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# **Actinidin Treatment and *Sous Vide* Cooking: Effects on Tenderness and *In Vitro* Protein Digestibility of Beef Brisket**

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# Abstract

Actinidin from kiwifruit can tenderise meat and help to add value to low-value meat cuts. Compared with other traditional tenderisers (e.g. papain and bromelain) it is a promising way, due to its less intensive tenderisation effects on meat. But, as with other plant proteases, over-tenderisation of meat may occur if the reaction is not controlled. Therefore, the objectives of this study were (1) finding a suitable process to control the enzyme activity after desired meat tenderisation has been achieved; (2) optimising the dual processing conditions- actinidin pre-treatment followed by *sous vide* cooking to achieve the desired tenderisation in shorter processing times. The first part of the study focused on the thermal inactivation of actinidin in freshly-prepared kiwifruit extract (KE) or a commercially available green kiwifruit enzyme extract (CEE). The second part evaluated the effects of actinidin pre-treatment on texture and *in vitro* protein digestibility of *sous vide* cooked beef brisket steaks.

The results showed that actinidin in KE and CEE was inactivated at moderate temperatures (60 and 65 °C) in less than 5 min. However, the enzyme inactivation times increased considerably (up to 24 h at these temperatures) for KE/CEE-meat mixtures, compared with KE/CEE alone. The thermal inactivation kinetics were used as a guide for optimising actinidin application parameters during the second phase of the study.

For the final experiments, beef steaks were injected with 5 % (w/w, extract/meat) of CEE solution (3 mg/mL) followed by vacuum tumbling (at 4 °C for 15 min) and cooking

(at 70 °C for 30 min) under *sous vide* conditions. This cooking time was considerably less than usual *sous vide* cooking times used in the meat industry. The actinidin-treated meat had no change in pH and colour, but showed a lower instrumental shear force; and improved sensory scores for tenderness, juiciness and flavour than the untreated meat steaks when tested by a sensory panel. Improved tenderness agreed well with the Transmission Electron Microscopy (TEM) results that showed considerable breakdown of the myofibrillar structure, particularly around the Z line. The addition of actinidin enhanced the rate of breakdown of muscle proteins, as shown by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and led to an increase in both protein solubility and ninhydrin-reactive free amino N release, during simulated gastric digestion. These results demonstrate the positive effects of actinidin on meat tenderness and meat protein digestibility during gastric digestion *in vitro*.

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# Abbreviations

<b>ANZFSC</b>	Australia New Zealand Food Standards Code
<b>CA</b>	Commercial availability
<b>CBZ</b>	N- $\alpha$ -carbobenzoxy-L-lysine P-nitrophenyl ester hydrochloride
<b>CEE</b>	Commercial enzyme extract
<b>DTT</b>	DL-Dithiothreitol
<b>EA</b>	Enzyme activity
<b>FDA</b>	Food and Drug Administration of United States
<b>GRAS</b>	Generally Recognised as Safe
<b>KE</b>	Kiwifruit extract
<b>MPI</b>	Ministry for Primary Industries
<b>SGF</b>	Simulated gastric fluid
<b>SF</b>	Simulated salivary fluid
<b>SSF</b>	Slice shear force
<b>SDS-PAGE</b>	Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis
<b>TEM</b>	Transmission Electron Microscope
<b>WBSF</b>	Warner-Bratzler shear force

# Chapter 1. Introduction

The conception of meat quality is multi-dimensional (Bekhit, Hopkins, Geesink, Bekhit, & Franks, 2014) which is valued based on culture, religion, function and other factors. Sensory quality is the most important factor that determines meat quality and affects the meat market. Consumers are directly influenced in the purchase of meat by the colour, smell and the amount of visible fat (Mancini & Hunt, 2005). However, since the appearance of the meat is changed after cooking, the tenderness, juiciness and flavour/odour of meat after cooking will be the major factor that determines consumers' satisfaction and further purchase (Bekhit *et al.*, 2014). In some cultures, such as in Africa, people prefer to eat chewy meat. However, people in western countries prefer their meat to be tender, which is reflected in the price - the tenderer, the more expensive (Warriss, 2001). It has been reported that only 10 % of the carcass can be made into prime grilling tender cuts (Polkinghorne, Philpott, Gee, Doljanin, & Innes, 2008), which means that the rest (90 %) can potentially benefit from meat tenderisation methods, such as the use of exogenous enzymes, to achieve an improvement in tenderness, to economically reward the meat industry and to supply consumers with good quality red meat.

Traditional enzymes used in the industry, such as papain and bromelain, usually have a tendency to over-tenderise the meat and make it “mushy” (Bekhit, Han, Morton, & Sedcole, 2007; Maróstica & Pastore, 2010; Melendo, Beltrán, Jaime, Sancho, & Roncalés, 1996). The use of actinidin from kiwifruit has been studied on meat tenderisation in recent

years, due to its less intensive tenderisation ability (Toohey, Kerr, van de Ven, & Hopkins, 2011). However, over-tenderisation may continue to occur due to the ongoing reaction between actinidin and meat proteins. Information on manipulating actinidin activity to avoid mushy meat texture and achieve desired tenderisation conditions is lacking in the literature.

