Development of Tenderized Fermented Squid Product in the Form of Spread and Pieces Using Kiwifruit Protease

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Abstract

Squids are cephalopods widely used as food in many cultures, where they are customarily cooked as a means of enhancing nutritional value, and as a way of temporarily preserving squid from spoilage.

Modelled on a cooked, comminuted and fermented mussel product developed at AUT, this project primarily aimed to develop a tenderized fermented squid product in the form of spread or pieces. Squid mantle is chewy by virtue of its high collagen content. While this may appeal to some consumers, the New Zealand culinary tradition uses kiwifruit proteases to minimise this chewiness. Zyactinase, a standardized kiwifruit protease was used in this study as well as freshly prepared kiwifruit extract.

Various product forms such as minced, bowl chopped and chopstick-suitable pieces were compared. Irrespective of the use of enzyme, during the preparation of fermented product, minced and bowl chopped squid mantles expanded when the required evacuation was applied. This was undesirable. It was laborious to expel all the gas from the system necessary for the subsequent anaerobic fermentation. It was also noticed that the finer the cut the harder it was to achieve the required vacuum. Light and electron microscopy revealed the fine structure of squid mantle, which led to this packaging problem. Therefore the preparation of finely comminuted squid spread was discontinued. Chopstick-suitable fermented pieces were more promising because they could be easily vacuum packed.

The cooking step prior to evacuation and fermentation was thought to be a useful way of limiting the protease activity through enzyme denaturation. Over tenderized squid develops a mushy texture, and exposure to protease also led to a yellowing of squid mantle, possibly linked to the Maillard reaction. Thus the tenderizing effects of the kiwifruit protease preparations were examined under varying conditions of concentration, pH and incubation period.

Based on the earlier experiments with comminuted squid, 1% Zyactinase to the weight of the squid tissue was chosen as the starting point for tenderising the chopstick-suitable fermented product. However, it took overnight incubation to tenderise the mantle pieces. In contrast, freshly prepared kiwifruit was highly effective in tendering squid pieces when marinated for only five hours. Thus, the tendering ability of Zyactinase was questionable. The pH of the incubation medium can influence the texture of squid mantle,

and it was found that acidic conditions toughened the mantle pieces irrespective of the presence of protease. This could be linked to the denaturation effect of food acids in the same way that the raw fish becomes sushi on the addition of lemon juice or vinegar.

Fermentation was successful throughout the studies, with or without enzymes. The lactic acid produced might be anticipated to affect texture, but there were no significant difference in the textural values of squid pieces between after enzyme incubation and after fermentation, when enzyme incubations were conducted at ambient pH. In respect of tenderising, the springiness of the squid pieces was close to unity in most of the preparations with or without enzyme under varying pH and incubation times. However, a slight but significant drop in toughness was found for pieces after fermentation

In conclusion, cooked fermented squid pieces could be commercially produced in a commercial environment, but Zyactinase is not effective as a tenderiser.

Statement of Originality

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other institutions of higher learning, except where due acknowledgement is made in the acknowledgement".

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Chapter 1

Introduction

Fermentation and fermented foods

Fermentation is one of the oldest methods of food preparation and preservation. The term fermentation is derived from the Latin word 'fermentum' meaning 'to boil' (JyotiPrakash, Namrata, Buddhiman, Arun, & Rajen, 2015). Campbell-Platt (1987) described fermented foods as foods subjected to the activity of enzymes or microorganisms which in turn cause desirable biochemical changes in the food, and results in the significant modification of the food (Bamforth, 2008).

Fermentation was exploited as a low energy technique to preserve food, along with drying and salting before refrigeration, freezing and canning became possible. Preservation of food by fermentation occurs through the formation of inhibitory metabolites such as organic acids (lactic, acetic etc.), ethanol, carbon dioxide, diacetyl, reutrin, bacteriocins, etc., often in combination with a decrease of water activity by drying or use of salt (Ramesh & Joshi, 2014). Fermentation improves the safety of food through inhibition of harmful pathogens and removal of toxic compounds by metabolism. In addition to food preservation and safety, fermentation processes also give foods a variety of flavors, tastes, textures, sensory attributes, and therapeutic values (Leroy & De Vuyst, 2004). Thus, several fermentation techniques enhance the nutritional value, palatability and digestibility of the products compared to the raw form (Motarjemi, 2002; Van Veen & Steinkraus, 1970). All these attributes contributed to the extensive use of food fermentation in many cultures.

History of fermentation

The availability and acceptance of fermented food have had a very long history among different cultures. Acceptability varies depending upon the cultural habits; a product that is highly acceptable in one culture may not be acceptable in other cultures. The practice of fermentation is believed to have originated independently in the Middle East, the Indian subcontinent and the Far East (Caplice & Fitzgerald, 1999). Fermented bread and beer were known to people in Egypt and Babylonia as early as 6,000 and 5,000 years before present. In the sacred book of the Hindus, Rigveda (about 2,600 years before

present), there are references to the origin of fermentation techniques followed by observing fermentative changes in fruits and their juices (JyotiPrakash et al., 2015).

In spite of the long history of fermentation, there was little knowledge about the science governing it until 1857, when Louis Pasteur discovered the presence of bacteria in a milk fermentation(Singh, Pathak, & Verma, 2012). This was the first step of understanding the chemistry. He described the process using the term *la vie sans air*, or life without air. The role of enzymes in fermentation was identified in 1896 by Germen chemists Hans and Eduard Buchner. In 1907, Russian microbiologist Ellie Metchnikoff isolated *Lactobacillus* from fermented milk and Hans von Euler obtained the Nobel Prize for his work on the fermentation of sugars and fermentation enzymes in 1930 (Mehta, Kamal-Eldin, & Iwanski, 2012). Examples of variants of fermented products available today is huge, depending on the manufacturing process, food groups, form and characteristics of final product, kinds of ingredients used, cultural diversity etc.

At a strictly biochemical level, fermentation is a metabolic process that delivers metabolic energy from the organic compounds in foods without the involvement of any exogenous oxidizing agents like oxygen itself (Ramesh & Joshi, 2014). Although fermentation is an anaerobic process, its definition is also extended to include aerobic as well as some non-microbial processes such as those caused by enzymes not derived from the microbial agent but rather enzymes endogenous to the food.Therefore fermentation describes the whole process where the complex organic foods are catabolized to simpler compounds with the simultaneous production of chemical energy in the form of adenosine triphosphate (ATP). In this respect the process is similar to glycolysis in advance of aerobic respiration (Mehta et al., 2012).

Traditional and industrial fermentations

Fermentation can occur spontaneously due to adventitious microorganisms. In spontaneous fermentation the raw material is subjected to non-sterile handling that induces the growth of an indigenous microbiome. In most spontaneous fermentations, a microbial succession occurs with time of fermentation. Lactic acid bacteria (more on these in the next section) mostly dominate the process initially, and are followed by various yeast species (Yiu H Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004). The presence of oxygen is required for the growth of molds. Therefore their occurrence is limited to certain types of fermented foods. In the wide context, the lactic bacteria reduce the sugars component of the raw materials and inhibit the growth of harmful pathogens, whereas

yeast mostly produces alcohols and other aroma compounds. When molds are involved in fermentation, they generally liberate intra and extra cellular proteolytic and lipolytic enzymes that mainly contribute to the aroma and texture of the product (JyotiPrakash et al., 2015). As a variant of spontaneous fermentation some traditional fermented products are started with samples from the previous and hopefully successful fermentation. This technique is colourfully called 'back slopping', and is still successfully used for the production of foods like sourdough, some artisanal cheeses, wine, sauerkraut and some fermented sausages for private use (Leroy & De Vuyst, 2004).

With the identification of microorganisms, it was easier to understand and manage the process of fermentation. Availability of isolated and purified bacterial cultures revolutionized the field of fermentation, use of well-defined cultures became the industrial standard of breweries by 19th century (Yiu H Hui et al., 2004). Direct addition of selected starter cultures to raw materials allowed high degree of control over the process fermentation. Also, the pasteurization of raw materials before the inoculation enables the fermentation process with few variations and standardized end product (Caplice & Fitzgerald, 1999; Leroy & De Vuyst, 2004) .

By the 20th century other food industries like wine, dairy and meat also shifted their production procedures to well-characterized and defined starter cultures (JyotiPrakash et al., 2015). In the beginning these starter cultures were isolated from the previous fermentations and also maintained and propagated at the site. The application of microbiology and process technology revolutionized the production of fermented food. The quality improved to the extent that currently all of the significant fermented food production is industrially or at least professionally prepared. Even the small amount of home fermentation conducted in the form of baking, home brewing and private cheese making mainly rely on commercially available yeast and bacterial cultures. The maintenance of microbial cultures varies depending on the fermenting industries. Industries like breweries and vinegar fermenters maintain their own strains and inocula, whereas other industries such as dairy, meat and bakery obtain their cultures from suppliers dedicated to the production of high quality food ingredients (Yiu H Hui et al., 2004).

Microorganisms involved in fermentation

The microorganisms involved in fermentation are many and diverse and include representatives of bacteria, yeasts and molds.

Lactic acid bacteria like *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Oenococcus*, etc. are the most common bacteria in fermented foods. They are endemic to human environment and are present in or on wide variety foods like milk, grain crops, and vegetables. Lactic acid bacteria produce lactic acid from simple carbohydrates, most notably lactose from where the name arises. They play a crucial role in fermentation due to their physiological features such as metabolic capabilities and probiotic properties (Leroy & De Vuyst, 2004). Other important groups of bacterial fermenters are *Acetobacter* and *Bacillus* species. *Acetobacter* species oxidize alcohol to acetic acid in the presence of air, and are used in the production of grain and fruit vinegars. *Bacillus* species are mainly responsible for the so-called alkaline fermentations. *Bacillus subtilis* is the dominant species used, which hydrolyses of protein in to amino acids and peptides with the release of ammonia thereby increasing the alkalinity of the medium, and making the substrate unsuitable for the growth of competing pathogenic organisms (Ramesh & Joshi, 2014). Alkaline fermentation is mainly associated with protein-rich foods like soybean and other legumes, although a few products use plant seeds (Nigerian oriri from water melon and oriri saro from sesame) as substrate for alkaline fermentation (K. Steinkraus, 1995).

Yeasts are widely distributed in nature, and in some fermented foods play a major role in fermenting sugars, producing alcohols and carbon dioxide. In some fermentations they also inactivate the harmful effect of mycotoxins by degrading them to less harmful compounds (Bata & Lásztity, 1999). Alcohols and carbon dioxide are the major fermentation products in the making wines, beers and leavened bread. Yeasts from *Saccharomyces* family, especially *Saccharomyces cerevisiae* is the most beneficial yeast in terms of fermentation. Strains of *S. cerevisiae* are variously used in these roles: baker's yeast, brewer's yeast, inoculum for wine fermentation, food and feed additives and as a flavour generating culture in dairy and meat products. The annual production of bakers/s yeast is estimated be 1 million tonne, which exceeds the combined production of all other microorganisms by about two orders of magnitude (Yiu H Hui et al., 2004). *S. cerevisiae* var. *ellipsoideus* is employed extensively in wine making and *S. cerevisiae* var. *carlsbergensis* is dominant in beer production. Other species such as *Schizosaccharomyces pombe* and *Schizosaccharomyces boulderi* dominate the traditional rural beverage fermentations especially those from maize and millet. *Schizosaccharomyces pombe* also has the ability to metabolise malic acid to ethanol and carbon dioxide, and has been successfully used to lower the acidity in grapes and plum musts (Ramesh & Joshi, 2014).

Fungi, especially filamentous molds play an important role in some fermented foods and alcoholic beverages due to the production of flavor- and texture-enhancing intra- and extra-cellular proteolytic and lipolytic enzymes. They also improve the bioavailability of minerals by degrading antinutritive factors like phytic acid (Egounlety & Aworh, 2003). *Actinomucor, Amylomyces, Aspergillus, Monascus, Mucor, Neurospora, Penicillium, Rhizopus*, and *Ustilago* are some of the mold genuses found in fermented food and alcoholic beverages. Species like *Aspergillus* are used in production of citric acid from byproducts like apple pomace, *Penicillium* species are used for the ripening and flavor development of many of the cheeses (Evcan & Tari, 2015; Mrázek et al., 2016).

Classification of fermented foods

Fermented foods are produced all over the world, depending upon the substrate availability and food consumption pattern. Product that is highly acceptable in one culture would not be acceptable to others. For example fermented dairy products are highly acceptable in Western countries whereas fermented fish products are highly acceptable in South and Southeast Asia (Yiu H Hui & Evranuz, 2012a).

There are various ways of classifying fermented foods depending upon the substrate, end product, geographic region etc. Steinkraus classified fermented food into a number of categories based on end products: fermentations producing a meat-like textured food; high-salt savoury-flavoured amino acid/peptide sauces and pastes; fermentations involving production of ethanol; acetic acid; lactic acid; and alkaline fermentations (Keith H. Steinkraus, 1997).

Fermentation producing a meat-like textured food

Indonesian tempeh is the best examples of product in this category. The substrate used for the production of tempeh is soaked, dehulled and partially cooked soybeans. Typically the cooked soybeans are treated with vinegar to lower the pH of the substrate to 5 or below, which is inhibitory to many microbes, but highly advantageous for the growth of certain molds (Feng, Eriksson, & Schnürer, 2005) . The main microbe is aerobic *Rhizopus oligosporous* or related *Rhizopus* species, which knit the surface-dried cooked soybean cotyledons in to a compact cake that can be sliced thinly and deep fried or cut in to cubes and used in soups or other recipes as a protein rich meat substitute (Yiu H Hui et al., 2004; Nout & Kiers, 2005).

These molds have the ability to grow very rapidly and at very high temperatures, around 40°C. The low pH and high temperature of the fermenting bean mass enables the overgrowth of *Rhizopus* in soybean in 18 h. The principle is that when the substrate is overgrown with the desired organism it is resistant to the invasion by other organisms. The mold also uses the available oxygen and releases carbon dioxide that is also inhibitory to the growth of many other microorganisms(Keith H Steinkraus, 1986). Molds also appear to produce some compound(s) with antibiotic activity (K. H. Steinkraus, 2002). All these factors prevent the product from the invasion and spoilage of pathogenic microorganisms. Soybean tempeh has an excellent record of safety in Indonesia and Malaysia where eaten for centuries. Other products in this category are peanut ontjom and okara ontjom made with *Neurospora intermedia* (Matsuo & Yumoto, 1999).

High-salt savoury-flavoured amino acid/peptide sauces and pastes

Addition of high percentage of salt, ranging from 13% of the original weight or higher, to a raw protein-rich substrates results in a controlled form of protein hydrolysis that prevents the putrefaction and thus spoilage of food by bacteria like *Clostridium botulinum* and many others (Khem, 2009). These fermentations rely on salt tolerant microorganisms and/or endogenous proteolytic enzymes. The final products are meaty, savoury, amino acid/peptide-rich sauces and pastes, which serve as important nutritious condiments. Major foods from this kind of fermentation include Asian soy sauce, Japanese shoyul and miso, Indonesian kecap, and some fish sauces (Vietnamese nuocmam and Philippine patis) and pastes (Philippine bagoong and Vietnamese mam)(Keith H Steinkraus, 1985). These sauces are made by the fermentation of bycatch of small fish and crustaceans by their endogenous proteolytic gut enzymes (Keith H. Steinkraus, 1997).

Alcoholic fermentations

Fermentation involving the production of ethanol is usually safe in food and beverages for consumption. Major foods of this kind are European-style wines and beers, and more exotic Indonesian tape ketan, Chinese lao-chao, South African kaffir/sorghum beer and Mexican pulque, the last made from agave species (Savadogo, 2012). The process generally involves fermentation by the *Saccharomyces* yeast, but also some yeast-like molds (*Amylomycesrouxii*), mold-like yeasts (*Endomycopsis*) and bacteria such as *Zymomonas mobilis* (Keith H. Steinkraus, 1997). Substrates for alcohol fermentation are products rich in fermentable sugars such as diluted honey, sugar-cane juice, palm sap, fruit juices, germinated cereal grains or hydrolyzed starch (Lin & Tanaka, 2006). Carbon

dioxide is the co-product. It flushes out the oxygen to maintain an anaerobic fermentation medium, in which the yeast can grow and multiply easily whereas the other mainly aerobic microorganisms cannot grow. Ethanol is germicidal as well. Therefore, as long as the condition remains anaerobic the product is quite stable and preserved (Keith H. Steinkraus, 1997).

Alcoholic fermentation is one of the ancient fermentations known to humans, primitive beers and wines are quite different from the wines and beers available now, the early forms are cloudy and effervescent containing not only liquid but particles from the fermenting substrate, yeast cells and B vitamins (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003). (Interestingly, chewing cereal grains was a way to introduce saliva as a source of amylose to hydrolyze starch to fermentable sugars. One example is chica produced in the Andean region. Maize kernels were chewed, and the gobs are removed from the mouth, and sundried. When later placed in crocks covered with water, wild yeasts fermented the sugars to produce the cloudy-yellow chica, containing about 6% ethanol.)(Yiu H Hui & Evranuz, 2012b)

Acetic acid/vinegar fermentations

When the anaerobic condition in alcohol fermentation is lost, bacteria present in environment, mainly the *Acetobacter* genus, oxidizes a portion of the ethanol produced to acetic acid/vinegar (K. H. Steinkraus, 2002). Acetic acid is bacteriostatic to bactericidal depending upon the concentration. Generally fermentation results in the production of acetic acid are safe for consumption and acetic acid is a stronger preservative than ethanol (Blandino et al., 2003). Vinegar is an ancient condiment and exclusively used for the picking vegetables and even in medicine because of its germicidal properties (Entani, Asai, Tsujihata, Tsukamoto, & Ohta, 1998).

