14	27.70	14.89	17.54	33.45	19.06	24.81	21.75	4.90	16.11	2.54	33.37

Appendix C - Enhancing glutamic acid levels with glutaminase

Enhancing glutamic acid levels with glutaminase – Liquid

Flavorzyme 1000L + Glutaminase Combination: pH Values		
Time (h)	**Flavorzyme 1000L + 0.25% Glutaminase (P4095)**	**Flavorzyme 1000L + 0.50% Glutaminase (P4096)**
0	7.59	7.59
1	6.85	6.82
2	6.78	6.79
3	6.78	6.78
5	6.76	6.75
7	6.75	6.73
22	6.72	6.72
23	6.72	6.72
24	6.72	6.72

Flavorzyme 1000L + Glutaminase Combination: Brix Values (°Brix)		
Time (h)	**Flavorzyme 1000L + 0.25% Glutaminase (P4095)**	**Flavorzyme 1000L + 0.50% Glutaminase (P4096)**
0	12.8	12.8
1	9.2	8.9
2	10.2	10.1
3	10.1	10.1
5	10.6	10.6
7	10.9	11.1
22	13.1	13.1
23	13.2	13.1
24	13.1	13.2

Flavorzyme 1000L + Glutaminase Combination: Osmolality Values (mOsm/kg)		
Time (h)	**Flavorzyme 1000L + 0.25% Glutaminase (P4095)**	**Flavorzyme 1000L + 0.50% Glutaminase (P4096)**
0	50	50
1	228	231
2	270	272
3	294	294
5	332	346
7	368	381
22	480	489
23	479	492
24	481	495

Enhancing glutamic acid levels with glutaminase – Final powder

Enzymes/Parameters	Ash (%)	DH (%)	Protein (%)	Moisture (%)	FFA (mg/g of Protein)
Flavorzyme 1000L (P4010)	4.5	35.2	84.4	3.6	375
Flavorzyme 1000L + 0.25% Glutaminase (P4095)	4.2	36.2	82.7	3.8	399
Flavorzyme 1000L + 0.50% Glutaminase (P4096)	4.3	36.7	82.6	4.2	401

Free amino acid profile of Flavorzyme 1000L + Glutaminase Combinations

Sample ID (mg/g)	Control (Average)	Flavorzyme 1000L (P4010)	Flavorzyme 1000L + 0.25% Glutaminase (P4095)	Flavorzyme 1000L + 0.50% Glutaminase (P4096)
L-Aspartic Acid	6.59	5.69	6.22	6.40
L-Threonine	16.52	18.58	18.95	18.60
L-Serine	10.26	15.71	15.63	15.54
L-Asparagine	7.72	12.46	12.20	12.24
L-Glutamic Acid	31.95	12.74	46.61	48.01
L-Glutamine	1.38	20.71	0.80	0.76
L-Proline	10.08	0.96	0.67	0.80
Glycine	1.95	2.62	2.67	2.64
L-Alanine	9.71	14.12	14.76	14.48
L-Valine	23.41	22.35	23.68	23.75
L-Cystine	0.39	1.14	0.00	0.00
L-Methionine	14.19	12.80	13.28	13.35
L-Isoleucine	17.96	20.23	21.43	21.95
L-Leucine	60.87	59.60	62.24	61.91
L-Tyrosine	28.73	33.47	34.98	35.71
L-Phenylalanine	28.56	29.23	30.40	31.59
L-Tryptophan	5.30	12.93	10.87	11.14
L-Lysine	44.69	45.07	47.67	46.76
L-Histidine	13.81	14.42	14.74	14.58
L-Arginine	23.92	19.95	21.19	20.91
Total mg/100g	357.98	374.76	398.97	401.13
L-Glu + L-Gln	33.33	33.45	47.41	48.78

Appendix D – Test parameter methodologies

Test Parameter methodologies

Test parameters	Method reference	Methodology
Degree of hydrolysis	Tatua internal – LCHPM2/4.14/2	Confidential
Protein content (TN x 6.47)	Tatua internal – LCHPM/3.30/20	Confidential
Free amino acid profile	New Zealand AgResearch method based on AOAC988.15 and 994.12	Confidential
Molecular weight profile	Tatua internal - LCHPM2/13.3/2	Confidential
Ash	Tatua internal - LCHPM	Confidential
Moisture	Tatua internal - LCHPM	Confidential