Kiwifruit is a niche fruit, which only accounts for approximately 0.22 % of the international fruit market. However, during the last decade, the global production of kiwifruit has increased by more than 50 % (Ward & Courtney, 2013), and New Zealand accounts for approx. 30% of the global export volume of kiwifruit (Ward & Courtney, 2013). Kiwifruit applications as a digestibility enhancer have also attracted interest recently. The positive effects of actinidin on *in vitro* and *in vivo* digestion of different food proteins including muscle proteins have confirmed by many studies (Kaur, Rutherford, Moughan, Drummond, & Boland, 2010a, 2010b; Rutherford *et al.*, 2011).

Brisket, which is the cut from the lower chest, made up of superficial pectoral and deep pectoral muscles, is used to support about 60 % of the animal body weight and is usually treated as a low-value cut due to its toughness. A longer cooking time is required to degrade the relatively large amount of connective tissue (0.1 %) from brisket to make it tender (Torrescano, Sánchez-Escalante, Giménez, Roncalés, & Beltrán, 2003). Christensen *et al.* (2009) have provided evidence that actinidin from kiwifruit could degrade the myofibrils and connective tissue, indicating its potential application for value addition of brisket muscles. Information on the effects of pre-treatment with actinidin on *sous vide* cooked beef brisket steaks on meat microstructure and protein digestibility is

lacking in the literature.

The main objectives of this project were: 1) developing a suitable process to control the enzyme activity after desired meat tenderisation has been achieved; 2) optimising the dual processing conditions- actinidin pretreatment followed by *sous vide* cooking to achieve desired tenderisation; 3) analysing the effects of dual processing on appearance, microstructure, texture, and *in vitro* protein digestibility of beef brisket steaks.

# **Chapter 2. Review of Literature**

## **2.1. Toughness of meat**

The quality of meat is usually used to describe the appeal of meat products to the customers (Dinh, 2006). Good quality and acceptable price of meat products are the main demands of most consumers. These demands constantly give force to the development of meat industry with value-addition to poor-quality meat cuts and higher efficiency of carcass utilisation (Lantto *et al.*, 2009).

Factors determining meat quality include safety requirements, animal welfare and also the sensory appeal of meat, e.g. palatability (Dinh, 2006). Once a meat product is cooked, the tenderness, juiciness, flavour and aroma have to meet consumer requirements (Aaslyng, 2002). Flavour and aroma may be easily changed through the addition of spices, and juiciness may be improved through the use of different cooking methods. However, tenderness depends on variable factors, such as meat structure, composition of meat, post-mortem ageing, freezing-thawing, and cooking temperature and time (Dinh, 2006).

### **2.1.1. Muscle composition and structure**

The basic tenderness of meat is the most unpredictable trait, but it is also the most important parameter of meat quality impacting consumers' choice. It is based on the ease of chewing, and the fibrous nature of muscle contributes to the chewing resistance of meat (Gerrard & Grant, 2003).

Meat tenderness mainly depends on two components of meat structure, connective tissue and myofibrillar proteins (Marsh & Leet, 1966; Nishimura, Hattori, & Takahashi, 1995; Smith & Judge, 1991). The former, commonly known as the “background toughness” is mainly contributed by the amount and type of collagen that provides support for the muscles at different hierarchies, while the latter is mainly associated with myofibrillar structure, which includes the cytoskeletal framework (Bekhit *et al.*, 2014; Ouali, 1990). Tender muscles were found to be lower in collagen than tough muscle (Keith, Vol, Miles, Bechtel, & Carr, 1985). Torrescano *et al.* (2003) analysed the relationship between shear force values, collagen content and sarcomere length (composition of myofibrils) of 14 different bovine muscles (**Table 1**) and demonstrated that shear force was positively correlated with the amounts of total and insoluble collagen. Decrease of sarcomere length can increase fibre diameter and results in a decrease of tenderness (Herring, Cassens, & Risky, 1965).



**Table 1.** Ranking of shear force values of 14 bovine muscles and their relationship with total collagen, insoluble collagen and sarcomere length