Alkaline fermentations

These fermentations results in alkaline products those are safe for consumption. Africa has a number of fermented products of this category, they are used as flavor enhancers in soups and stews as well as serves as the low cost protein source in their diet. The main microbes involved in alkaline fermentations are proteolytic *Bacillus subtilis* or related bacilli, which hydrolyse protein in various seeds to peptides and amino acids, and also deaminating the latter, specifically the α-amino group. Ammonia is released and the pH rises to 8 or higher. The combined effect of free ammonia, high pH along with rapidly growing essential microorganism at a relatively high temperature (above 40°C) makes the

invasion of spoilage microbes less likely (K. H. Steinkraus, 2002). Therefore the finished product is quite stable and well preserved especially when dried even though prepared in not so hygienic environment.

Products such as Nigerian dawadawa, Ivory Coast soumbara and West African iru are produced by fermenting soaked, cooked locust bean *Parkia biglobosa* seeds with *Bacillus* species typically *Bacillus subtilis*. Several other alkaline fermented foods are derived from melon seeds (Nigerian ogiri), oil bean (Nigerian ugba), sesame seed (Leone ogiri-saro), castor bean seeds (Nigerian ogiri-igbo) and pumpkin bean seeds (Nigerian ogiri-nwan). Pans in which they are prepared act as a source for the required spores that germinate and overgrow the seeds forming a sticky mucilaginous gum on the surface of the beans/seeds. Locust bean is substituted with soybean in other part of the world and alkaline fermented. Product such as Japanese natto, Thai thua-nao and Indian kinema are all produced by alkaline fermented soybean (Keith H. Steinkraus, 1997).

Lactic acid fermentation

Lactic acid fermentation is the most ancient and one of the important fermentations in the world history that enabled the humans to survive and thrive. Humans have known of this technique since the domestication and milking of mammals. In lactic acid fermentation lactic acid bacteria such as *Leuconostoc mesenteroides, Lactobacillus brevis, Lactobacillus plantarum, Pediocccus cerevisiae, Streptococcus thermophilus, Streptococcus lactis, Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus citrovorum* and *Bifidobacterium bifidus* convert sugars into lactic acid, with no gas production or other loss on constitutive C, H or O atoms (Axelsson, 2004). (The fermentation can thus be viewed as a structural isomerization reaction.)(Narayanan, Roychoudhury, & Srivastava, 2004)

The oldest form of lactic acid fermentation is likely to be the production of fermented sour milk that still occurs in the form of yogurt to date. Primitive cheese is the residual curd resulting from the evaporation or removal of whey from the sour milk. In addition to product safety lactic acid also provide lots of flavor, aroma and texture to the product. The quantity and variety of fermented milk and cheese products available today are countless but are beyond the scope this review.

Due to the acid accumulation, unpasteurized milk will sour rapidly. The final product is safe for consumption because of the lactic acid and associated low pH, which together limit the invasion and growth of disease causing microorganisms. In addition, many of the lactic acid bacteria produce bacteriocins.

These are bactericidal, proteinaceous lantibiotics (Bali, Panesar, Bera, & Kennedy, 2014), represented by nisin in Figure 1.

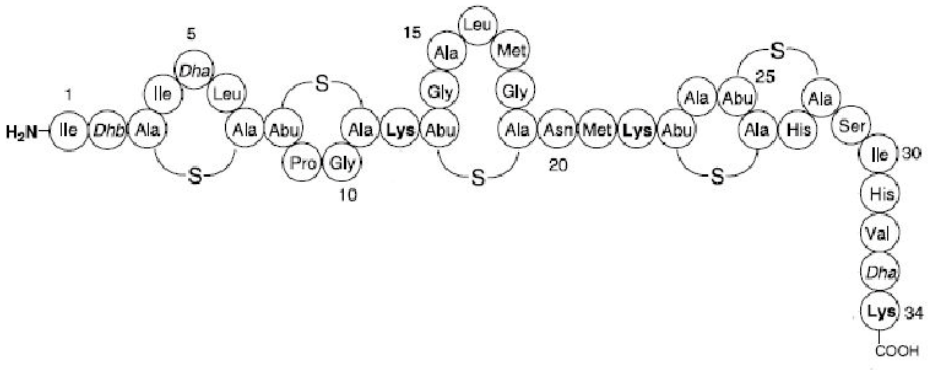

Figure 1 Primary structure of nisin produced by Lactobacillus lactis. Dhb is β-methyldehydroalanine; Ala-S-Ala is lanthionine; Dha is dehydroalanine; Abu-S-Ala is β-methyllanthionine (Karam et al., 2013)

The main difference between lantibiotics and classic antibiotics is that bacteriocins are active against strains of species related to the productive species and particularly to strains of the same species, whereas antibiotics are active against a wide spectrum of bacteria and other microorganisms. Also, bacteriocins are ribosomally synthesized and produced during the primary growth stage of bacteria unlike classic antibiotics, which are usually the secondary metabolites. Bacteriocins have low in molecular weights and undergo posttranslational modification as seen in Figure 1. They can be digested easily by the proteases of the mammalian gastrointestinal tract, which makes them safe for human consumption (Woraprayote et al., 2016). The uses of bacteriocin in food preservation especially on dairy, egg, vegetable and meat products have been widely investigated. Among the lactic acid bacteriocins, nisin is highly effective against microorganisms causing food poisoning, e.g. *Listeria monocytogenes*, and spoilage (Zacharof & Lovitt, 2012). Moreover nisin is one of the few bacteriocins that have been officially approved to use in food industry worldwide.

Lactic acid fermentation is highly energy efficient, generally requiring no heating or cooking either before or after fermentation. It has also been used as a medium for preserving vegetables for centuries throughout the world. The classic lactic acid fermented vegetable product is German sauerkraut (literally sour cabbage) in which fresh cabbage leaves are anaerobically fermented in the presence of about 2% salt. A kinetic

sequence of lactic acid bacteria is responsible for the production of final product, first removing oxygen, and then producing lactic acid (Gardner, Savard, Obermeier, Caldwell, & Champagne, 2001). Korean kimchi is also lactic acid fermented similar to sauerkraut but it contains radishes, red peppers, ginger and garlic in addition to cabbage (Y. H. Hui, 2012). Generally, any plant food can be fermented and very many are throughout the world. Appendix 1 lists a range of common lactic acid fermentations with plant foods.

Fermented meat products

Meat based fermentation is one of the oldest techniques used for meat processing and preservation which provides a relatively stable meat product with acceptable sensory attributes and safety. Fermented meat is produced by action of natural or added microbial cultures on the meat mixed with different condiments. Microbes involved in fermentation are complex and diverse depending upon the ripening process of the meat and the fermented product. Lactic acid bacteria present in the meat and the environment mainly dominate the fermentation process (Samelis, Maurogenakis, & Metaxopoulos, 1994). Acids and alcohols are liberated as a result of fermentation process leading to the decrease in the pH of the medium. Proteins hydrolysis to peptides and amino acids were also taken place and the finished fermented product with special flavors, palatability, colour, aroma, extended shelf life and microbial safety (S.-n. Liu, Han, & Zhou, 2011).

Fermented meat products like chorizo, salami and pepperoni are highly popular throughout the world especially in Europe and United states. Nowadays about 700,000 tonne of fermented sausages are consumed annually in European Union countries, particularly in Germany, Italy, Spain and France (Lücke, 1994). Although fermented meat products such as Cantonese fermented sausage, Chinese bacon, jinhua ham, sucuk, nem chua and som moo are traditionally made in several Asian countries, fermented fish is more popular (S.-n. Liu et al., 2011).

Fermented sausages are defined as minced meat mixed with salt, spices and curing agents (nitrite), stuffed into casings and subjected to anaerobic microbial fermentation (Leroy, Verluyten, & De Vuyst, 2006). Most of the fermented sausages are dried to lower the water activity and can be kept with minimal cooling (Lücke, 1994). There are many types of sausages available in the market depending upon the ingredients, microorganisms employed – of which there are 100s, and the degree of drying ranging from 10 to 30% weight loss.

In commercial environments, sausages are mainly fermented with lactic acid bacteria and coagulase negative cocci starter cultures in a controlled environment of temperature and humidity (Leroy et al., 2006).

The range of temperature used varies depending upon the type of fermentation technology used and the starter culture. Lactic acid bacteria dominate the fermentation process and metabolize sugars to lactic acid in about 48 hours. As a result pH drops and stabilizes the product making it increasingly resistant to spoilage. Coagulase negative cocci are important due to their various biochemical and metabolic properties such as lipolytic and nitrate reduction ability. The main species of lactic acid bacteria used in sausage fermentation are *Lactobacillus sakei*, *Lactobacillus curvatus, Pediococcus pentosaceus* and *Pediococcus acidilactici* (Singh et al., 2012). In case the of coagulase negative cocci, Staphylococcus xylosus is the most predominant but other strains such as *Staphylococcus carnosus, Staphylococcus simulans, Staphylococcus equorum* and *Staphylococcus saprophyticus* found to be useful as well (Talon, Leroy, & Lebert, 2007). (A *Pediococcus pentosaceus* and *Staphylococcus carnosus* mixture has been used in the present research.)

Ham is another widely available meat product in fermented form. Ham is basically dry-cured cut of raw pork meat mainly preserved by salting and drying. Typical aroma and tenderness of the ham is developed during aging due to the activity of meat enzymes. Bacterial strains are mainly used to reduce nitrate, a popular curing agent used in preparation. Bacterial activity enhances the flavor and also lowers the pH and improves the safety of the product (Lücke, 1994). Taiwanese dry ham is one of the highly popular fermented hams, in which the unique flavor is mainly due the high proportion of free amino acids and nucleotides liberated during the long curing process as well as the fermentation of product by the bacteria, yeast and molds present on the surface of the ham (Tu, Wu, Lock, & Chen, 2010).

Fermented fish and seafood products

Fermented fish plays an important role in the history and the nutrition requirements of many of the Asian and Pacific Rim countries. Fermented seafood can be usefully classified under two categories, high salt and low salt fermented products. High-salt fermented products mainly include fermented fish sauces and shrimp pastes (Yiu H Hui et al., 2004), which are prepared as described earlier: *High-salt savoury-flavoured amino acid/peptide sauces and pastes'.*

A good example of a low salt fermented fish product is Thailand indigenous fish sausage, som-fug. The main ingredients are fish mince (78 to 80%), salt (2 to 5%), ground, steamed rice (2 to12%) and minced garlic (4%). The largely domestic process usually involves mincing of fish meat, mixing with other ingredients at ambient temperature and tightly packing into plastic bags or packing in banana leaves, in either case to exclude air as much as possible. The mixture is fermented at ambient tropical temperature for 2 to 5 days (Riebroy, Benjakul, Visessanguan, & Tanaka, 2005). Som-fu fermentation is mainly dominated by lactic acid bacteria especially *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Pediococcus pentosaceus*, which can either hydrolyse the cooked rice to fermented carbohydrates or rely on simple sugars liberated by the rice cooking (Riebroy, Benjakul, & Visessanguan, 2008). Fermentation of simple sugars to lactic acid reduces the pH of the mixture; the final product will be slightly sour and salty with a firm and springy texture. The texture and colour of the product varies with the fish species, the other ingredients and the preparation methods. The colour of traditional som-fug varies from white to brown to red.

Khem (2009) & Khem, Young, Robertson, and Brooks (2013) described the preparation of a som-fug equivalent made from New Zealand marine species, using glucose as the source of carbohydrate. This pioneering research was the inspiration for low salt fermentation of other New Zealand seafoods. The iconic green shell mussel was the obvious choice. When fermentation was attempted on the raw mussel using a commercial starter culture, the result was a disaster (Dsa, 2013). Although the pH fell to acceptable levels, endogenous biological activities from the gut enzymes and microflora resulted in a rotten slurry (Figure 2).

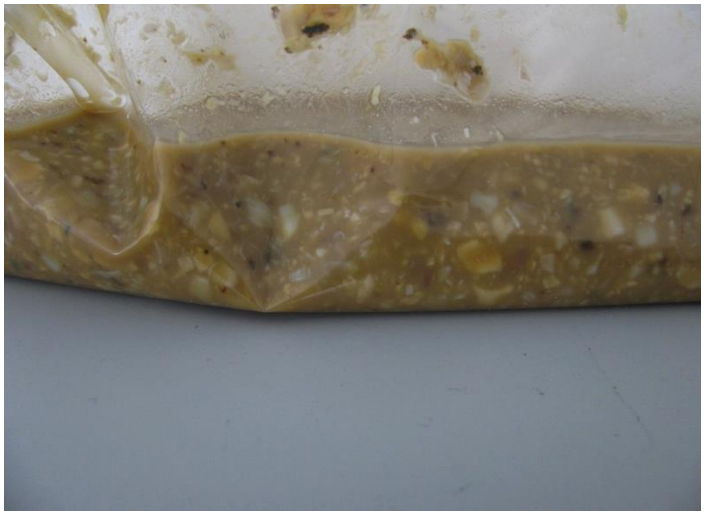

Figure 2 Comminuted raw mussel fermented with a commercial culture

The putrefaction problem was solved by prior cooking of the mussels leading to a confidential report by Dsa (2013) which is the basis of a commercialization venture by AUT Enterprises Limited. The technology is called cook-then-ferment technology. Cooking of raw materials before fermentation is sometimes practiced in fermented foods especially in case of fermented milk products and plant products. In most industrial preparation of fermented milk products, only pasteurized milk is used and cooking of plant ingredients for the preparation of soy sauce and other products are seen in fermented food category; *High-salt savoury-flavoured amino acid/peptide sauces and pastes* and *alkaline fermentations etc* ("Introduction," 2012; Keith H. Steinkraus, 1997).

But, cook-then-ferment technology is applied in traditionally fermented meat and fish products. Raw meat and fish products are usually cured with salt, then directly fermented and often dried, where the overall process creates many hurdles to pathogen growth. Unlike mussel with the gut included, squid mantle – the subject of this thesis – is likely to be colonized by a limited microbiota. Therefore it should be possible to create a fermented product without cooking. However, cook-then-ferment technology was adopted for two reasons. Product safety would be enhanced, and cooking allowed the control of the activity of a tenderizing plant protease, which will be described in detail later in this chapter.

Squid

Squids are molluscs, under the class Cephalopoda, featuring an elongated tapered streamlined body, a distinct head, eight arms and two longer tentacles around the mouth. There are more than 300 identified squid species and even more are believed to exist in various ocean bodies. They reside both in fresh and salt water and also found to be in varying temperatures from warm tropical waters to very cold and from shallow to the deep sea levels depending upon the species. The average size of squids varies from species to species. Most fully-grown squids average about 20 cm in length (Gilbert, Adelman, & Arnold, 1990; Z. E. Sikorski & Kołodziejska, 1986; Keith H Steinkraus, 1986).

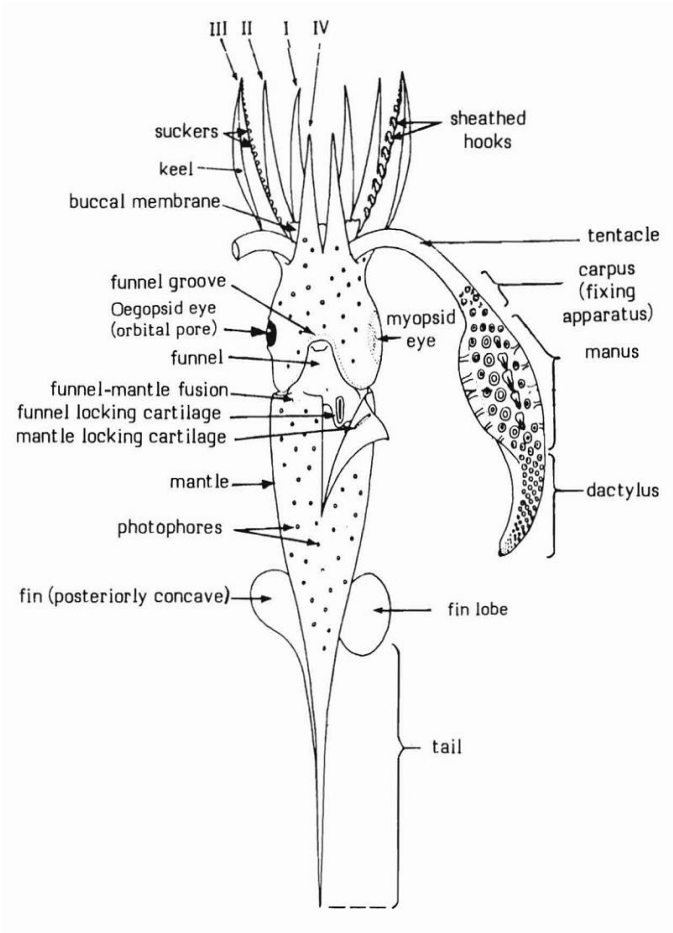

Figure 3 Anatomical picture of a squid (Vecchione & Sweeney, 1987)

In squid, the characteristic molluscan shell is reduced to a horny plate shaped like a quill pen and buried under the mantle (Figure 3). The mantle is the main body cavity in which the body mass is enclosed. As the mantle relaxes and contracts, two valves enable the squid to move by jet propulsion. In this form of locomotion water is expelled in jets from the muscular funnel located just below the head, propelling the squid backwards in an abrupt jet like motions (Boyle & Rodhouse, 2008).

Major squid products

Squid is popularly marketed by the Italian name 'calamari'. Many species are popular in cuisines as diverse as Chinese, Greek, Turkish, American, Japanese, Portuguese, Italian, Spanish, Korean, Vietnamese, Philippian, and Japanese. The tentacles, arms, mantle (also called the tube), fins, ink are all edible, in fact; the only parts that are not consumed are the beak and the gladius (pen). Squid is a good source of zinc, manganese, copper, and selenium. It may be cooked in various ways, the whole tube can be stuffed and baked, cut it in to rings, which are then crumbled and fried. The flesh only takes a few minutes to cook and the colour changes from translucent ivory to opaque, milky white. It has a delicate

shellfish taste but becomes rubbery and unpalatable if overcooked (Collignan & Montet, 1998). This is an enduring problem with squid but can be mitigated by enzymatic tenderization as will be discussed in the next section.