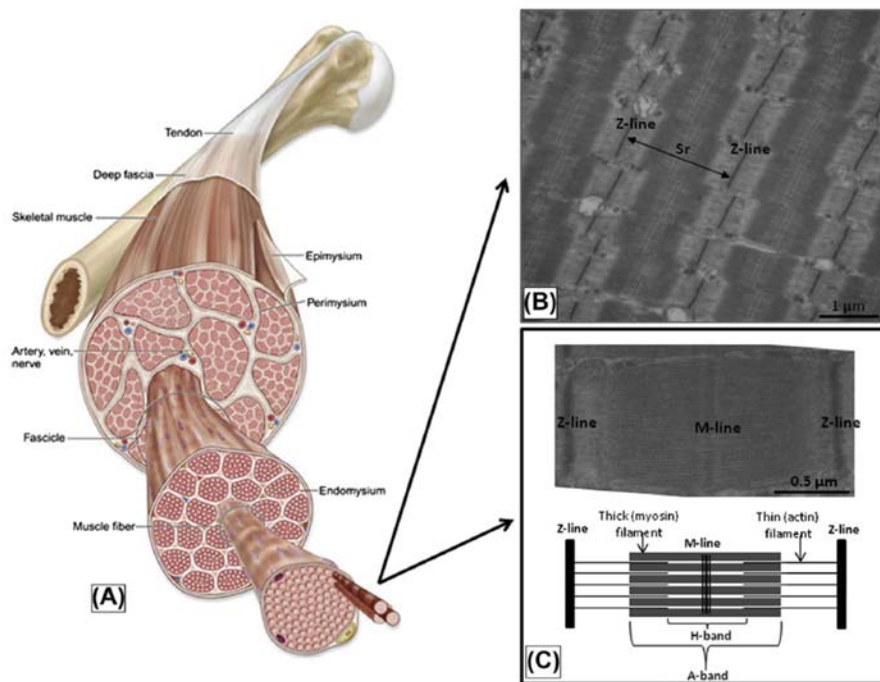
Muscle rank	Warner–Bratzler shear force (kg)	Total collagen (mgHyp/g wet tissue)	Insoluble collagen (mgHyp/g wet tissue)	Sarcomere length ( $\mu\text{m}$ )
1	<i>psoas major</i> 2.11a $\pm$ 0.39	<i>psoas major</i> 0.31a $\pm$ 0.04	<i>psoas major</i> 0.18a $\pm$ 0.01	<i>l. lumborum</i> 1.57a $\pm$ 0.3
2	<i>diaphragm</i> 2.24a $\pm$ 0.90	<i>l. thorecis</i> 0.51b $\pm$ 0.04	<i>l. thoracis</i> 0.38b $\pm$ 0.03	<i>gluteus medius</i> 1.59ab $\pm$ 0.2
3	<i>l. thoracis</i> 2.29a $\pm$ 0.91	<i>diaphragm</i> 0.53c $\pm$ 0.08	<i>diaphragm</i> 0.39b $\pm$ 0.10	<i>biceps femoris</i> 1.6ab $\pm$ 0.2
4	<i>l. lumborum</i> 2.35a $\pm$ 0.59	<i>gluteus medius</i> 0.59d $\pm$ 0.05	<i>l. lumborum</i> 0.42c $\pm$ 0.03	<i>quadriceps femoris</i> 1.71b $\pm$ 0.4
5	<i>gluteus medius</i> 3.68b $\pm$ 0.51	<i>semimembranosus</i> 0.60de $\pm$ 0.06	<i>gluteus medius</i> 0.45d $\pm$ 0.04	<i>diaphragm</i> 1.84c $\pm$ 0.2
6	<i>semimembranosus</i> 4.10c $\pm$ 0.79	<i>l. lumborum</i> 0.61e $\pm$ 0.09	<i>semimembranosus</i> 0.49e $\pm$ 0.02	<i>triceps brachii</i> 1.87c $\pm$ 0.1
7	<i>infraspinatus</i> 4.24c $\pm$ 0.93	<i>quadriceps femoris</i> 0.68f $\pm$ 0.09	<i>flexor digitorum</i> 0.51f $\pm$ 0.06	<i>semimembranosus</i> 1.93cd $\pm$ 0.2
8	<i>quadriceps femoris</i> 4.72d $\pm$ 0.89	<i>biceps femoris</i> 0.72g $\pm$ 0.05	<i>quadriceps femoris</i> 0.52f $\pm$ 0.06	<i>l. thoracis</i> 1.95cd $\pm$ 0.5
9	<i>semitendinosus</i> 4.79d $\pm$ 0.58	<i>semitendinosus</i> 0.77h $\pm$ 0.09	<i>sternomandibularis</i> 0.57g $\pm$ 0.06	<i>semitendinosus</i> 2.05d $\pm$ 0.2
10	<i>triceps brachii</i> 5.42e $\pm$ 0.82	<i>sternomandibularis</i> 0.81i $\pm$ 0.07	<i>biceps femoris</i> 0.60h $\pm$ 0.04	<i>infraspinatus</i> 2.21e $\pm$ 0.3
11	<i>biceps femoris</i> 5.46e $\pm$ 0.84	<i>triceps brachii</i> 0.94j $\pm$ 0.08	<i>semitendinosus</i> 0.64i $\pm$ 0.03	<i>flexor digitorum</i> 2.39f $\pm$ 0.2
12	<i>flexor digitorum</i> 5.93f $\pm$ 0.92	<i>pectoralis profundus</i> 0.96k $\pm$ 0.09	<i>pectoralis profundus</i> 0.71j $\pm$ 0.04	<i>sternomandibularis</i> 2.55g $\pm$ 0.2
13	<i>sternomandibularis</i> 6.32g $\pm$ 0.72	<i>infraspinatus</i> 0.98k $\pm$ 0.05	<i>infraspinatus</i> 0.76k $\pm$ 0.08	<i>pectoralis profundus</i> 2.65g $\pm$ 0.3
14	<i>pectoralis profundus</i> 6.66h $\pm$ 0.66	<i>flexor digitorum</i> 1.15l $\pm$ 0.03	<i>triceps brachii</i> 0.78l $\pm$ 0.02	<i>psoas major</i> 3.42h $\pm$ 0.3

Means within a column with a common letter are not significantly different ( $p < 0.05$ ). Hyp = hydroxyproline. *Pectoralis profundus* is beef brisket. Reprinted from Torresco et al. (2003), © Elsevier, with permission from Elsevier.

Meat comes from the skeletal muscle of animals. The composition of skeletal muscle is around 75 % water, 19 % protein, 2.5 % intramuscular fat, 1.2 % carbohydrates, and 2.3 % other soluble non-protein substances (Astruc, 2014). Skeletal muscles are mainly composed of separated cells known as muscle fibres, which make up 90 % of the whole muscle volume. Those muscle fibres are enclosed in a layer of connective tissue called the endomysium, and those multiple packed muscle fibres are re-ensheathed in another layer of connective tissue called the perimysium to constitute fibre bundles, which are covered by the third kind of connective tissue called the epimysium (Astruc, 2014). Muscle fibres are predominantly consisted of proteins, which are categorised as sarcoplasmic, myofibrillar and stromal proteins (Dinh, 2006). A myofibril is composed of a succession of sarcomeres (Sr) placed between Z lines. A sarcomere is composed of actin and myosin myofilaments, which work together to result in muscle contraction. The muscle structure is presented in **Figure 1**.

Connective tissues, accounting for 1.5-10 % of the dry weight of muscle (depending on the muscle function), give the muscle strength, but also toughness. Previous research of Bailey and Light (1989) and Purslow (2005) showed that the content and composition of connective tissue significantly influenced the technical and organoleptic properties of meat and meat products, i.e. water-holding capacity and meat texture. There is 90 % collagen in meat connective tissue, which means meat tenderness based on connective tissue is mainly ascribed to collagen fibres (Dinh, 2006; Lantto *et al.*, 2009). Although there are 28 different types of collagen, and a high proportion of type III collagen may lead to higher toughness, and the extent of the type of cross-linking within different types

of collagen is also important. The increase in cross-linking and collagen insolubility is related to age of the animal: the older the animal is, the tougher the meat will be (Gerrard & Grant, 2003).



**Figure 1.** Structure of muscle

(A) The general muscle structure, (B) the longitudinal section of a muscle fibre showing the myofibrils organisation, and (C) the organisation of a sarcomere. Reprinted from Astruc (2014), © Elsevier, with permission from Elsevier.

Additionally, the type of muscle plays an important role. It has been reported that tenderness was negatively related to the type II (fast-twitch glycolytic) muscle and positively related to the type I (slow-twitch oxidative) muscle (Dinh, 2006; Lawrence, Fowler, & Novakofski, 2012). The ratio of different muscle types differs within animal

breeds, crosses and even within the same breeds.

### **2.1.2. Post-mortem conditions and the effects of endogenous proteases in meat**

Another factor influencing meat tenderness is the contractile system of muscle. Muscle begins to convert to meat when the animal is dead. The structure of muscle predominantly consists of fibrous structure, which gives the contraction function to muscle. The contractile function of a living muscle fibre is enabled by many enzymes acting in concert and is supported by the energy derived from ATP hydrolysis. When the animal is slaughtered, fibres continue metabolism in an anaerobic situation. To maintain the ATP level, the muscle polysaccharide glycogen is degraded and subsequent anaerobic glycolysis happens, which leads to lactic acid production and a decrease in muscle pH. Decreased pH and temperature result in a decrease of glycolytic enzyme activities and finally a decrease in the ATP level. As a result, the main contractile protein complex (filaments), normally kept separated by ATP in living muscle, combine irreversibly and make the meat tough, which is known as *rigor mortis* (Lantto *et al.*, 2009). The pH of muscle decreases from a normal value of 7.2 to about 5.5 during this post-mortem situation (normally 24 hours after slaughter) (Brewer, Zhu, Bidner, Meisinger, & McKeith, 2001; Varnam & Sutherland, 1996), due to the generation of lactic acid. This change in pH causes the inactivation of metabolic enzymes and results in the termination of ATP energy metabolism (Muchenje, Dzama, Chimonyo, Strydom, & Raats, 2009), which

terminates further toughening and softening starts to take the dominant role.

After rigor mortis, meat starts to get softer during the following several days due to the action of endogenous muscle proteases, i.e. calpains, collagenases and cathepsins. These enzymes are active at post-mortem pH (favourable environment) and start to hydrolyse contractile muscle proteins (Astruc, 2014; Dinh, 2006; Lawrie, 1974). Cathepsins are considered to be active at low pH, which usually plays a major role in pale, soft and exudative (PSE) meat, such as fish muscle, while calpains are more reactive at higher pH, which means the calpains are more important than cathepsins in red meat (pH around 5.5) or poultry tenderisation (Dransfield, 1996; Du Toit & Oguttu, 2013; Varnam & Sutherland, 1996; Warriss, 2001). The activities and target proteins of calpains and cathepsins are shown in **Table 2**.