Of the derived squid products, shiokara and jeotgal are the two main fermented squid products available in the market. Shiokara is a popular Japanese food prepared from marine animals by adding about 10% salt and 30% malted rice. The product is fermented for up to a month and sold in glass or plastic containers. Micrococci and staphylococci are the major group of microorganisms that dominate the ripening process of shiokara. The microbes produce organic acids especially lactic and acetic acid during the fermentation process, which contribute to the odour and texture of shiokara and also prevent the invasion of harmful pathogens (Fujii, Wu, Suzuki, & Kimura, 1999). Jeotgal is a traditional fermented Korean food made by adding 20 to 30 % salt to various types of seafood (Guan, Cho, & Lee, 2011).

Tenderization of squid

As noted in the previous section, there can be negative consumer opinion about the eating quality of squid meat due to its characteristic rather tough texture. Squid mantle texture is dependent on its musculature and connective tissue features. Muscle fibers show both radial and circular arrangements, and supported by connective tissue in radial, circular and longitudinal orientations (Melendo, Beltrán, & Roncalés, 1997). Texture studies have revealed that collagen highly contributes to the longitudinal mantle toughness, whereas muscle fibres are more important transversely. Another factor that is linked with the toughness of squid is the formation of protein linking agent, dimethylamides, by the enzymatic conversion of trimethylamine oxide (Collignan & Montet, 1998). In addition to the mantle structure, cooking methods also influence the texture of the squid (Z. E. Sikorski & Kołodziejska, 1986) . Rheological experiments have shown that cooking for more than 1 minute at 100°C can gelatinize the collagen and make the squid more tender, but cooking more than 5 minute leads to excessive toughening (Collignan & Montet, 1998).

In meat industry several physical and chemical tendering techniques such as blade tenderization, electrical stimulation and addition of enzymes to the meat are used in order to tenderize the structure of the meat and there by enhancing the consumer acceptability of the meat (Bolumar, Enneking, Toepfl, & Heinz, 2013).

Chemical tenderization includes the infusion or addition of chemicals of biochemical such as calcium chloride and microbial or plant proteases. Calcium ions tenderize meat by the activation of the endogenous protease system (Bekhit, Hopkins, Geesink, Bekhit, & Franks, 2014; Gerelt, Ikeuchi, Nishiumi, & Suzuki, 2002). In certain concentrations sodium chloride has the ability to soften connective tissue and tenderize meat; brine injection is the common way to do this (Bolumar et al., 2013).

Tenderization of muscle foods with exogenous proteolytic enzyme has a long history in human nutrition (Bekhit et al., 2014). In industrial meat tenderization plant proteases are commercially more acceptable to the consumers than the microbial proteases due to their plant origin (Ketnawa & Rawdkuen, 2011). Importantly however, protease activity needs to be inactivated at some point of production; otherwise excessive proteolysis of muscle proteins can lead to undesirable texture and flavour of the product (Bolumar et al., 2013).

The most commonly used proteases are papain from papaya, bromelain from pineapple and ficin from figs, which are highly similar in their primary chemical structure (Turk, Turk, & Turk, 1996). There were several studies on the tendering ability of these proteases, dominating the literature before 1980. They come under the category of cysteine proteases (often referred to as thiol or sulfhydryl proteases) with a molecular weight ranging from 21 to 30 kDa. They are endopeptidases with low substrate specificity and enhance the hydrolysis of wide range of chemical bonds present in proteins such as peptide, amide, ester, and thiol ester and thiono ester bonds (Bekhit et al., 2014). Other proteases like actinidin from kiwifruit, zingibain from ginger have also gained popularity in the last few decades (C. Liu, Xiong, & Rentfrow, 2011) (Naveena, Mendiratta, & Anjaneyulu, 2004).

Actinidin, a mixture of six proteases with similar molecular weights around 23.5 kDa, is extracted from the common kiwifruit, *Actinidia deliciosa*. The enzyme activity greatly varies between different cultivars (Bekhit et al., 2014). Regulations on the use of actinidin varies according to the laws in different countries although there is a growing interest in the use of actinidin due to its less intensive tenderizing effect on meat when compared with other papain-like proteases (Ha, Bekhit, Carne, & Hopkins, 2012; Han, Morton, Bekhit, & Sedcole, 2009). Though actinidin exerts higher proteolytic activity towards collagen, it can also hydrolyze myofibrillar and connective tissue protein (Bekhit et al., 2014). These reports are based on work with of concentrated commercial forms and

also the freshly prepared kiwifruit extract. Zyactinase (Vital Food Processors Ltd., Auckland, New Zealand), a concentrated commercial form of actinidin, is used in the present study because its activity is defined, unlike the activity of an extract that will vary with fruit, age, cultivar and other factors. However, as will be shown freshly extracted kiwifruit extract is used, for reasons to be explained in the body of the thesis.

Several enzymatic and non-enzymatic methods have been used for the tenderization of squids. Major ones include the use of either endogenous or exogenous enzymes. After the identification of existence of number of endogenous proteases in squid mantle, autolytic tenderization of the squid mantle by up-regulation of endogenous proteases was observed (Melendo et al., 1997). The activity of these endogenous enzymes mainly depends upon the pH, time and temperature factors (Collignan & Montet, 1998). Another popular technique is the addition of papain-like proteases and other proteases (see for example (Ketnawa & Rawdkuen, 2011)). A technique using bromelain and a bovine spleen lysosomal-enriched extract, employed transversally cut squid mantles (rings) marinated with the enzymes at pH 7 at 30°C for 30 minutes prior to cooking (Melendo et al., 1997). Ultrasound waves of varying frequency have also used in the tenderization of squid (Hu et al., 2014). Although ultrasonic techniques can achieve a satisfactory tenderization, the use of ultrasound waves in food products is not highly accepted by consumers due to safety reasons.

The New Zealand squid industry

The New Zealand squid fishery is based on two closely related species of arrow squid, *Nototodarus gouldi* and *Nototodarus sloanii,* both found thoughout the continental shelf in waters up to 500 m in depth though they are most commonly found in waters shallower than 300 m. They have a colouration that can flash irridescent red or green when excited, fading to pale brown/grey upon death (Figure 4). Bot species live for a year, growing up to 30 cm in length and weighing just over a kilogram (Smith, Mattlin, Roeleveld, & Okutanp, 1987) (*Ministry for Primary Industries*, 2014). The squid fishery is concentrated around the South Island and the sub Antarctic Auckland Islands, mainly from January through to May, usually by mid water trawling but sometimes by jigging. Squid fishing is typically a low value, high volume industry targeted predominantly by Korean and Ukrainian vessels chartered by New Zealand companies (Uozumi, 1998). Because of natural variability of squid wild harvest, the export revenue from the industry varies from year to year. According to the seafood export data, around 14840 tonne of squid with a

value of $40 million was exported from NZ in 2015 (*Export Statistics*, 2015). Most of the squid is exported frozen and the major overseas market are China, Greece, Korea, USA, Taiwan, Spain and Italy (*Ministry for Primary Industries*, 2014).

Figure 4 Arrow squid, *Nototodarus sloanii* fade to pale brown colour after caught from water (*New Zealand Seafood*, 2016)

In the domestic market, squid rings cut from mantle are popular and are typically deep fried or used in sautéed forms in salads and stir-fries. Stuffed squid mantles in baked forms are also used in dinner recipes. Deep fried squid tentacles are also favoured in the domestic Asian community.

Stemming from the success of a fully cooked, comminuted, fermented mussel product developed at AUT, pilot studies showed that a unique shelf stable product might also be made from cooked squid through lactic fermentation after application of a vacuum. That is the topic of this thesis.

Objectives of the study

1) To develop a fully cooked and successfully fermented squid product in the form of spread, pate and chopstick-suitable pieces.
2) To characterise the tenderising effect of Zyactinase on squid mantle under a range of concentration, pH and time conditions.
3) To compare the tenderising effects of Zyactinase and freshly prepared kiwifruit extract.

Chapter 2

Product development with minced squid

Introduction

This initial aim of this project was to develop a lactic-fermented squid product in the form of a spread or pâté[1]. It is modelled on a fully cooked, comminuted, fermented mussel product developed at AUT (Dsa, 2013). Pilot studies by, Kassid (2013)showed that a shelf stable product might also be made from cooked squid. Squid is considered chewy because of its high proteinaceous connective tissue content, and the act of comminution by conventional mincing might also solve this problem to some degree. Also New Zealand culinary tradition uses kiwifruit proteases to reduce the chewiness problem and this enzyme in the form of an extract called Zyactinase was also used in this study. Zyactinase (Udani & Bloom, 2013) is a kiwifruit extract with a defined specific activity, and is sold in ambient-stable foil pouches.

Unlike mussels, raw squid mantle and tentacles do not contain a high population of enteric bacteria, nor the squid digestive enzymes. Therefore it would be possible to create a fermented product from raw squid. However, the cook-then-ferment technology initially developed for mussels gives an extra level of food safety. Moreover proteases added to squid might or would continue to be active during cooking and after fermentation, perhaps resulting in an inedible slurry. This approach was not tested. The idea was that protease would be added to raw squid, and after a defined incubation period, the subsequent cooking would destroy unwanted future protease activity.

Only squid mantles are used in this project. The cooked, comminuted (minced) squid is mixed with low concentrations of glucose, and salt (NaCl), and very low concentrations of a lactic-fermentation culture, the details of which will be discussed later.

After mixing air is removed with a vacuum packer. This is done because minced squid traps air, which is unfavourable for the successful anaerobic lactic fermentation. The mixture sealed in a vacuum barrier bag and is held for four days at 30°C, followed refrigerated storage. The success of fermentation is judged on the basis of a fall in pH to

[1] The final product is likely to contain added vegetable oil to aid spreading, but recipe development is not within the scope of this thesis.

around 4.0 to 4.5, conditions that are generally fatal for pathogens, and at the same time provide a desirable lactic tartness.

In the event the fermentation was always successful, but what was not so successful was the vacuum packing and the proteolysis. All this is now described. The outcome variables of interest to be reported are ease of packing, pH, colour and texture.

Materials and Methods

Equipment, chemicals and starter culture

Salt (NaCl) and glucose monohydrate used for fermentation was sourced from AUT laboratories. Starter culture Bactoflavour® (BFL-F02) was donated by Chr.-Hansen Ltd., Australia. This culture consists of *Pediococcus pentosaceus* and *Staphylococcus carnosus*, and its use was suggested by Chr.-Hansen. (It is a fast fermenting culture for meat fermentation, where the main role *Pediococcus* is lactic acid creation, and the role of the *Staphylococcus* is flavor development.). Zyactinase was donated by Dr Bruce Donaldson of AIT University.

Squid tubes were minced using a 4-mm plate Kenwood mincer (KM 200, Kenwood, Hampshire, U.K.) around 5 to 10 minutes. The pH value of the ferments were analysed using portable pH meter (Meterlab PHM-201, U.K.) fitted with a conventional glass electrode. Colour of the fermented squid mince was measured using a colour meter (Hunter Lab ColorFlex Model 45/0, Reston, Virginia, U.S.A.). Texture was measured with a TA.XT Plus Texture Analyser (Stable Microsystems, U.K using a 5-mm spherical probe of diameter in compression mode. Rectangular, plastic, colourless, nominally transparent domestic storage containers of 200 mL capacity were purchased at retail.

Vacuum barrier bags (240 x 250 mm) were bought from Duuninghams Ltd., Auckland. Evacuation of containers and barrier bags was accomplished with a benchtop-scale vacuum packer (Model DZ of obscure Chinese origin).

Other equipment included conventional laboratory and kitchen items.

Fermented squid mince preparation

Frozen squid mantle tubes from the local retail outlets were used throughout the project. The package labels identify the source as *Nototodarus* spp. (arrow squid), which mainly comprises two species harvested in New Zealand waters, *Nototodarus sloanii* and *Nototodarus gouldi.*

About 1 Kg of frozen mantles were thawed and cut in to two to three portions suitable for a domestic pot, and cooked in vigorously boiling water for exactly two minutes, followed by rapid cooling in cold tap water. The only variation to this method was addition of varying concentrations of Zyactinase before cooking or after rapid cooling, as discussed in the next paragraph. Pieces were then minced using a 4-mm plate Kenwood mincer (KM 200, Kenwood, Hampshire, U.K.) with 2% salt, 2% glucose, and 0.01% of the BFL-F02 lactic acid starter culture, as added quantities. This mixture is now abbreviated as SGC. The mixture was then transferred to 200 mL plastic containers and the lid was applied but leaving an unsealed edge. The idea was to provide a way for air to escape during evacuation; when the vacuum chamber was re-exposed to atmospheric pressure at the end of the vacuum cycle, the barrier bag forces the lid to close. The sealed bags were held at 30°C for 96 hours for fermentation. Experience has shown that fermentation is typically complete as judged by pH at 72 hours (Dsa, 2013).

Studies were carried out to determine the tenderizing effect of Zyactinase when added before and after cooking. Table 1 summarises the experiments done.

Table 1 Concentration of Zyactinase used and the steps followed in the pilot and formal trials.

Trial	Squid state	Zyactinase concn. (%) before cooking	Cooked and minced	Zyactinase concn. (%) after cooking	SGC and fermentation	Inspection	pH analysis	Colour and texture measure-ments
Pilot	Thawed	0	Yes	5	Yes	Yes	Yes	No
	Thawed	0	Yes	0	Yes	Yes	Yes	No
	Thawed	1	Yes	0	Yes	Yes	Yes	No
Formal	Thawed	0	Yes	1	Yes	Yes	Yes	Yes
	Thawed	0	Yes	0	Yes	Yes	Yes	Yes
	Thawed	1	Yes	0	Yes	Yes	Yes	Yes

In the pilot study, a high concentration of Zyactinase – 5% of powdered Zyactinase – was first added to minced cooked squid and incubated for 2 hours before conventional SGC addition and fermentation (Row 1, Table 1). Its control (Row 2) had no added Zyactinase. An additional but imperfect control was a 1% addition for 2 hours before cooking (Row 3). In the formal equivalent trial where colour and texture measurements were made, Zyactinase concentrations were limited to 1%.

Analysis by inspection

Fermented samples were inspected for colour, texture by touch and smell. Results were photographed.

Measurement of pH

pH of the fermented squid were determined by the method of Benjakul, Seymour, Morrissey, & An (1997). In outline, 2 g of fermented sample is dispersed with 18 mL of deionised water and the pH was measured.

Measurement of colour

The colour of the fermented squid was measured after fermentation through the base of the container. Colour was measured in L*, a*, b* colour space which indicates the lightness, redness/greenness, and yellowness/blueness, respectively. L* represents the lightness that is the measurement of reflectance. The minimum value of L* is zero which corresponds to no reflected light, and the maximum value is 100 which means all light is reflected. Positive a* values to +60 indicates redness and negative values to -60 indicate green. Similarly positive values of the b* indicates yellowness and negative as blue. An increase in the values of a* and b* irrespective of sign represents an increase in the intensity of the particular colour.

Containers full of product were placed on the light source of the machine and covered with a black box to avoid stray light. Four readings of L*, a*, b* were taken for each treatment. The equivalent mean values for and empty containers were subtracted from the raw data before means and standard deviations were calculated.

Texture analysis of minced fermented squid

Containers were placed on the platform base of the TA.XT and the probe descended to compress the product once followed by a retreat to the origin. Products were probed at five different locations, one in the middle and four towards the four corners of the rectangular container. All the textual parameters were measured at room temperature with the test conditions described in Table 2. Force, distance and time data were monitored and processed using the proprietary software, Texture Exponent 32 (Stable Micro Systems, U.K.), yielding texture data described by Bourne (1978) .

Table 2 Compression settings used for the textural analysis of fermented minced squid with a spherical probe.

Mode	Compression
Probe	Spherical, 5 mm diameter
Pretest speed	5.00 mm sec^{-1}
Trigger type to begin data collection	Auto force
Trigger force	0.0200 N
Test speed	5.00 mm sec^{-1}
Distance to record data over	18.00 mm
Post-test speed	5.00 mm sec^{-1}

Data analysis for this and later chapters

Data was marshalled and statistically analysed for means and standard deviations by routines in Microsoft Excel and Texture Exponent 32. Graphics were prepared with Excel. Analysis of variance was done with ANOVA and ANCOVA routines in XLSTAT (AddinSoft.com)

Results and discussion

Pilot trial

A problem that persisted throughout this work, irrespective of the Zyactinase treatment, was that it was difficult to achieve an evacuation of the squid mince such that the mass of mince would compress into a firm solid. When the vacuum chamber was progressively evacuated, the squid mince expanded beyond the bounds of the container. To overcome this the vacuum had to be applied several times until the expansion was very limited. In the likely event that much of the entrained gas is simply air, its retention would jeopardize successful lactic fermentation, which requires an anaerobic environment. This expansion phenomenon prompted the microscopy study in Chapter 4.

By repeated evacuations adequate packaging was achieved as judged by final pH (Table 3). Irrespective of the treatment, pH of the squid pieces before fermentation, around 8 dropped to 4 after fermentation. Whether added before or after cooking, Zyactinase had no effect on fermentation as judged by pH alone. However, it was noted that the treatments where Zyactinase was added before cooking and the control had a squid-like smell, whereas the squid with Zyactinase added after cooking had an unpleasant fruity odour. The reason for this was not explored.

Table 3 Properties of fermented samples with varying concentration of Zyactinase added at different stages of preparation in the pilot trial (details in Table 1).