**Table 2.** Activity and target proteins of calpain and cathepsin (Varnam & Sutherland, 1996)

<b>Category</b>	<b>Endogenous Protease</b>	<b>Activity and Target Proteins</b>
Sarcoplasmic	Calpain I	Releasing $\alpha$ -actinin, Z-nin
	Calpain II	Degrading desmin, filamin, nebulin, troponins, tropomyosin, C- and M-proteins
Lysosomal	Cathepsin B	Degrading myosin, actin, troponin T and collagen
	Cathepsin L	Degrading myosin, actin, troponins, tropomyosin, $\alpha$ -actinin and collagen
	Cathepsin D	Degrading myosin, actin, $\alpha$ -actinin, troponins, tropomyosin and collagen

### **2.1.2.1. Calpains**

The extent of activity of Ca<sup>2+</sup>-activated calpains, including calpain I and calpain II, depends on activators (i.e. pH, temperature and the level of calcium ion) and an inactivator (i.e. calpastatin). When the content of calcium is still low, calpain I will be activated first (pH 6.3), then the increased calpain I level activates calpain II (Dransfield, 1996). During the degradation of myofibrillar proteins by calpains (Livisay, Xiong, & Moody, 1996), structural protein calpastatins are degraded by calpains and meanwhile calpains degrade themselves in the long run (Lantto *et al.*, 2009) because both of the calpains seem to be unstable at a temperature higher than 4 °C (Dransfield, 1996). The calpain II: calpastatin ratio is a good index to describe the efficiency of the endogenous proteolytic system. Ouali and Talmant (1990) have reported that the conditioning (ageing) rate might be positively related to this ratio and negatively related to the concentration of calpastatin.

### **2.1.2.2. Cathepsins**

Cathepsins are located in lysosomes in the sarcoplasm and probably are released from lysosomes post-mortem. The optimal activity of cathepsins is achieved under moderately acidic conditions. All cathepsins (B, D & L) are irreversibly inactivated above pH 7 (Ogino & Fujihara, 1992). They are known to degrade myofibrillar proteins such as troponin and myosin-F-actin (Penny, 1980) and -collagen cross-links (Warriss, 2001). However, the degradation of myosin and actin seems to happen at a pH lower than 5,

which is unlikely to occur in normal meat (Warriss, 2001), but likely in fish muscle.

### **2.1.2.3. Collagenase**

Collagen molecules (connective tissue), located between or within muscles, are degraded by collagenase. Once they are cleaved, the denatured collagen and myofibril can be further degraded by other broad-spectrum proteases (e.g. papain) to peptide and amino acid level. This degradation process is very slow during natural ageing of meat (Bailey & Light, 1989). The addition of exogenous proteases, such as plant, microbial or animal enzymes, could speed up the tenderisation process.

## **2.2. Techniques for improvement of meat tenderness**

In industry, the unprocessed meat after slaughtering is normally kept at a temperature above the freezing point for different lengths of time to develop its flavour and texture (Astruc, 2014; Etherington, 1987) and this procedure is referred to as “ageing” or “conditioning”. Other traditional or novel techniques to improve tenderness of pre- and post-rigor meat are classified into physical, chemical and enzymatic methods.

### **2.2.1. Physical processing**

The physical processes include electrical stimulation (Hwang, Devine, & Hopkins, 2003), high power ultrasound (Jayasooriya, Torley, D’arcy, & Bhandari, 2007), ageing, freezing-thawing cycle (Lagerstedt, Enfält, Johansson, & Lundström, 2008; Leygonie, Britz, & Hoffman, 2012), shockwave processing (Bolumar, Enneking, Toepfl, & Heinz,

2013), high hydrostatic pressure (Jung, de Lamballerie-Anton, & Ghoul, 2000) and mechanical methods (e.g. flaking, pounding, piercing and mincing tenderisation).

### **2.2.2. Chemical processing**

Chemical methods have played an important role in tenderness improvement in past industrial processing (Bekhit *et al.*, 2014). These methods usually refer to marinating, injection and infusion with chemical solutions (e.g. calcium salts, sodium salts and phosphate salts) or commercial marinate solutions containing maltodextrin and starch. Calcium could activate endogenous enzymes (i.e. calpains). Sodium chloride enhances protein solubilisation while phosphate buffer improves the water retention of meat. However, for some muscles with high-density connective tissue from older animals, the endogenous proteolytic enzyme capacity might not be sufficient (Veiseth, Shackelford, Wheeler, & Koohmaraie, 2004). In this situation, that chemicals cannot help, this kind of muscle could be effectively tenderised through the use of exogenous proteases.

### **2.2.3. Enzymatic processing**

Some enzymes have the ability to hydrolyse meat proteins playing the main role in meat tenderness. Enzymatic intervention is the application of exogenous enzyme from plants, microbes and animals by the method of injection, marinating or infusion. The commonly used plant-derived enzymes include papain (EC 3.4.22.2) from papaya, bromelain (EC 3.4.22.33) from pineapple, ficin (EC 3.4.22.3) from fig and actinidin (EC 3.4.22.14) from kiwifruit.



Usually, the cost of physical processing is quite high, especially for some of the novel techniques. The traditional physical methods (particularly mechanical processes) could require long-time processing (ageing) or result in flavour loss (Leak, Kemp, Fox, & Langlois, 1987). Chemicals may change the pH and colour (Killefer, 2004), increase the saltiness to cause adverse health effects, and shorten the shelf-life of meat products (Robbins *et al.*, 2002). The use of exogenous enzymes is promising due to the different tenderising specificities of the different proteases (from various sources) towards certain meat proteins (Bekhit *et al.*, 2014). For example, zingibain has a higher specificity towards collagen than actomyosin (Thompson, Wolf, & Allen, 1973), and a *Bacillus subtilis* protease preferentially degrades myofibrillar proteins over collagen proteins (Sullivan & Calkins, 2010).

### **2.2.3.1. Methods of enzyme application**

Enzymes used for meat tenderisation are not easy to distribute evenly into meat, due to the biochemical consistency and structure of meat. The current commonly used application methods include dipping, marinating, injection and spraying.

Dipping and marinating could be the same method, which means meat is dipped and marinated in the enzyme solution. Spraying includes spraying enzyme powder or solution on the surface of meat. These methods have been widely used (Mendiratta, Sharma, Narayan, & Mane, 2010; Naveena & Mendiratta, 2004; Quaglia, Lombardi, Sinesio, Bertone, & Menesatti, 1992) but they have the drawback that poor penetration of the enzyme solution contributes to the over-tenderised mushy meat surface with an

inadequately-tenderised interior. Therefore, these methods are usually limited to relatively thin cuts. Several procedures have been used to obtain more even distribution of enzyme throughout the tissues, such as using a fork to pierce the meat steak either prior to or following the enzyme application.

Compared with marinating, the injection method needs a much lower dosage of the enzyme to achieve the same level of tenderisation. The potential reason is due to the increase in the contact area between enzyme and meat protein (Ashie, Sorensen, & Nielsen, 2002). For big pieces of meat, such as ham or round for roasting, the injection method is more often used, but the enzyme distribution is still relatively poor, leading to mushy pockets at the injection location. Techniques such as needleless hypodermic injectors or spray tenderiser have been explored (Wattenbarger, 1965). Another procedure to improve the uniform distribution is tumbling. Research has shown a more uniform distribution of enzyme and a greater breakdown of structural proteins for papain-treated beef muscle when vacuum tumbling was used after enzyme application (Huerta-Montauti *et al.*, 2008).

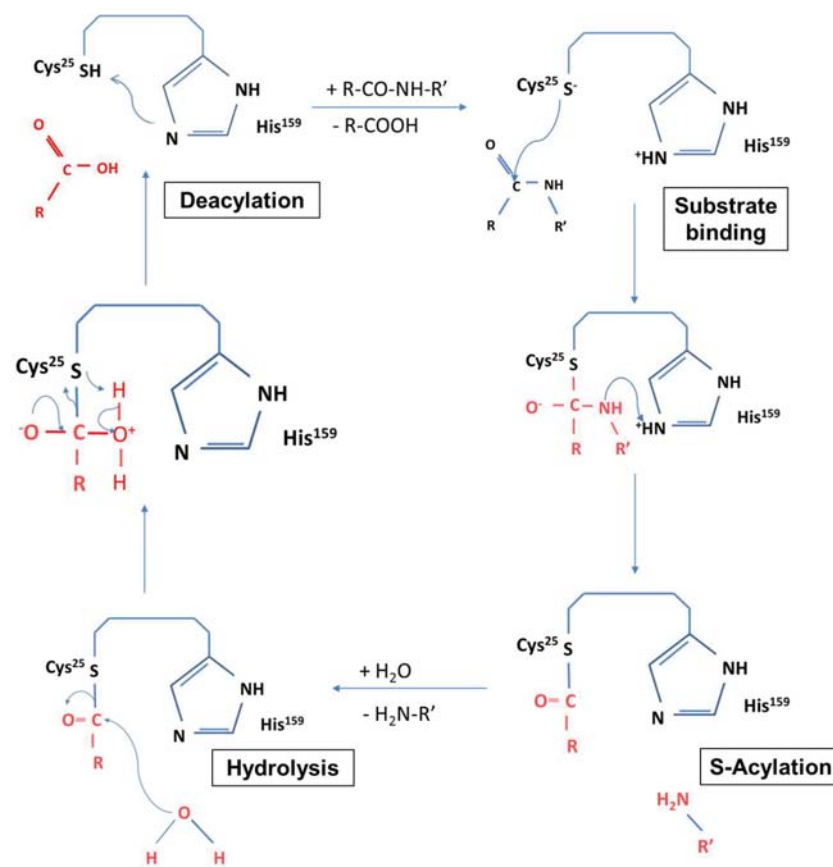
## **2.3. Exogenous proteases**

### **2.3.1. Plant-derived enzymes**

The application of plant-derived proteases has been extensively studied. Commonly used enzymes are cysteine proteases (also known as thiol proteases) and have broad-spectrum specificities, which results in indiscriminate breakdown or degradation of

myofibrillar proteins and collagen (Ashie *et al.*, 2002; Foegeding & Larick, 1986; Miller, Strange, & Whiting, 1989). The information about pH and temperature for optimal activity, application regulation status, target protein types, commercial availability, advantages and disadvantages of the normal plant proteases is summarised in **Table 3**. A recent paper reported some promising candidates from fruits and vegetable resources for plant-derived protease production, such as broccoli, ginger, leek and red pepper, which indicated the potential use of many unexplored plant resources (Sun, Zhang, Yan, & Jiang, 2016).