Treatment details (abbreviated)					
Zyactinase concn. (%) before cooking	Zyactinase concn. (%) after cooking	Final pH	Final smell	Final colour	Figure
0	5	3.98	Fruity	Brown	Figure 5
0	0	3.98	Squid-like	Creamy white	Figure 6
1	0	4.06	Squid-like	Creamy white	Figure 7

Figure 5 Fermented mince with 5% Zyactinase added after cooking the squid

Figure 6 Fermented mince without Zyactinase (control)

Figure 7 Fermented mince with 1% Zyactinase added before cooking the squid

Although the colour in Figure 7 was slightly more yellow than the colour in Figure 6, both were much less coloure than the squid in Figure 5. The likely explanation for the browning in Figure 5 is that the Maillard reaction between peptides and amino acids – continually liberated by (5%) Zyactinase's protease activity – and added glucose was unrestrained in this treatment. In contrast, where 1% Zyactinase was added before cooking, only two hours were available for peptide/amino acid liberation. Further, cooking denatures most proteins, which then become even more susceptible to protease activities (Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008). Another observation fits with this interpretation: the product in Figure 5 appeared wet, suggesting over-tenderization due to proteolysis, and the 5% addition rate would have greatly contributed to this effect. It seems likely that if a protease is used it should be used before cooking, not after.

Formal trial

In the light of pilot studies, that a fermented squid sample could be successfully made using Zyactinase, a formal trial was carried out using 1% Zyactinase both before and after cooking (Table 1).

Table 4 Properties of fermented samples with one concentration of Zyactinase added at different stages of preparation in the formal trial (details in Table 1).

Treatment details (abbreviated)				
Zyactinase concn. (%) before cooking	Zyactinase concn. (%) after cooking	Final pH	Final smell	Final colour
0	1	4.03	Neutral	Creamy yellow
0	0	4.19	Squid-like	Creamy white
1	0	4.23	Squid-like	Creamy white

Fermentation was successful as judged by final pH (Table 4). Unlike the pilot trial, there were few differences in the smell and appearance of fermented squid. However, where 1% Zyactinase was added after cooking the smell was neutral rather than unpleasantly fruity as shown in Table 3 with 5% Zyactinase. All three fermented products were creamy white, although the 1% after-cooking treatment had a yellow tinge prompting formal colour measurements (Table 5).

Table 5 Mean and standard deviation of corrected L*, a* and b* values of fermented squid mince with 1% Zyactinase added before or after cooking.

Treatment	L*	a*	b*
Fermented squid with 1% Zyactinase added after cooking	43.6 ± 0.4	1.3 ± 0.1	22.2 ± 0.7
Fermented squid without any Zyactinase (control)	50.9 ± 0.1	1.3 ± 0.0	18.6 ± 0.1
Fermented mince with 1% Zyactinase added before cooking for 2 hours	45.7 ± 0.5	1.4 ± 0.0	19.0 ± 0.1

There were minimal differences in the colour values of squid mince treated with Zyactinase before cooking compared to that of control (Rows 3 and 2, Table 5). The Control treatment without any enzyme was the brightest out of three as judged by L*

values. The yellowest squid was where Zyactinase was added after cooking (highest b* value), colour development attributed to the Maillard reaction. Force data in the compression test using the TA.XT texture analyser are given in Table 6

Table 6 Mean peak compression force of fermented squid mince with 1% Zyactinase added before and after cooking.

Treatment	Force (N)
Fermented mince with 1% Zyactinase added after cooking	1.5 ± 0.3
Fermented mince without any Zyactinase (control)	9.1 ± 0.8
Fermented mince with 1% Zyactinase added before cooking	7.7 ± 0.8

According to Bourne (1978) hardness of a sample is proportional to the peak force exerted by the sample on the probe during the texture analysis. On this basis Zyactinase was highly effective in tenderizing squid when applied after cooking, causing a huge reduction in force from 9.1 N to 1.5 N. This squid is likely to be over-tenderized. As well, undesirable yellowing was demonstrated in the pilot and formal trials. Clearly addition of Zyactinase after cooking is a risky choice. Zyactinase addition before cooking cause a more modest reduction in peak force, and has the major advantage that its tenderizing effect can be stopped at will by the cooking step. It is easy to control tenderization by controlling, temperature, time and enzyme concentration.

Conclusion

The addition of a fruit extract like Zyactinase – which is the kiwifruit form of actinidin –or equivalent, was effective in tenderizing the squid meat. Addition of the enzyme before cooking is highly recommended because the heating stop is used to limit the enzyme activity, which could otherwise continue uncontrolled. However, the enzyme was linked to yellowing probably by way of the Maillard reaction, which would be undesirable where a pure white product form was wanted.

Although the minced form of the fermented squid mantle could be successfully produced, this format was very difficult to work with because of the difficulty in achieving the proper vacuum packaging required for the adequate anaerobic fermentation. The minced squid expanded during the evacuation step. This phenomenon prompted two studies reported in the next two chapters: other forms of the fermented squid (sliced and bowl chopped) are explored in Chapter 3; and the cause of the undesirable expansion is studied by microscopy in Chapter 4.

Chapter 3

Development of various forms of fermented squid

Introduction

Following the problems of evacuation experiences with minced squid, the manner and degree of comminution was explored in the search for a suitable product format. In this chapter, comminution ranges from pieces suitable for consumption with chopsticks through to finely bowl chopped pieces resembling a pâté. Similar to previous chapter, Zyactinase continued to be used as the tenderizing agent. In Chapter 2, addition of enzyme after cooking had led to the development of undesirable characters in the final product. Therefore in this work the use of enzyme was limited to before cooking only. In addition to the degree of comminution a different packaging was also examined; 50 mL cylindrical plastic specimens vials, and in the case of squid pieces, vacuum bags. This experiment mainly aimed to see the alternative forms of comminution were effective in controlling the expansion phenomenon, but other data were also gathered, pH, colour and texture.

Materials and Methods

There were three comminution methods, a minced form previously described in Chapter 2, a very finely cut form prepared with a domestic bowl chopper (2-mm plate Kenwood food processor) and a sliced form with pieces typically 10 x 30 mm (chopstick-suitable), prepared with a kitchen knife.

The experimental design is summarized in Table 7, which includes the three product forms, and their respective no-Zyactinase controls, and various packaging formats.

Table 7 Experimental layout of preparation various comminuted forms (minced, bowl chopped and sliced) of fermented squid.

Event	Minced control	Minced 1% Zyactinase	Bowl chopped control	Bowl chopped 1% Zyactinase	Sliced vial control	Sliced vial 1% Zyactinase	Sliced bag control	Sliced bag 1% Zyactinase
Cut up into medium pieces	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Enzyme treatment (2h) before cooking	No	Yes	No	Yes	No	Yes	No	Yes
Cook	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Comminution	Mincer	Mincer	Bowl chopper	Bowl chopper	Knife	Knife	Knife	Knife
Add SGC	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Packaging type	Vial	Vial	Vial	Vial	Vial	Vial	Bag	Bag
Fermented at 30° C for 4 days	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
pH determination	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Colour analysis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Texture analysis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Preparation of bowl chopped treatments

These were prepared exactly as described for minced treatments in Chapter 2, except that a bowl chopper was used rather than a mincer, resulting in a very finely comminuted product after about two minutes.

Preparation of sliced squid treatments

200 g of thawed squid mantles were immersed in a 1% Zyactinase solution in tap water. After two hours of incubation the tube was sliced in to small chopstick-suitable pieces using a sharp kitchen knife. Only the flat portions of the mantle were used because the edges would be unsuitable for texture analysis because of their variable shapes. The pieces were then cooked in boiling water for one minute while held in a wire colander. Cooked pieces were then mixed with SGC. The pieces were packed in two different ways. 10 to 15 pieces were placed in 50 mL plastic vials, which in turn were placed in a vacuum barrier bag (240 x 250 mm) and vacuum-packed. The second method was to vacuum pack in plastic bags. Both formats were fermented at 30°C for four days.

pH, colour and texture analysis

pH, colour and textural values of all the three treatments were measured as described in Chapter 2. In outline, pH was measured with pH probe electrode, and colour data were corrected for the container blank. However, only one texture data point could possibly be obtained from the vials containing minced or bowl chopped squid, so data were not collected (with the spherical probe described in Table 2. Data were collected for replicates of the slices using settings described in Table 8.

Table 8 Compression setting used for the textural analysis of fermented sliced squid with a wide cylindrical probe.

Mode	Compression
Probe	Cylindrical, 50 mm diameter
Pretest speed	2.00 mm sec^{-1}
Trigger type to begin data collection	Auto force
Trigger force	0.0400 N
Test speed	3.00 mm sec^{-1}
Distance to record data over	2.00 mm
Post-test speed	2.00 mm sec^{-1}

Results and Discussion

First comparing the evacuation effect on different forms of squid mantle, it was found that sliced squid showed a very limited expansion when a vacuum was applied. Therefore it could be easily packed, whereas minced and bowl chopped squid expanded greatly on evacuation. And it was also noted that the finer the cut of the squid greater the expansion. Thus, bowl chopped squid was more difficult to vacuum pack than the minced squid.

All the treatments were successfully fermented with a range of pH values 3.95 to 4.76, as shown in Table 9. Photographs of minced, bowl chopped and sliced squid treatments after fermentation are given in Figures 8, 9, 10 and 11.

There was little difference between the appearance of minced and bowl chopped treatments. Unlike the previous experiments (Chapter 2) where Zyactinase was added after cooking development of yellowness was not significant in any of the squid ferments prepared in this experiment. This was confirmed by the rather static b* values in Table 9, where positive b* values are a measure of yellowness. This confirms that tenderizing with Zyactinase before cooking is much more useful than tenderizing after cooking. Sliced squid was the most visually appealing format (Figures 10 & 11).

Figure 8 Fermented minced squid with 1% Zyactinase added before cooking

Figure 9 Fermented bowl chopped squid with1% Zyctinase added before cooking

Figure 10 Fermented sliced squid pieces with 1% Zyactinase added before cooking in vial

Figure 11 Fermented sliced squid pieces with 1% Zyactinase added before cooking in bag

One of the disadvantages of using the small vials for texture analysis was that it is possible to take only one reading for the minced and processed samples from a single container. With no opportunity to perform a statistical analysis, data were not collected. In case of sliced squid, 5 pieces from each treatment was texture analysed. It was of interest to see is the short distance to record data over, 2 mm (Table 8), could yield data. The mean value of their peak force (N) and standard deviation are shown in Table 9. The standard deviations were very high and it was realized that in future the size of the squid slices would have to be controlled and randomized, and a greater number of replicates would have to be tested to get meaningful data. However the parameters selected in Table 8 were useful and for future chapters that was the starting point for parameter selection.

Table 9 Results of physicochemical analysis of various comminuted forms (minced, bowl chopped and sliced) of fermented squid.

Event	Minced control	Minced 1% Zyactinase	Bowl chopped control	Bowl chopped 1% Zyactinase	Sliced vial control	Sliced vial 1% Zyactinase	Sliced bag control	Sliced bag 1% Zyactinase
pH	4.08	4.26	3.95	4.48	4.76	4.54	4.38	4.08
Colour								
L*	73.5 ± 0.1	71.82 ± 0.3	80.57 ± 0.0	78.3 ± 0.1	70.98 ± 0.2	74.16 ± 0.3	73.11 ± 0.5	76.14 ± 0.4
a*	1.61 ± 0.3	0.77 ± 0.2	0.21 ± 0.0	-0.17 ±0.0	-0.84 ± 0.0	-0.23 ± 0.1	-0.83 ± 0.1	-0.29 ± 0.2
b*	17.1 ± 1.1	14.31 ± 0.2	12.85 ± 0.1	12.86 ± 0.1	14.74 ± 0.3	14.79 ± 0.1	13.96 ± 0.0	14.81 ± 0.0
Peak force (N)					4.8 ± 3.9	5.4 ± 3.6	5.0 ± 4.7	12.7 ± 9.8

Conclusion

Sliced squid pieces were highly promising on the grounds of appearance and ease of vacuum packaging. The finer the cut the more difficult it was to remove air/gas from the squid. This could be that more cuts facilitate the release of more air/gas. Whatever the cause, the sliced format adopted for all future work, although the next chapter used microscopy to describe the undesirable expansion phenomenon. It is undesirable because vacuum packaging is a crucial step in the development of fermented squid.

Chapter 4

Microscopy

Introduction

The difficult-to-control expansion of minced squid meat on the application of a vacuum, suggested the presence of trapped air or some other gas inside the mantle meat. Because vacuum packaging is a highly desirable step prior to anaerobic fermentation, microscopic analysis on the structure of squid products were carried out to understand the nature of the expansion and also a possible solution. The structure of different preparations of squid, including structure before and after cooking were viewed using conventional transmission light microscopy, and scanning electron microscopy. Neither kiwifruit extract nor Zyactinase, the commercial tenderizing protease, was used in this work.

Materials and methods

Light microscopy

No vacuum was applied in this work. Minced and bowl-chopped samples were cooked after thawing, and comminuted as described in Chapter 2. For the sliced pieces, mantles were cooked in one piece after opening the mantle into a butterfly form by a single long cut along one fold. Chopstick-suitable pieces were cut axially and radially. All product forms were mounted in an embedding medium (Tissue-Tek OCT Compound) and rapidly frozen using a freon freeze spray. Five-micrometer sections were cut using a cryomicrotome (Microm Zeiss HM 350 SV, Zeiss Germany), and stained with 0.5% toluidine blue dye that binds to the nucleic acid material of tissues. The sections were viewed using a transmission light microscope fitted with a digital camera under a 4 times objective lens.

Electron microscopy

An electron microscopic analysis of squid samples were carried out using Hitachi SU-70 FESEM, field emission gun scanning electron microscope (SEM), which generates the images of samples by scanning it with a focused beam of electrons.

Three preparation types were examined (Table 10), all of which were freeze dried because gases (water vapour, air, other gas) cannot be present in the specimen chamber of a SEM. The freeze-drying temperature was -20°C, whereas dessication was done at room temperature over dried silica gel. This latter step was found to be necessary because the SEM took hours to pump down to the required vacuum when cooked mantle was inspected.

Table 10 Sample preparations for SEM work with squid mantle.

	Action 1	Action 2	Action 3	Action 4
Cooked thawed mantle	All of minced; bowl chopped; sliced pieces	Freeze-dried for two days		Select small sample and view
Raw fresh mantle	Sliced pieces	Freeze-dried for two days	Dessicated for five days	Select small sample and view
Raw thawed mantle	Sliced pieces	Freeze-dried for two days	Dessicated for five days	Select small sample and view

This study is based on a cook-then-ferment technology therefore the work is mainly focussed on the characteristics of cooked squid. At the same time it was essential to discover that the act of cooking is the cause of long lasting expansion phenomenon. Therefore other raw forms of squid- raw as fresh and thawed raw forms of squid were also examined.

Results

Light microscopy

The toluidine blue stained well, revealing numerous, large cavities within the cooked mantle structure as seen under a 4 x objective for both axially and radially-cut pieces, Figure 12, 13, and 14. Higher power objectives revealed no more detail.

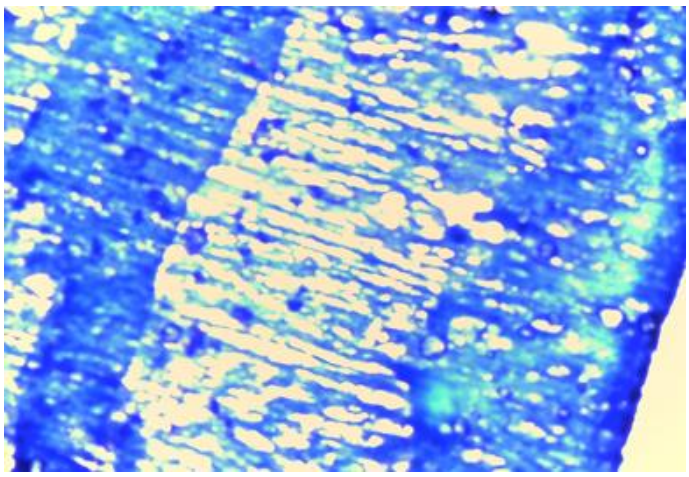

Figure 12 An axially-cut piece of squid mantle (sliced) stained with toluidine blue and viewed under a 4 x objective. Note the cavities

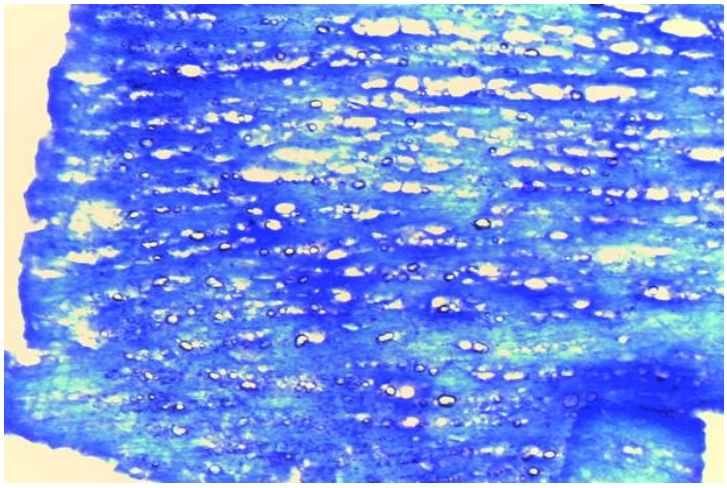

Figure 13 A radially-cut piece of squid mantle (sliced) stained with toluidine blue and viewed under a 4 x objective. Note the cavities

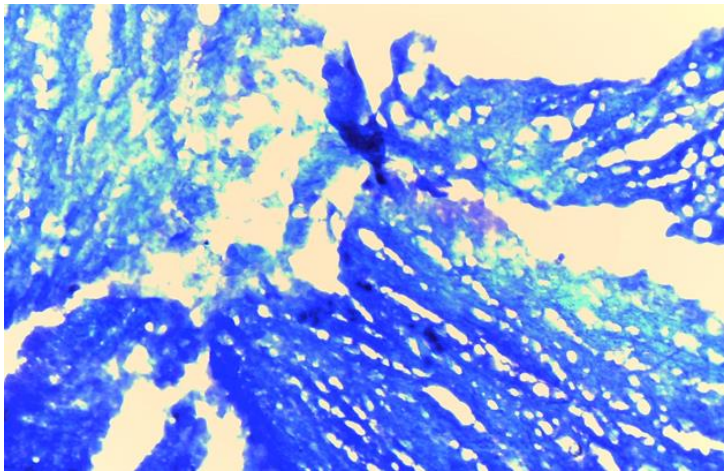

Figure 14 A minced piece of squid mantle stained with toluidine blue and viewed under a 4 x objective. Note the cavities

The light microscopy images suggested a layered porous structure for the squid that could account for expansion on evacuation. It was thought that SEM might reveal these structures in more detail.

Electron microscopy

Of the three preparation types of cooked thawed mantle – bowl chopped, minced, and sliced (Table 10) – the first two took the longest to pump down. Although gas – air, water vapour or otherwise – was difficult to remove from cooked thawed mantle, useful images were eventually obtained (Figure 15 and 16), showing a somewhat random porous structure in the case of bowl chopped and minced.

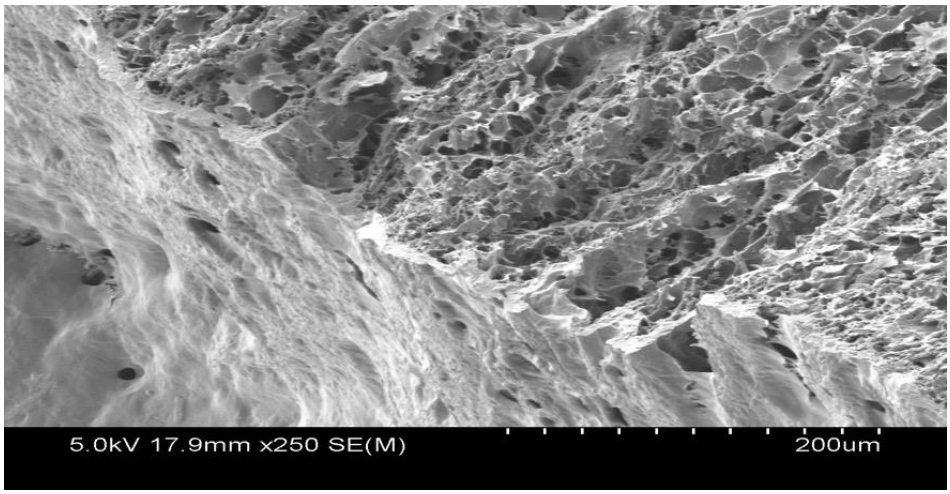

Figure 15 Bowl chopped cooked thawed mantle under SEM. A porous structure was evident

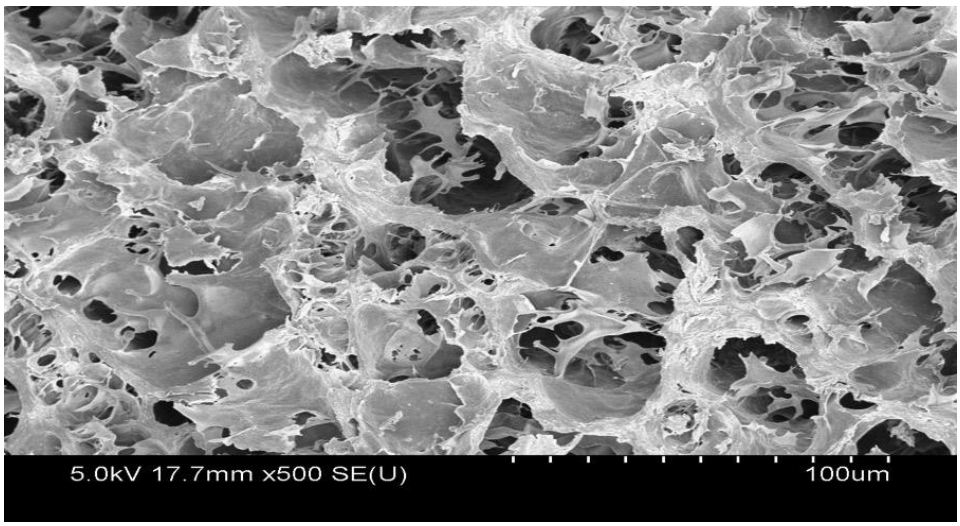

Figure 16 Detailed structure of bowl chopped cooked thawed mantle

Images of minced cooked thawed mantle similarly revealed a porous structure (Figure 17), but the most revealing images were of sliced pieces (Figure 18 and Figure 19). Although Figure 17 and Figure 18 are not on quite the same scale, it appears that the physical disruption cause by mincing was responsible for the highly porous nature in Figure 17. If so, the fully porous structure is incipient in Figure 18.

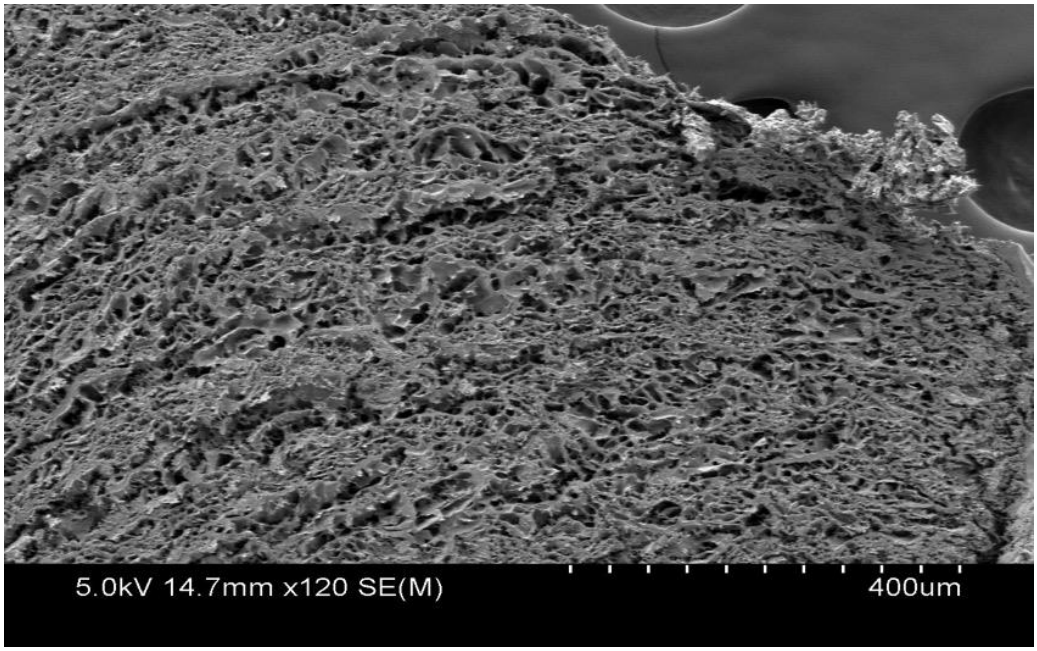

Figure 17 A low magnification cut-surface of minced mantle, showing the porous structure

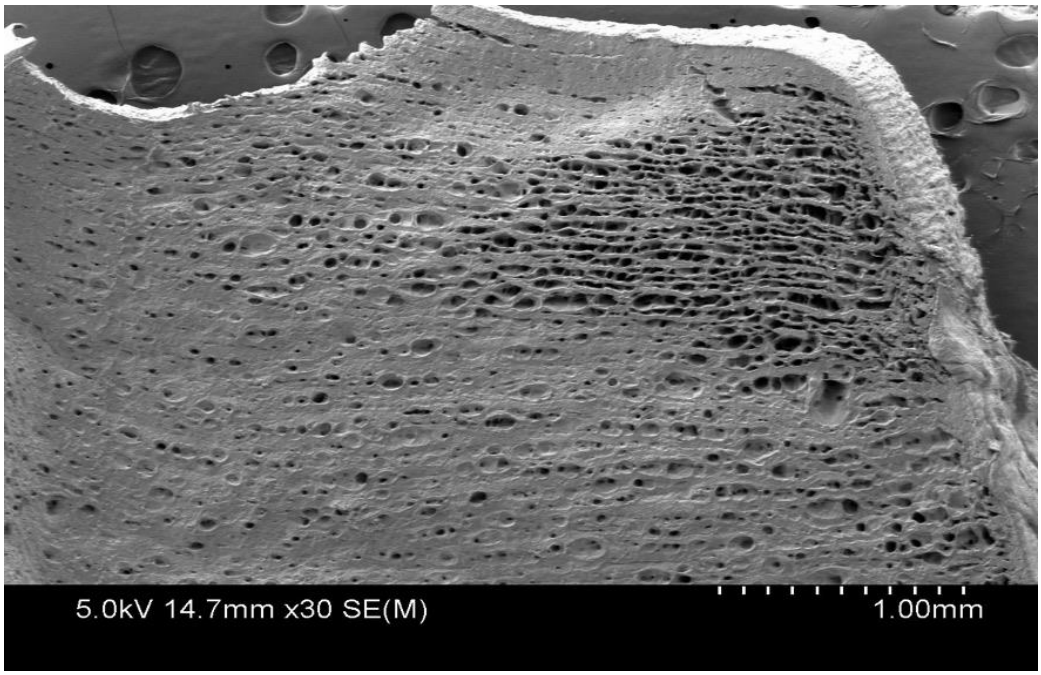

Figure 18 A low magnification cut-surface of sliced mantle, showing a layered porous structure

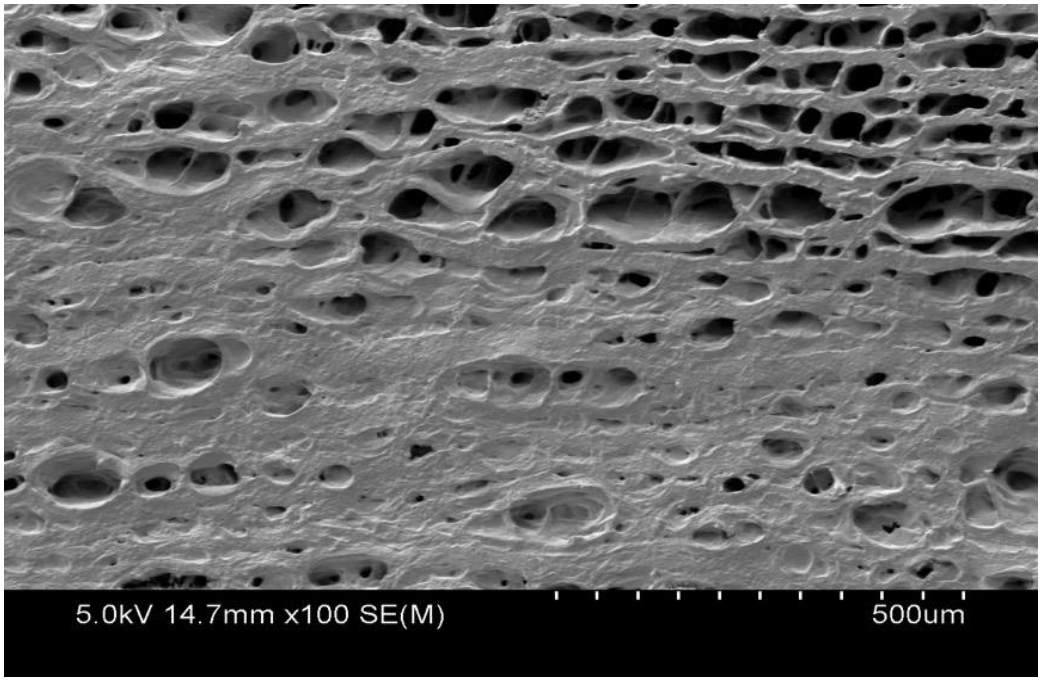

Figure 19 A higher magnification cut-surface of sliced mantle showing a layered porous structure on a 500 µm scale

In summary, cooked thawed mantle work revealed a porous structure that may account for the behaviour of cooked squid mantle under vacuum. Because images of sliced pieces were the most revealing, further work was restricted to that format.

Sliced raw fresh mantle (Table 10) showed another type of structure, which was more difficult to interpret Figure 20 and 21. The structures were clearly fibrous, but the pores that were clear in Figure 18 and 19 were not evident. Comparing Figure 19 and 20 revels this better as they are equally scaled.

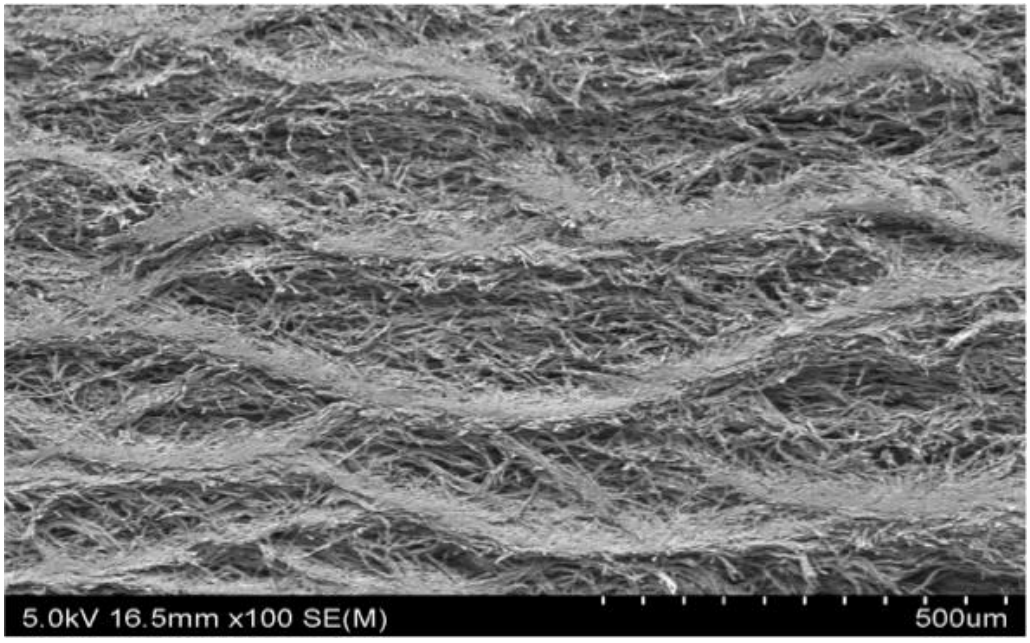

Figure 20 Fibrous layers in raw fresh mantle under SEM on a 500 μm scale

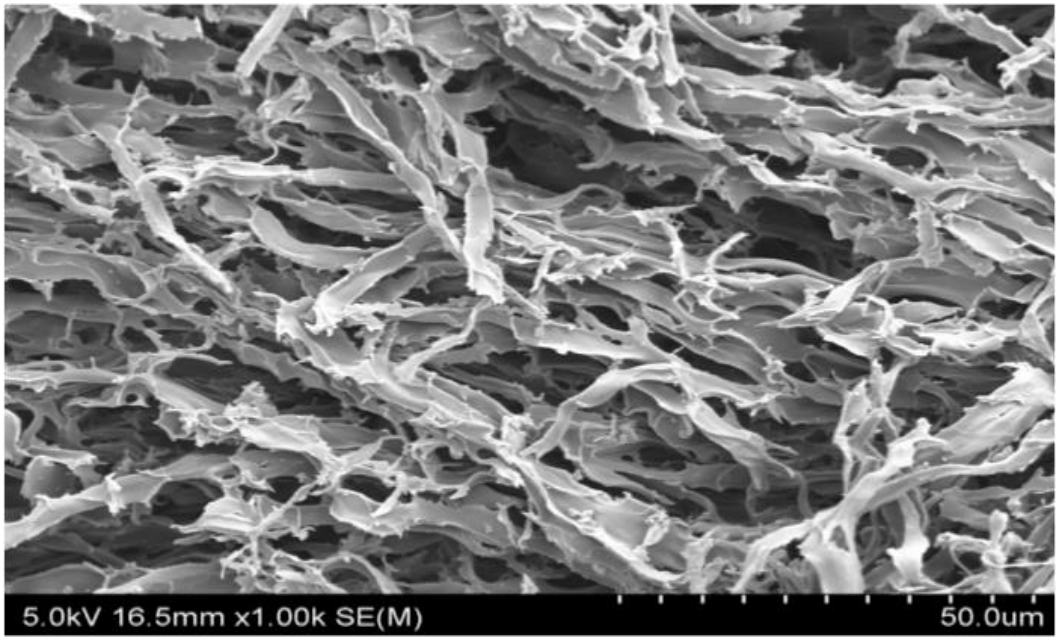

Figure 21 A higher magnification view of raw fresh mantle Figure 20

The final preparation technique was to add a freezing/thawing step to the raw mantle (Table 10), thus giving a control technique to cooked thawed mantle. The two techniques differ only by the action of cooking (Figure 22 and Figure 23). Thus Figure 19 has a raw mantle control in Figure 22. A layered structure was evident but was much more organized with clear pores in Figure 19.