The catalytic mechanism (**Figure 2**) of papain has been studied as a model enzyme of the cysteine proteases. Maximum activity of papain is observed when in a reducing and acidic environment because the thiol group of protease has to be protonated to be active. The Cys25 residue on the enzyme forms a covalent bond with the substrate. The formation of an intermediate S-acyl enzyme moiety, releases the C-terminal fragment of the cleaved substrate. Then the hydrolysis and deacylation of the intermediate happen, and the regenerated cysteine protease begins a new catalytic cycle (Grzonka, Kasprzykowski, & Wiczak, 2007; Storer & Ménard, 1994).



**Figure 2.** The catalytic mechanism of papain (Grzonka *et al.*, 2007)

**Table 3.** Different plant-derived enzymes and their properties

<b>Enzyme</b>	<b>pH</b>	<b>Optimal Temperature</b>	<b>Target protein</b>	<b>CA<sup>1</sup></b>	<b>Advantages</b>	<b>Disadvantages</b>	<b>Reference</b>
<b>Papain (Papaya)</b>	5-8 (Active)	65°C	Connective tissues Myofibrillar proteins	Y	1. GRAS 2. Broad-spectrum enzymatic activity	1. Over- tenderisation 2. Mushy texture	Ha, Bekhit, Carne, and Hopkins (2012) Bekhit <i>et al.</i> (2014)
<b>Bromelain (Pineapple)</b>	5-7 (Active)	50°C	Collagen more than contractile proteins	Y	1. GRAS 2. Broad-spectrum enzymatic activity	1. Over- tenderisation 2. Mushy texture	Ha <i>et al.</i> (2012) Bekhit <i>et al.</i> (2014) Sullivan and Calkins (2010) Barrett, Woessner, and Rawlings (2012)
<b>Ficin (Fig)</b>	5-8 (Optimal)	45-55°C	Balanced degradation of collagen and myofibrillar proteins	Y	1. GRAS 2. Broad specificity	1. Over- tenderised meat 2. Mushy texture	Ha <i>et al.</i> (2012) Schwimmer (1981) Bekhit <i>et al.</i> (2014) Sullivan and Calkins (2010)

<b>Actinidin</b>	5-7	45°C	Myofibrillar proteins	Y	1. Less intensive tenderising effect in meat	1. Not approved as GRAS <sup>2</sup> by FDA <sup>3</sup>	Christensen <i>et al.</i> (2009)
<b>(Kiwifruit)</b>	(Optimal)		Connective tissues				Ha <i>et al.</i> (2012)
			Higher proteolytic activity to beef myofibril proteins		2. Do not affect flavour and juiciness.	2. Permitted by ANZFS <sup>4</sup>	Bekhit <i>et al.</i> (2014)
					3. Regulation varies depending on countries		
<b>Zingibain</b>	6-7	60°C	Higher specificity to collagen	Y	1. Higher specificity toward connective tissue	1. Several fractions of ginger have been given GRAS except zingibain	Bekhit <i>et al.</i> (2014)
<b>(Ginger)</b>	(Optimal)		compared with actomyosin				Adulyatham and Owusu-Apenten (2005)
							Bhaskar, Sachindra, Modi, Sakhare, and Mahendrakar (2006)

<sup>1</sup>CA, commercial availability; <sup>2</sup>GRAS, Generally Recognised as Safe; <sup>3</sup>FDA, Food and Drug Administration of United States; <sup>4</sup>ANZFS, Australia New Zealand Food Standards Code.

**Papain** (EC 3.4.22.2) is a cysteine protease extracted from the latex of the papaya plant. It has a broad spectrum enzymatic activity over pH 5-8 and an optimal activity at a high temperature of 65 °C (Smith & Hong-Shum, 2003). Papain has the greatest ability to improve tenderness compared with bromelain, ficin, homogenised fresh ginger and some microbial enzymes, but also has a negative effect on juiciness and textural changes (Sullivan & Calkins, 2010).

**Bromelain** represents a mixture of cysteine proteases from pineapple, including the enzyme obtained from pineapple stem (EC3.4.22.32) and fruit (EC3.4.22.33). It has a narrower pH range (pH 5-7) and shows maximum activity at a lower temperature of 50 °C compared with papain. Although both the enzymes are glycosylated single-chain proteins, the fruit bromelain has higher enzyme activity and a greater specificity for peptide bonds compared to stem bromelain (Barrett *et al.*, 2012; Grzonka *et al.*, 2007). When used in meat, bromelain increases tenderness and degrades collagen more than the contractile proteins (Sullivan & Calkins, 2010).