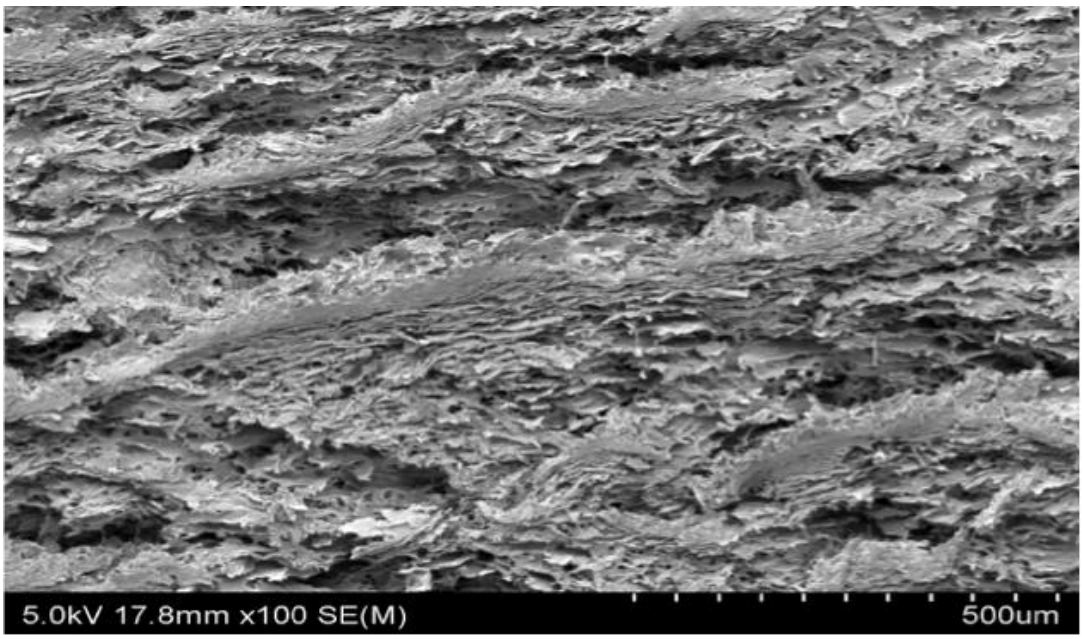

Figure 22 Layered structure of raw thawed mantle under SEM on a 500 µm scale

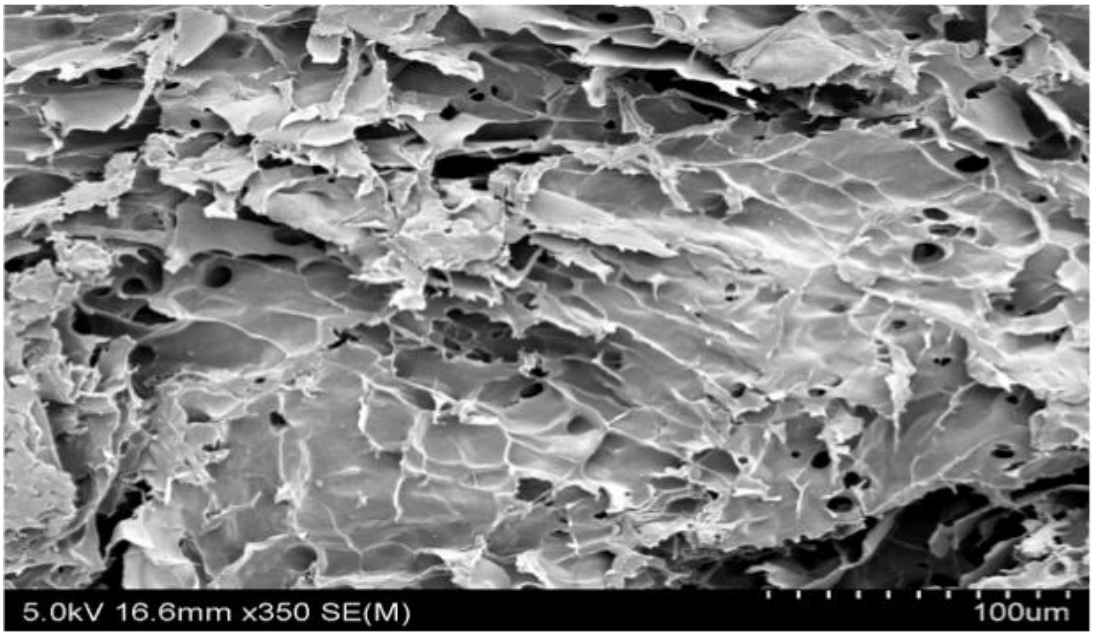

Figure 23 A higher magnification view of raw thawed mantle, Figure 22

Discussion

Electron microscopy of samples confirmed the presence of pores in the squid mantle. It was evident in all the samples even before and after cooking. SEM also revealed a fibrous layered structure in raw squid. There was some change in structure noticed in thawed sample compared to the raw fresh specimen, which indicates that the freezing has some effect on the squid structure. This is to be expected because the act of freezing can

create cavities or freeze artifacts in specimens. Freezing can also affect the functional characteristics of the proteins. The process is called freeze denaturation (Z. Sikorski & Kołakowska, 1995). Cooking massively changed the structure of raw squid, and clearly causes of gelling of squid mantle as is clearly seen in Figure 19.

Even after the samples were freeze-dried for two days, it took an unusually long time to create sufficient vacuum for the SEM to operate. Freeze drying was only partially successful in removing the air or gas from the sample. This suggests that air/gas is trapped within the gelled squid mantle. If so, it is difficult to explain how exposure to high shear stress (bowl chopped and minced) could result in a longer time to pump down; the fine structure in Figure 15 – 17 is clearly more porous than Figure 18 and 19.

What gases are involved? Water vapour and air are obvious gases, but another likely gas is ammonia or similar ammoniated compounds (Voight, Pörtner, & O'Dor, 1995). These have long been known to be associated with squids, and have roles in the buoyancy of squid in water (squids lack swim bladders). Squids can be classed in two groups depending on their habitat in the seawater column. Some squids are much denser than seawater; they must swim to maintain their level in the column, while others are neutrally buoyant. Studies shown the presence of large quantities of ammonia in these neutrally buoyant squids, though the ammonia is not uniformly distributed throughout the body (Clarke, Denton, & Gilpin-Brown, 1979). The species used in my work was *Nototodarus sloanii*, a member of the Ommastrephidae (Boyle, Rodhouse 2005). This family is neutrally buoyant due to ammonia, and as found in Chapter 5, is in sufficient quantities to result a mantle pH of around 8.

Chapter 5

Effect of pH and incubation time on Zyactinase activity in tenderising squid pieces

Introduction

In Chapter 2 it was established that Zyactinase – an enzyme source of known activity –could tenderize cooked minced squid mantle. However, the kinetics of its activity and the factors affecting its activity such as pH are unknown. The pH optimum of actinidin, the cysteine family protease(s) active in Zyactinase, is controversial.

In a study that explored the optimum temperature (35, 40, 45 and 55°C) and pH (1 to 8) of Zyactinase in hydrolysing meat protein, Zyactinase was found to have the maximum protein solubility and therefore hydrolysis at pH 2 (Y. Liu, 2011). But in another study at pH values simulating gastric and duodenal conditions, Zyactinase was found to have the highest activity at pH 3.1 and very limited capacity above pH 6 (Donaldson, Rush, Young, & Winger, 2014). Analyses using the relatively unpurified enzyme (actinidin) on various food proteins under gastric and intestinal conditions, the optimum pH was found to vary depending upon the food protein. The enzyme was active over a wide pH range from 3 to 6 with an optimum of 4 when the substrate was gelatin. Most other food proteins tested hydrolysed best between pH 3 and 4.5, whereas the range for the meat protein myosin was from 3 to 8 (Kaur & Boland, 2013). In respect of squid mantle, its composition is dominated by the contractile protein myosin and supporting connective tissue dominated by collagen (the native form of gelatin) (Macgillivray, Anderson, Wright, & Demont, 1999; Z. E. Sikorski & Kołodziejska, 1986). This suggests that the pH range of 3 to 8 might be useful.

Based on the literature, it was reported in Chapter 4 that the gas associated with squid mantle was likely to be ammonia, which is alkaline in nature. The unpublished results of AUT undergraduate student Nassir Nu were consistent with the involvement of ammonia, because he found the pH of squid mantle to be about pH 8. It therefore became important to test Zyactinase – applied only to raw mantle pieces – under a range of pH conditions, and incubation times. That is the topic of this chapter, where the experimental outputs were changes in weight and dimension, pH and texture. Also, in this chapter is an

experiment comparing Zyactinase activity with that of a freshly prepared kiwifruit extract, because the Zyactinase performed more poorly than anticipated. In all this work, fermentation of pieces was not carried out, but is the topic of Chapter 6.

Materials and Methods

This chapter comprises three experiments. The first two experiments evaluate the effect of varying pH on the texture of squid pieces - incubated with Zyactinase before cooking the squid meat. The third explores the effect of incubation time ranging from zero hours to overnight incubation, again before cooking the squid meat. In addition to Zyactinase effect of freshly prepared kiwifruit on the tenderization of squid was also analysed in the third part of the experiment. In the text that follows the design of the three experiments are detailed, but before than the methods of preparing squid mantle and cooking, and of analysing outputs are described, because these are largely common to the three experiments.

Slicing of squid and cooking

Sliced squid pieces were prepared as described previously in Chapter 3. In outline, frozen squid mantles were thawed and cut in to small roughly equally sized square pieces (10 x 10 mm) using a sharp kitchen knife. Pieces were then mixed in a large bowl and 5 to 10 pieces were randomly selected for each treatment. After incubations the pieces were cooked by immersing a small colander in rolling boiling water for 1 minute, followed by cooling and analysis.

Measurement of weights and dimensions

The weight of each squid piece was measured after cooking in a standard weighing scale. Height was measured in two ways. Using a Vernier micrometer, each piece was gently squeezed between the jaws and the reading taken. This was laborious and the data were highly dependent on the operator's consistency because the pieces had a soft/rubbery texture. The second method of calculation of height was also used in experiment 3. The method was to program the machine to record the distance at which the probe hit the squid and calculate the value of height. The total distance between the probe and the machine platform was set to be 30 mm in all the experiments and also every compression test probe pre-travel for 20 mm and start the measuring processes. Therefore the height of each sample will be equal to the distance at which the probe hit the piece plus 20 mm reduced from 30 mm (total height of probe and platform).

Measurement of pH

The pH of squid pieces under each treatment was measured using a calibrated pH meter, by dipping the electrode in the squid pieces and buffer/enzyme mixture after the required incubation.

Preparation of buffers

An estimated 0.1 M sodium phosphate buffer of pH 6.9 was used in the first experiment. The pH 4 and 6 was adjusted by adding drops of NaOH/HCl to the phosphate buffer. A citrate/phosphate buffer at pH 3, 80 mM with respect to citrate, was used for the second experiment, and made by mixing 79.5 mL of 0.1 M citric acid with 20.6 mL of 0.2 M Na_2HPO_4 (Dawson, 1986).

Measurement of texture

The texture of cooked squid pieces was determined using the TAXT texture analyser in double compression test mode using a 50 mm cylindrical probe (Table 11).

Table 11 Compression settings used for the textural analysis of cooked sliced squid with a 50 mm cylindrical probe.

Mode	Compression
Probe	Cylindrical, 50 mm diameter
Count	2
Distance of probe above the ground zero	30 mm
Pretest travel distance	20 mm
Contact force (minimum)	0.1 N
Test mode	Distance
Test speed	2.00 mm s^{-1}
Target distance	8.00 mm
Post first compression test return speed	2.00 mm s^{-1}
Return speed of probe at test end	20 mm s^{-1}

The squid mantle pieces of each treatment were placed on the analysis platform, the machine was calibrated (height and force) and set to descend from 30 mm above the ground zero as described in Table 11. The machine was set to travel for 20 mm without recording data. The test could begin at any distance after that as determined by the trigger force, 0.1 N. The maximum distance of test travel was 8 mm, meaning that each piece was compressed to 2mm and no more. The program was set to do a double compression test, such that after the first compression the probe returned to the origin and the test was repeated. Values of the first and second peak compression force were recorded and retrieved using the dedicated software.

Experiment 1

The 1% Zyactinase solution was used prepared by mixing 1 g of Zyactinase powder to 100 mL of tap water. A detailed experimental layout is given in Table 12. To check the effect of pH, 10 mL of buffer was also added to pieces with 10 mL Zyactinase solution and incubated for 2 hours. This resulted in a halved Zyactinase concentration. There were two controls, a zero time ambient pH control and a 2-hour ambient pH control both prepared with no buffer. These are greyed in Table 12. There were 5 replicate mantle slices in each of the seven treatments.

Table 12 Experimental design for the effect of varying pH on the activity of Zyactinase in squid mantle.

Treatment and final Zyactinase concn.	Zyactinase soln. (mL)	Water (mL)	Buffer pH 4 (mL)	Buffer pH 6 (mL)	Time of incubation (h)
Ambient pH and water control	0	10	0	0	0
Ambient pH					
Plus Zyac (1%)	10	0	0	0	2
Water	0	10	0	0	2
pH 4					
Plus Zyac (0.5%)	10	0	10	0	2
Water	0	10	10	0	2
pH 6					
Plus Zyac (0.5%)	10	0	0	10	2
Water	0	10	0	10	2

Experiment 2

Because Experiment 1 did not result in any measurable tenderization, it was thought that the pH may have been too high (Y. Liu, 2011). Therefore, the effect of pH on the texture of squid mantle pieces was investigated using a more acidic pH 3 buffer. Unlike the previous experiment, the no-Zyactinase control was a heat-denatured Zyactinase solution instead of water. A 1% Zyactinase solution was prepared and enzyme activity was denatured by attaining boiling point on a hot plate for about 1 minute (Klibanov, 1983). Heat-denatured Zyactinase solution was expected to be a better control than water as the activity of enzyme was destroyed due to heat and the other contents of the enzyme remains the same. Also the experimental design was refined with only one enzyme concentration, 0.5% Zyactinase was used in all treatments. The design is shown in Table 13.

Table 13 Experimental design for the effect of a pH 3 citrate/phosphate buffer on the activity of Zyactinase (0.5%) on squid mantle pieces.

Treatment	Native Zyac soln. (mL)	Heated Zyac soln. (mL)	Water (mL)	Buffer pH 3 (mL)	Time of incubation (h)
Ambient pH					
Heated Zyac	0	10	10	0	0
Heated Zyac	0	10	10	0	2
Native Zayc	10	0	10	0	0
Native Zayc	10	0	10	0	2
pH 3					
Heated Zyac	0	10	0	10	0
Heated Zyac	0	10	0	10	2
Native Zayc	10	0	0	10	0
Native Zayc	10	0	0	10	2

Experiment 3

According to the two previous experiments the effect of Zyactinase in tendering the squid meat was very limited when used before cooking. In those experiments squid pieces were incubated with Zyactinase for no more than two hours. It was thought that a longer incubation time could be more effective. Therefore in this experiment longer incubation times were examined. Moreover, also the tenderizing ability of Zyactinase was suspect by this time so an additional tenderizing method was tested. This was freshly extracted (green) kiwifruit juice, but realizing that the extract's activity was unknown and would be variable for many biological reasons.

One percent Zyactinase was used in this experiment (Table 14). The kiwifruit extract was prepared by blending two peeled kiwifruits (around 100 g) in 400 mL of water. And the pulp was removed by straining through a colander. Inactive controls of kiwifruit extract and Zyactinase were made by boiling the native forms. A water control was also included.

Table 14 Experimental design of textural analysis design of squid pieces incubated for 0, 5 and 24 hours.

Treatment	Native kiwifruit extract (mL)	Heated kiwifruit extract (mL)	Water (mL)	Native Zyactinase solution (mL)	Heated Zyactinase solution (mL)	Time (h)
Kiwi extract						
Native	10	0	0	0	0	0
Native	10	0	0	0	0	5
Native	10	0	0	0	0	24
Heated	0	10	0	0	0	0
Heated	0	10	0	0	0	5
Heated	0	10	0	0	0	24
Water						
Ambient	0	0	10	0	0	0
Ambient	0	0	10	0	0	5
Ambient	0	0	10	0	0	24
Zyactinase (1%)						
Native	0	0	0	10	0	0
Native	0	0	0	10	0	5
Native	0	0	0	10	0	24
Heated	0	0	0	0	10	0
Heated	0	0	0	0	10	5
Heated	0	0	0	0	10	24

Squid pieces were inoculated with 10 mL of water of other enzyme preparation for 0, 5 and 24 hours and pH was measured after incubation. Pieces were then cooked in boiling water as described previously and refrigerated overnight. Texture analysis of all the samples was carried out on the next day. Texture analysis and the interpretation of data was performed as described previously, expect that all 2 mm s^{-1} were increased to 5 mm s^{-1} to greatly shorten analysis time.

Results

Experiment 1

Squid pieces of all treatments had pH values ranging from 6.8 to 8.3 (Table 15), and that the desired pH values of 4 and 6 were not attained probably because of the alkalinity associated with the ammonia in squid and the inherent buffering capacity of protein. Also, in hindsight it was recognized the phosphate buffer would have very low buffering capacity at pH 4 and at best it was a replicate of the pH 6 treatments. Although many

means were numerically different, the weight and height were unaffected by the treatments ($P > 0.05$). The mean heights were numerically greater where Zyactinase had been included. This was unexpected, because a protease – if active – would be expected to erode squid pieces. No explanation is offered for this.

Table 15 Mean pH, weight, height and peak force values of squid pieces in ambient and buffered solution (pH 4 and 6) for 2 hours. For clarity standard deviations have not been included. However, the full data set is in Appendix 2.

Treatment and final Zyac concn.	Time (h)	pH after incubation	Weight (g)	Height (mm)	First peak force (N)	Second peak force (N)	First peak/ Second peak
Ambient pH and water control	0	8.1	1.03	3.3	50.4	45.9	1.10
Ambient pH							
Plus Zyac (1%)	2	8.3	1.14	4.0	50.3	45.7	1.10
Water	2	8.0	1.10	3.2	55.7	53.2	1.05
pH 4							
Plus Zyac (0.5%)	2	6.8	1.11	3.6	52.3	48.2	1.08
Water	2	7.2	0.96	3.2	51.6	48.0	1.08
pH 6							
Plus Zyac (0.5%)	2	7.9	0.95	4.2	46.2	42.0	1.10
Water	2	8.2	1.03	3.4	47.6	42.6	1.12

From the textural data (Table 15), Zyactinase had negligible numerical effects on the texture of squid pieces, and the minor differences were statistically insignificant (data not shown). Importantly the ratios of the 1st peak to the 2nd peak were close to unity, indicating that the springiness of the squid pieces was unchanged. Thus Zyactinase had no effect under these conditions of concentration, temperature and time.

Experiment 2

The pH of all treatments, ambient and buffered, increased with incubation with the 2-hour incubation period, especially when unbuffered (Table 16), presumably in response to the buffering capacity of squid proteins and the likely presence of ammonia.

Table 16 Mean mass, pH and peak force values of squid pieces in ambient and pH 3-buffered solution for 2 hours. For clarity standard deviations have not been included. However, the full data set is in Appendix 3.

Treatment	Time of incubation (h)	pH after incubation	Weight (g)	Height (mm)	First peak force (N)	Second peak force (N)	First peak/ second peak
Ambient pH							
Heated Zyac	0	6.3	1.03	2.9	61.9	54.2	1.14
Heated Zyac	2	8.4	0.93	3.1	51.2	44.8	1.14
Native Zyac	0	5.7	0.95	3.1	73.1	64.1	1.14
Native Zyac	2	8.2	1.02	3.7	59.8	52.3	1.15
pH 3							
Heated Zyac	0	3.4	0.89	3.5	71.2	63.4	1.12
Heated Zyac	2	4.7	0.77	3.8	82.2	73.1	1.12
Native Zayc	0	3.3	0.81	3.4	66.7	58.3	1.14
Native Zayc	2	4.8	0.90	4.1	65.9	56.2	1.17

Numerically, the mean weight of squid pieces at ambient pH was higher than that of squid pieces incubated in pH 3 buffer ($P < 0.001$), but was unaffected by enzyme activity and incubation time ($P = 0.773$ and 0.713 respectively). The reduction in weight in pH 3-buffered solution is discussed in a later paragraph. In contrast, the height of the squid pieces slightly increased at pH 3 ($P = 0.032$), suggesting that the combined effect of pH 3 and cooking caused significant changes in dimensions. The enzyme had no effect ($P = 0.214$) and the effect of incubation time was marginal ($P = 0.051$), such that the pieces were higher after 2 hours.

At ambient pH squid pieces were numerically harder in the first compression at 0 hours than at 2 hours (67.5 versus 55.5 N), but the difference was insignificant ($P = 0.134$), and denaturing the reportedly active Zyactinase by heating likewise had no effect. In pH 3 buffer, there was an indication that native Zyactinase was effective (66.3 versus 76.7 N) but this difference was statistically insignificant as well ($P = 0.457$). Thus there was no obvious effect of Zyactinase at any pH. This view was reinforced by the ratio generated by the second compression, which varied minimally between 1.12 and 1.17.

Returning to the matter of weight reduction at pH 3, there are two possible causes of this. Proteins have different water binding properties at different pH values. Water binding is least at the isoelectric point of proteins (reference), unknown in the case of squid mantle. However, it was also noticed that the pieces in pH 3 buffer were whiter than those at ambient pH. This was reflected in the compression force values at 2 hours, which were higher from the resulting pH 4.7 and 4.8 than from the resulting pH 8.4 and 8.2

(second results column Table 16) (P value 0.028). This is likely to be caused by denaturation of squid proteins at low pH in the matter of raw fish treated with vinegar or lemon juice at room temperature in the manner of sushi.

Experiment 3

There were more marked changes on incubation for 24 hours. Data are shown in Table 17.

Table 17 Average of pH, mass and peak textural force of squid pieces incubated for 0, 5 and 24 hours. For clarity standard deviations have not been included. However, the full data set is in Appendix 4.

Treatment	Time (h)	pH	Weight (g)	Height (mm)	First peak force (N)	Second peak Force (N)	First peak/ second peak
Kiwi extract							
Native	0	3.9	0.56	3.0	49.0	42.0	1.17
Native	5	8.0	0.43	2.9	28.7	24.9	1.15
Native	24	6.2	0.36	1.9	8.4	7.0	1.21
Heated	0	4.7	0.73	3.8	66.1	53.0	1.25
Heated	5	7.3	0.46	3.6	58.5	52.0	1.12
Heated	24	6.3	0.42	3.0	60.2	55.5	1.09
Water							
	0	8.9	0.73	3.2	63.2	51.2	1.23
	5	9.3	0.70	3.7	68.0	57.1	1.19
	24	8.7	0.61	2.7	50.5	42.8	1.18
Zyactinase							
Native	0	4.1	0.74	3.8	67.9	54.5	1.25
Native	5	9.0	0.62	3.9	66.8	57.2	1.17
Native	24	6.6	0.37	2.4	33.5	30.4	1.10
Heated	0	4.6	0.65	3.2	51.8	41.8	1.24
Heated	5	8.9	0.72	3.8	71.0	60.2	1.18
Heated	24	8.2	0.41	2.6	50.5	46.4	1.09

The pH of pieces in most treatments rose after 5 hours and then dropped after 24 hours (Table 17). There was no significant variation in the pH values of samples in water; the three conditions were alkaline around pH 9 due to squid itself. Also the pH of pieces in heated Zyactinase raised from 4.6 at 0 hour to 8.9 at 5 hours and remained same 8.2 even after overnight incubation unlike the enzymatic treatments where pH was markedly raised after 5 hours and then markedly lowered. Clearly, the kiwi extract and the Zyactinase suspensions/solutions were both acidic, and this was confirmed by measuring those pH values; both were around 3. The question may be posed: what causes the increase followed by a decrease? The increase was probably cause by the buffering capacity of the

squid proteins, and the decrease was probably due to protein hydrolysis tending to the isoelectric point of the proteins being hydrolysed (Adler-Nissen, 1986), although unknown for squid mantle proteins. If these arguments are true, the pH in the water controls should be largely unvarying and it was.

In respect of weight, and considering all 15 treatments, there was a significant decrease (P =0.001), but it was difficult to assign this to enzymatic activity, because the decrease also occurred with heated enzyme treatments (P values significant but variable). Perhaps the enzymes were not fully denatured. An important point here is the water holding capacity of proteins at their isoelectric points, where it is lowest, perhaps resulting in least weight, independent of weight loss due to protein hydrolysis (Huff-Lonergan & Lonergan, 2005). The value of the water controls was shown here as no significant weight loss even though there was a numerical decrease from a mean of 0.73 g at 0 hours to 0.61 g at 24 hours (but different pieces).

There was a slight decrease in the mean height of the squid pieces – with varying degrees of significance – but the causes of this are unknown given the uncertainties about isoelectric point and enzyme activities.

Whereas the changes in weight and height were difficult to interpret, the effect of native enzyme activity on texture were largely understandable (Table 17). Native kiwifruit extract was very effective in tendering squid meat (P value < 0.001) even after 5 hours of incubation. The first peak force value of squid pieces incubated with native kiwifruit extract dropped from 48 N at 0 h to 28 N after 5 hours and then to 8 N by overnight incubation. In case of native Zyactinase, 5 hours of incubation made no significant difference to texture (P = 0.982), whereas overnight incubation made the pieces tender (P < 0.001 between 0 (67.9 N) and 24 hours (33.5 N)). Texture changes within the two heated controls and the water control were nearly always insignificant. The same patterns were observed with the second peak with similar significance (data not shown in detail).

Though there was a significant reduction in the peak force of enzymatic treatment with time, the springiness of squid pieces calculated from the ratio of the first peak to the second was close to unity. If the springiness was strongly affected by enzyme activity, the ratio of first divided by second should be markedly greater than unity. The changes observed had no particular pattern (Table 17).

A comparison of height measurement by two methods

The use of a Vernier microtome to determine height was subjective because the squid piece had some springiness. In an attempt to overcome this problem, it was decided to use the trigger setting as the signal to confirm the squid piece had been contacted. Knowing the initial starting point above ground zero and the travel of the probe it was a simple matter to calculate the height of the piece. Therefore, I had two independent measures of height that could be plotted to determine correlation (Figure 24).

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The two values were poorly correlated and importantly, the projected graph did not pass through the origin. Clearly, the height measurements are uncertain. Which should be used, the height by texture analyser or the height by vernier? The vernier data was chosen because that was routinely measured.

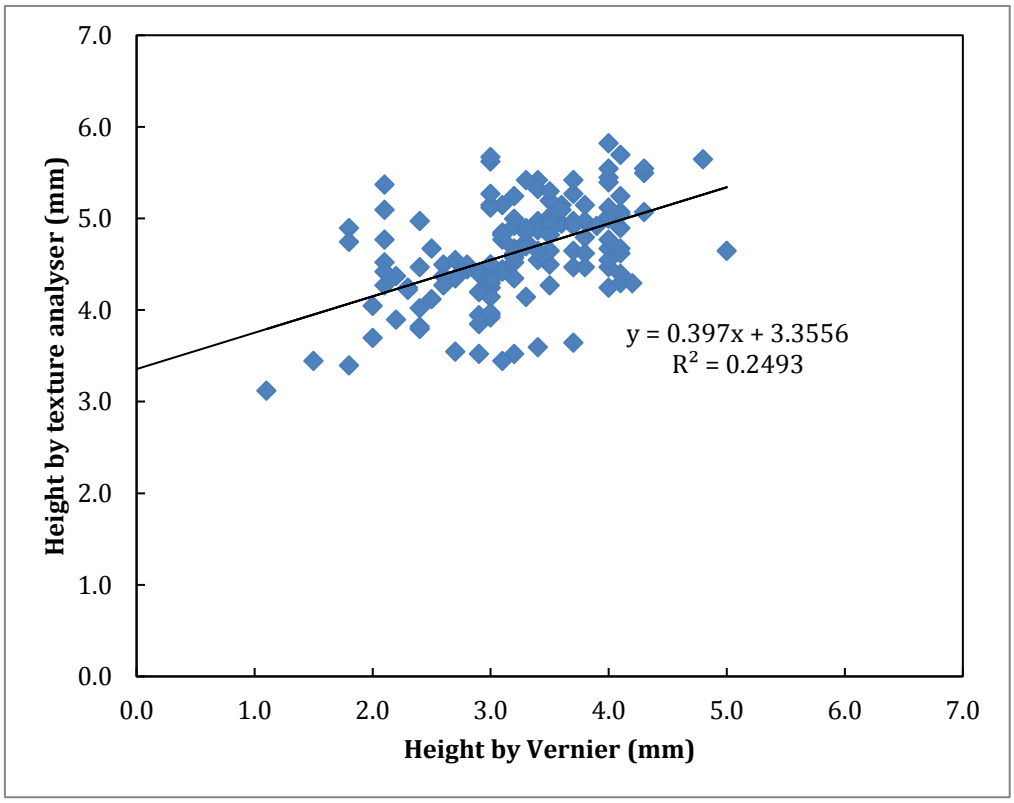

Figure 24 Correlation plot of height measured using a Vernier and calculated from the texture analyser

Conclusion

Although there is disagreement regarding the optimum pH of Zyactinase activity, the experience here was that in pH 3, 4 and 6 buffers, Zyactinase had no marked effect on squid texture within a 2-hour incubation. The Zyactinase as supplied was thus useless

under these conditions. However it was also observed that the pieces in pH 3 and pH 4 buffer for 2 hours were whiter than pieces in pH 6 buffers and water. Moreover, the peak force value of squid pieces in buffer 3 solutions for 2 hours was significantly higher than that of the respective ambient squid pieces ($P = 0.028$). This toughness in squid pieces was probably be due to the denaturation of squid mantle proteins at low pH, similar to the treatment of raw fish with vinegar or lemon juice in the manner of foods like sushi.

Comparing the texture of squid mantle pieces incubated with Zyactinase and fresh kiwifruit extract for varying incubation times, Zyactinase has the ability to tenderise squid pieces if only incubated for a long period of time. Of 0, 2, 5 and 24 hours incubation treatments with Zyactinase, only 24 hours of incubation with 1% Zyactinase was effective in tendering squid pieces (P value <0.001). Freshly prepared kiwifruit extract was much more effective than Zyactinase, and could tenderize the squid pieces in as few 5 hours (P value <0.001). The weight and height of squid pieces with both enzymatic forms were reduced significantly after 24 hours of incubation.

According to the data obtained from the various treatments of squid, kiwifruit extract is effective in tendering squid mantle tissue – as is found in domestic experience – whereas Zyactinase was relatively ineffective. The initial idea was that by using Zyactinase, natural variability in enzyme activity from different kiwifruit would be overcome. That idea was not realized.

The final experimental chapter now addresses the effect of kiwifruit extract and Zyactinase when the incubation protocol is continued through to the final fermented product.

Chapter 6

Comparison of mass and textural characteristics of squid pieces after incubation and fermentation

The previous chapter determined the effect of enzyme source, pH and incubation time on weight loss, and texture of squid pieces. It surprisingly showed that the purported Zyactinase was relatively ineffective compared with a fresh kiwifruit extract, but realizing that Zyactinase was initially chosen because a commercial preparation had a known claimed activity. Fresh kiwifruit extracts will always have variable activity and would thus be less suited to commercial application. Knowing the differences between the two enzyme sources, it remained to complete product preparation by progressing to the final fermentation. In this chapter, I have analyzed the effect of fermentation on the texture of squid pieces by comparing the textural parameters before and after fermentation.

Materials and Methods

Squid pieces of various enzyme treatments were prepared and analysed as explained in Chapter 5. In outline, thawed squid mantles were sliced in to small chop stick -suitable pieces of similar dimensions, incubated with the chosen enzyme and cooked in boiling water for 1 minute to inactivate the enzyme. Both kiwifruit extract and Zyactinase were used. The concentration of Zyactinase was fixed at 1%. Zyactinase requires a longer period of incubation to tenderize the squid (Chapter 5). Therefore pieces were analyzed pre-fermentation after 0 hours and 24 hours. Two lots of squid pieces were prepared, one for analysis after incubation and the second for analysis after fermentation for 96 hours followed by overnight chilled storage. Squid pieces for fermentation were incubated with enzyme, cooked and mixed with 2% salt, 2% glucose and 0.02% bacterial culture (BFL-F02) and fermented at 30°C under vacuum for four days. The pH, weight, height and texture were measured as described previously in Chapter 5. The experimental design is shown in Table 18. Buffer was not used.

Table 18 Experimental design of textural analysis of squid pieces incubated for 0 and 24 hours.[2]

Treatment	Native kiwifruit extract (mL)	Heated kiwifruit extract (mL)	Water (mL)	Native Zyactinase solution (mL)	Heated Zyactinase solution (mL)	Time (h)
Kiwi extract						
Native	10	0	0	0	0	0
Native	10	0	0	0	0	24
Heated	0	10	0	0	0	0
Heated	0	10	0	0	0	24
Water						
Ambient	0	0	10	0	0	0
Ambient	0	0	10	0	0	24
Zyactinase						
Native	0	0	0	10	0	0
Native	0	0	0	10	0	24
Heated	0	0	0	0	10	0
Heated	0	0	0	0	10	24

Results

The average mass and textural values of squid pieces before and after fermentation are shown in Table 19. As before, standard deviations are not shown for clarity, but are shown in Appendix 4. Consistent with the results in Chapter 5, the pH at 0 hours of incubation was acidic where enzyme had been added (upper data Table 19), but after 24 hours the pH values had moved much closer to the pH recorded for squid pieces in water alone, 8.3 and 8.4. This was due to the buffering capacity of the squid proteins and ammonia, and the tendency for the pH to tend toward the (unknown) isoelectric point on proteolysis (Adler-Nissen, 1986). All the squid treatments destined for fermentation were successfully fermented as judged by the low pH achieved, 3.8 to 4.9 (lower data Table 19).

[2] Subsequently, half the pieces were analysed post-incubation and the other half were fermented with salt, glucose and culture for 96 hours, and then analysed.

Table 19 Average of pH, weight, height and peak textural force of squid pieces incubated for 0 and 24 hours before and after incubation. For clarity standard deviations have not been included. However, the full data set is in Appendix 5.

Treatment	Incubation time (h)	pH	Weight (g)	Height (mm)	First peak force (N)	Second peak force (N)	1st Peak/ 2nd Peak
			Non-fermented pieces				
Kiwi extract							
Native	0	3.2	0.92	5.1	70.5	57.5	1.22
Native	24	6.5	0.81	4.8	15.8	12.7	1.25
Heated	0	3.2	1.04	4.8	80.0	69.1	1.15
Heated	24	5.9	0.73	5.2	64.2	55.6	1.16
Water							
Ambient	0	8.3	0.98	4.8	66.5	54.1	1.23
Ambient	24	8.4	1.14	5.0	85.2	67.5	1.26
Zyactinase							
Native	0	4.8	1.09	5.0	77.9	64	1.22
Native	24	7.5	0.93	4.8	68.5	58.3	1.17
Heated	0	3.7	0.99	5.2	72.4	53.5	1.35
Heated	24	7.1	0.95	5.2	83.6	66.5	1.26
			Fermented pieces				
Kiwi extract							
Native	0	4.8	0.59	4.4	35.0	30.7	1.14
Native	24	3.8	0.56	4.1	41.2	36.6	1.13
Heated	0	4.6	0.59	4.4	76.9	71.7	1.07
Heated	24	4.5	0.45	4.2	85.7	80.8	1.06
Water							
Ambient	0	4.7	0.61	3.8	64.9	61.8	1.05
Ambient	24	4.8	0.73	4.5	91.6	85.2	1.08
Zyactinase							
Native	0	4.5	0.61	4.0	42.8	37.6	1.14
Native	24	4.9	0.71	4.3	78.2	71.9	1.09
Heated	0	4.5	0.58	3.9	72.0	64.5	1.12
Heated	24	4.4	0.66	4.6	77.8	72.8	1.07

Considering the upper textural data in Table 19, the native kiwifruit extract was particularly effective in tenderizing the squid, much more so than the Zyactinase, thus confirming and extending the equivalent results of Experiment 3 Chapter 5. Thus for the first and second peaks of native kiwifruit extract, the values fell from 70.5 and 57.5 N to 15.8 N and 12.7 N respectively ($P = <0.001$). The equivalent changes with native Zyactinase were not significant ($P = 0.274$ & 0.381). The heated kiwifruit extract results suggested some residual activity after the heating but the differences were not significant ($P = 0.08$ and 0.07).

All other textural changes between 0 and 24 hours in the upper data set were not significant. Similar to the textural data of Experiment 3 Chapter 5, there was not much difference in the springiness ratio of squid pieces of all the treatments, which was almost close to unity. The weight and height data were complicated by the pH changes between 0 and 24 hours, but were not very different, and are not considered further.

Consider now the fermented squid pieces (lower data). Except for the native Zyactinase treatment, there had been no significant difference between the texture of pieces between 0 and 24 hours. For native Zyactinase, the mean first and second peak forces of squid pieces increased from 42.8 N and 37.6 N to 78.2 N and 71.9 ($P = 0.03$ and $P = 0.02$) respectively. No explanation is offered to this.

Now comparing between treatments irrespective of time, fermented squid pieces incubated with native kiwifruit were tenderer than those incubated with its heated control or with water ($P < 0.001$). This indicates the effectiveness of native kiwifruit enzyme in tendering the squid meat and the effect is evident even after the complete process of fermentation. In contrast, the texture of pieces fermented with native Zyactinase were insignificantly different from pieces incubated with heated Zyactinase ($P = 0.18$ and $P = 0.14$ comparing the first and second peaks force of the native and heated treatments).

Although different pieces were measured for non-fermented and fermented there was marked variation in the weight and height of the pieces has noticed before and after fermentation. Both weight and height of the squid pieces were significantly reduced after fermentation probably due lost in water content of the squid pieces. The overall mean weight of squid pieces before fermentation, 0.96 g, became 0.61 g after fermentation ($P < 0.001$). Slight reductions in the height of the pieces were also noticed after fermentation. The equivalent means were 5.0 mm and 4.2 mm respectively ($P < 0.001$).

Comparing the overall compression data of both non-fermented and fermented treatments, there was no significant variation in the average first peak forces before and after fermentation ($P = 0.60$). Although the textural values of squid pieces incubated with native kiwifruit and native Zyactinase (0 and 24 hours) after fermentation were numerically different to the values before fermentation, the differences were statistically insignificant with respective P values of 0.39 and 0.15. Also, the average first peak values of all the fermented treatments at 0 hours were slightly lesser than the corresponding fermented treatments at 24 hours ($P = 0.009$). This could be due to the effect of low pH as in the case of treatment of raw fish with lemon or vinegar as explained in previous Chapter

5, due to the accumulation of lactic acid as well as the acidic enzymatic solutions. The second peak force values of all the treatments followed a pattern similar to the first peak value; individual values are shown in Table 16. Although the springiness of all the squid pieces both before and after fermentation were close to unity, a small but highly significant increase[3] ($P = 0.001$) in the average springiness of all the treatments was observed, 1.23 before and 1.10 after. The reasons for this are unknown.

Conclusion

Overall there were no marked differences between the texture of squid pieces before and after fermentation. Pieces of all treatments with or without enzyme were successfully fermented as judged by the lower pH. The average weight and height of all the squid pieces decreased after fermentation. Native kiwifruit extract was found to be highly effective in tendering squid mantle, and this effect was not lost on subsequent fermentation. Zyactinase showed minimal effect on the texture of squid pieces both before and after fermentation. The ability of Zyactinase in tendering squid pieces under these experimental conditions was very questionable. Fermentation slightly reduced the springiness of squid pieces.

Final statement

The outcome of all this work was not always successful, but it has shown what works and what does not work. This is essential information for any commercial application of this simple technology.

Fine comminution does not work well and the idea of creating a spread is unrealistic.

Chopstick-suitable pieces is a simple and potentially useful product form and would be suitable for major markets in East Asia. (Flavours would be adjusted to suit different markets, but was beyond the scope of this research.)

The commercial enzyme preparation Zyactinase did not work well to tenderize mantle pieces, contrasting with the efficacy of fresh kiwifruit extract. However, the latter is likely to be variable in activity and that would have to be routinely quantified with some probably colorimetric assay

[3] It is not immediately obvious, but springiness increases as the ratio approaches unity.

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Appendix 1

Common lactic acid fermentations with plant foods.

Lactic acid fermentation	Major food type	Method
Pit fermentations	Breadfruit, taro, banana and cassava tubers.	Fermented pastes or whole fruits are placed in leaf-lined pits, pits are then covered with leaves and sealed. Low pH and anaerobic conditions keep the food stable for a long period of time.
	Ethiopian kocho is a flat bread made from mush formed from pit fermnted banana pulp	
Rice/shrimp/fish mixtures	Baloa baloa (Phillippines)	Baloa baloa is prepared by mixing boiled rice with raw shrimp and solar salt (about 3% w/w). It is packed in anaerobic containers and fermented for several days or weeks. Low pH and anaerobiosis preserve the food until the container is opened and the product must be cooked and consumed.
Yogurt/cereal mixtures:	Kishk (Egyptian) , trahanas (Greek) and tarhanas (Turkish)	These products are basically parboiled wheat and yogurt mixtures that combine both the nutritional value of wheat and milk while attaining excellent storage capacities. At first the milk is fermented to yogurt, after that both the yogurt and wheat are mixed and boiled together until the mixture is highly viscous. The mixture is then cooled and formed in to biscuits using hand and sundried. Trahanas can be kept in kitchen shelf for years and mainly used as a base for the nutritive soups. Tomatoes, onions and other vegetables are also combined with wheat and yogurt in the production of Egyptian kishk biscuits.
Cereal/legume sour gruels/porridges/beverages	Ogi (Nigerian), uji (Kenyan), mahewu/magou (South African) and tairu (Malaysian)	For producing Nigerian ogi maize, millet or sorghum grains are washed and steeped for 24 to 72 hours. Lactic acid fermentation takes place during this process. The mixture is then drained and wet milled and wet sieved to get fine, smooth slurry of about 8% solid contents. Kenyan uji is a similar product except that the grains are ground to flour before mixing with water and fermenting.

		Mageu/mahewu/magou is a traditional sour, non-alcoholic maize beverage popular among the Bantu people of South Africa, in which corn flour is slurried with water, boiled, cooled and inoculated with 5% w/w wheat flour in house hold fermentations (serves as a source of microorganisms) or *Lactobacillus delbrueckii* at 45°C in industrial processes. Slurry is fermented to a pH of 3.5 to 3.9 and consumed directly.
Cereal/legume steamed breads and pancakes	Idli, dosa, dhokla (India), enjera (Ethiopian)	Polished rice and black gram dahl are soaked in various proportions and grounded with added water to yield a batter of desired consistency. A small quantity of salt is also added and fermented overnight. During this period microbes present naturally in grains and in the environment such as Leuconostoc mesenteroides and Streptococcus faecalis grow rapidly and out number the initial contaminants and dominate the fermentation. Released CO_2 makes the batter anaerobic and leavens the mixture. The batter is then steamed in to white muffin like Idli or pancake like dosa form. Black gram could be substituted with soybean cotyledons, green gram or bengal gram. Indian dhokla is prepared by replacing rice with wheat or maize. A closely related fermented product called enjera is prepared in Ethiopian, which is a large pancake that serves as the center of the meal in Ethiopian food.
Leavened bread fermentation	Western yeast, sourdough breads and Middle East breads	Bread is mainly produced from wheat or rye flour by yeast fermentation generally *Saccharomyces cerevisiae*. Carbon dioxide produced by fermentation leavens the surface of the beard and also maintains an anaerobic condition. Baking produces a dry surface resistant to the bacterial invasion. It destroys many pathogenic microorganisms present in the raw material itself and some ethanol is also released as a by-product of fermentation. Sourdough is produced by fermentation with lactic acid bacteria and yeast.

Adapted from (Keith H. Steinkraus, 1997)

Appendix 2

Full summary data for Table 15. Mean pH, weight, height and peak force values of squid pieces in ambient and buffered solution (pH 4 and 6) for 2 hours.

Treatment and final Zyac concn.	Time (h)	pH after incubation	Weight (g)	Height (mm)	First peak force (N)	Second peak force (N)	First peak/ second peak
Ambient pH and water control	0	8.1	1.03 ± 0.19	3.3 ± 0.7	50.4 ± 8.17	45.9 ± 7.07	1.10 ± 0.04
Ambient Ph							
Plus Zyac (1%)	2	8.3	1.14 ± 0.15	4.0 ± 1.1	50.3 ± 7.20	45.7 ± 7.30	1.10 ± 0.06
Water	2	8.0	1.10 ± 0.20	3.2 ± 0.8	55.7 ± 5.14	53.2 ± 5.12	1.05 ± 0.05
pH 4							
Plus Zyac (0.5%)	2	6.8	1.11 ± 0.10	3.6 ± 0.6	52.3 ± 9.36	48.2 ± 9.55	1.08 ± 0.10
Water	2	7.2	0.96 ± 0.21	3.2 ± 0.7	51.6 ± 9.89	48.0 ± 8.90	1.08 ± 0.05
pH 6							
Plus Zyac (0.5%)	2	7.9	0.95 ± 0.17	4.2 ± 0.7	46.2 ± 11.50	42.0 ± 11.15	1.10 ± 0.03
Water	2	8.2	1.03 ± 0.24	3.4 ± 0.7	47.6 ± 8.22	42.6 ± 7.01	1.12 ± 0.02

Appendix 3

Full summary data for Table 16. Mean pH, weight, height and peak force values of squid pieces in ambient and pH 3-buffered solution for 2 hours.

Treatment	Time of incubation (h)	pH after incubation	Weight (g)	Height (mm)	First peak force (N)	Second peak force (N)	First peak/ second peak
Ambient pH							
Heated Zyac	0	6.3	1.03 ± 0.12	2.9 ± 0.7	61.9 ± 13.4	54.2 ± 12.7	1.14 ± 0.05
Heated Zyac	2	8.4	0.93 ± 0.09	3.1 ± 0.8	51.2 ± 19.8	44.8 ± 17.4	1.14 ± 0.03
Native Zyac	0	5.7	0.95 ± 0.12	3.1 ± 1.1	73.1 ± 15.3	64.1 ± 13.1	1.14 ± 0.07
Native Zyac	2	8.2	1.02 ± 0.13	3.7 ± 0.6	59.8 ± 21.2	52.3 ± 17.9	1.15 ± 0.03
pH 3							
Heated Zyac	0	3.4	0.89 ± 0.21	3.5 ± 0.9	71.2 ± 23.5	63.4 ± 18.7	1.12 ± 0.04
Heated Zyac	2	4.7	0.77 ± 0.10	3.8 ± 1.0	82.2 ± 10.8	73.1 ± 8.4	1.12 ± 0.05
Native Zayc	0	3.3	0.81 ± 0.18	3.4 ± 1.2	66.7 ± 21.7	58.3 ± 19.8	1.14 ± 0.06
Native Zayc	2	4.8	0.90 ± 0.26	4.1 ± 0.9	65.9 ± 25.5	56.2 ± 21.6	1.17 ± 0.06

Appendix 4

Full summary data for Table 17. Mean pH, weight, height and peak force values of squid pieces incubated for 0, 5 and 24 hours.

Treatment	Time (h)	pH	Weight (g)	Height (mm)	First peak force (N)	Second peak force (N)	First peak/ second peak
Kiwi extract							
Native	0	3.9	0.56 ± 0.14	3.0 ± 0.8	49.0 ± 16.2	42.0 ± 11.7	1.17 ± 0.09
Native	5	8.0	0.43 ± 0.12	2.9 ± 1.0	28.7 ± 14.8	24.9 ± 11.4	1.15 ± 0.06
Native	24	6.2	0.36 ± 0.05	1.9 ± 0.5	8.4 ± 2.7	7.0 ± 2.8	1.21 ± 0.24
Heated	0	4.7	0.73 ± 0.08	3.8 ± 0.6	66.1 ± 9.9	53.0 ± 8.9	1.25 ± 0.06
Heated	5	7.3	0.46 ± 0.12	3.6 ± 1.0	58.5 ± 24.2	52.0 ± 20.7	1.12 ± 0.08
Heated	24	6.3	0.42 ± 0.13	3.0 ± 0.5	60.2 ± 17.9	55.5 ± 15.5	1.09 ± 0.03
Water							
Ambient	0	8.9	0.73 ± 0.09	3.2 ± 0.9	63.2 ± 10.2	51.2 ± 5.9	1.23 ± 0.10
Ambient	5	9.3	0.70 ± 0.10	3.7 ± 0.6	68.0 ± 15.5	57.1 ± 12.0	1.19 ± 0.08
Ambient	24	8.7	0.61 ± 0.11	2.7 ± 0.7	50.5 ± 10.2	42.8 ± 8.4	1.18 ± 0.13
Zyactinase							
Native	0	4.1	0.74 ± 0.10	3.8 ± 0.8	67.9 ± 15.1	54.5 ± 11.2	1.25 ± 0.06
Native	5	9.0	0.62 ± 0.12	3.9 ± 0.8	66.8 ± 17.1	57.2 ± 14.1	1.17 ± 0.07
Native	24	6.6	0.37 ± 0.08	2.4 ± 0.4	33.5 ± 8.5	30.4 ± 7.3	1.10 ± 0.05
Heated	0	4.6	0.65 ± 0.12	3.2 ± 0.5	51.8 ± 7.7	41.8 ± 5.7	1.24 ± 0.07
Heated	5	8.9	0.72 ± 0.07	3.8 ± 0.7	71.0 ± 13.1	60.2 ± 10.3	1.18 ± 0.06
Heated	24	8.2	0.41 ± 0.08	2.6 ± 0.6	50.5 ± 11.6	46.4 ± 10.1	1.09 ± 0.04

Appendix 5

Full summary data for Table 19. Average of pH, weight, height and peak textural force of squid pieces incubated for 0 and 24 hours before and after incubation.

Treatment	Time (h)	pH	Weight (g)	Height (mm)	First peak (N)	2nd Peak Force (N)	1st Peak/ 2nd Peak
Non fermented pieces							
Kiwi extract							
Native	0	3.2	0.92 ± 0.16	5.1 ± 0.5	70.5 ± 16.5	57.5 ± 16.0	1.22 ± 0.08
Native	24	6.5	0.81 ± 0.17	4.8 ± 0.5	15.8 ± 6.4	12.7 ± 5.0	1.25 ± 0.07
Heated	0	3.2	1.04 ± 0.27	4.8 ± 0.8	80.0 ± 15.2	69.1 ± 12.4	1.15 ± 0.10
Heated	24	5.9	0.73 ± 0.22	5.2 ± 0.8	64.2 ± 20.9	55.6 ± 18.2	1.16 ± 0.17
Water							
Ambient	0	8.3	0.98 ± 0.18	4.8 ± 0.9	66.5 ± 21.7	54.1 ± 17.9	1.23 ± 0.16
Ambient	24	8.4	1.14 ± 0.17	5.0 ± 0.6	85.2 ± 12.8	67.5 ± 9.9	1.26 ± 0.07
Zyactinase							
Native	0	4.8	1.09 ± 0.17	5.0 ± 0.8	77.9 ± 19.8	64.0 ± 15.7	1.22 ± 0.09
Native	24	7.5	0.93 ± 0.13	4.8 ± 0.3	68.5 ± 13.8	58.3 ± 9.5	1.17 ± 0.06
Heated	0	3.7	0.99 ± 0.09	5.2 ± 0.4	72.4 ± 20.2	53.5 ± 15.1	1.35 ± 0.85
Heated	24	7.1	0.95 ± 0.16	5.2 ± 0.4	83.6 ± 20.6	66.5 ± 15.2	1.26 ± 0.21
Fermented pieces							
Kiwi extract							
Native	0	4.8	0.59 ± 0.10	4.4 ± 0.7	35.0 ± 10.5	30.7 ± 9.1	1.14 ± 0.04
Native	24	3.8	0.56 ± 0.13	4.1 ± 0.9	41.2 ± 14.0	36.6 ± 11.9	1.13 ± 0.05
Heated	0	4.6	0.59 ± 0.13	4.4 ± 0.7	76.9 ± 23.1	71.7 ± 22.1	1.07 ± 0.04
Heated	24	4.5	0.45 ± 0.05	4.2 ± 0.4	85.7 ± 12.1	80.8 ± 12.2	1.06 ± 0.04
Water							

Ambient	0	4.7	0.61 ± 0.10	3.8 ± 0.6	64.9 ± 30.6	61.8 ± 29.1	1.05 ± 0.01
Ambient	24	4.8	0.73 ± 0.14	4.5 ± 0.6	91.6 ± 30.2	85.2 ± 28.2	1.08 ± 0.04
Zyactinase							
Native	0	4.5	0.61 ± 0.13	4.0 ± 0.5	42.8 ± 11.4	37.6 ± 9.2	1.14 ± 0.08
Native	24	4.9	0.71 ± 0.21	4.3 ± 0.8	78.2 ± 45.9	71.9 ± 39.9	1.08 ± 0.03
Heated	0	4.5	0.58 ± 0.10	3.9 ± 0.6	72.0 ± 39.3	64.5 ± 33.2	1.12 ± 0.13
Heated	24	4.4	0.66 ± 0.13	4.6 ± 0.6	77.8 ± 27.6	72.8 ± 24.9	1.07 ± 0.02