**Ficin** (EC 3.4.22.3) is extracted from the latex of fig plant and the crude ficin latex includes approximately 10 protease variants (Kramer & Whitaker, 1964). The maximum enzyme activity is obtained at pH 5-8 and 45-55 °C. As a cysteine protease, ficin has a broad specificity for the hydrophobic amino acid residues (Schwimmer, 1981). It gives the most balanced degradation of collagen and myofibrillar proteins compared with other traditional cysteine proteases (Sullivan & Calkins, 2010).

**Zingibain** contains two cysteine proteases in the crude form. The maximum activity is observed at pH 6-7 and 60 °C (Adulyatham & Owusu - Apenten, 2005; Bhaskar *et al.*,

2006). The positive effects of ground ginger on meat tenderisation have been demonstrated by Sullivan and Calkins (2010) but its application is limited due to flavour issues. The use of ginger and several fractions of ginger have been given GRAS (Generally Recognised as Safe) by Food and Drug Administration of United States (FDA) except zingibain. However, a growing interest of use of zingibain has been given because of its higher specificity to hydrolyse connective tissues (Ha *et al.*, 2012).

*Actinidin* (EC.3.4.22.14), or Actinidain, is extracted from kiwifruit. Different cultivars have different levels of actinidin. The maximal enzyme activity is obtained at 45 °C (Ha, Bekhit, Carne, & Hopkins, 2013) and at pH 5-7 (Boyes, Strübi, & Marsh, 1997). Except for kiwifruit concentrate, actinidin has not been approved as GRAS by FDA, but its usage is permitted by Australia New Zealand Food Standards Code (ANZFS). The application of actinidin in the industry is promising because of its less intensive tenderisation on meat and broader specificity compared with other traditional enzymes (Toohey *et al.*, 2011). It has shown the ability to hydrolyse myofibrillar proteins and connective tissues (Bekhit *et al.*, 2007; Christensen *et al.*, 2009; Han, Morton, Bekhit, & Sedcole, 2009; Lewis & Luh, 1988; Wada, Suzuki, Yaguti, & Hasegawa, 2002). Further research shows its higher proteolytic activity on beef myofibril proteins (Ha *et al.*, 2012).

### **2.3.2. Microbial and animal proteases**

Microbial proteases include neutral, alkaline and aspartic proteases from fungal and bacterial resources. The use of enzymes from fungal strains has been used in traditional food production (such as soy sauce and Miso) for a long time in history, and the



proteolytic activity of many bacterial strains has been studied for years. These enzymes usually have small effects on the myofibrillar proteins, but have an active proteolytic action against collagen and elastin (Bekhit *et al.*, 2014). Reported by Sullivan and Calkins (2010), a *Bacillus subtilis* protease and two *Aspergillus oryzae* proteases preferentially degrade myofibrillar proteins over collagen proteins, and contribute to good results of sensory testing. The hydrolytic ability of bacterial proteases towards collagen is intermediate between that of papain and bromelain (Yeh, Yang, & Tsai, 2002). Generally, the microbial proteases process actively over a wide pH range (such as the activation of aspartic protease in the pH range 2-7), have relatively specific activity (for example collagenase specifically degrades collagen) and are denatured at moderate temperatures (lower than 70 °C) (Ashie *et al.*, 2002; Bekhit *et al.*, 2014).

Some microbial and animal enzymes used on meat have been studied, such as the aspartic protease from *Aspergillus oryzae* (Ashie *et al.*, 2002), subtilisin and neutral protease from *Bacillus subtilis* and *Bacillus subtilis* var. *amyloliquefaciens*, alkaline elastase from *alkalophilic Bacillus sp. Strain Ya-B* (Takagi *et al.*, 1992), collagenase from *vibro B-30* (Miller *et al.*, 1989) and collagenase from porcine pancreatin (Pietrasik, Aalhus, Gibson, & Shand, 2010). Some of the microbial proteases have already been approved as GRAS status and are commercially available. However, due to the potential pathogenicity of microbial sources, those enzymes have not attracted much interest for this application (Ashie *et al.*, 2002).

## **2.4. Kiwifruit and Actinidin**

### **2.4.1. Kiwifruit**

The name ‘kiwifruit’ was proposed by the New Zealand company Turners & Growers Ltd. to replace the old name “Chinese gooseberry” in 1959, when they firstly exported *Actinidia deliciosa* fruit to America (Ferguson, 1990). There are hundreds of kiwifruit cultivars. However, only two cultivars, *Actinidia deliciosa* cv. ‘Hayward’ (green kiwifruit) and *Actinidia chinensis* cv. ‘SunGold’ (gold kiwifruit), are the main varieties on the international trading market. The composition of raw green kiwifruit (*Actinidia deliciosa*) includes approx. 83 % water, 9 % sugar, 3 % fibre, 1 % protein, and a minor amount of minerals (Drummond, 2013). Kiwifruit has a minor but important proportion of protein, which consists of both soluble and insoluble forms. Soluble kiwifruit protein from *Actinidia deliciosa* is mainly the proteolytic enzyme actinidin and its inactive forms (Boland, 2013). The enzyme activity of kiwifruit will vary depending on the cultivar, growing location, soil, weather and altitude. The total proteolytic enzyme activity of Hayward kiwifruit is approximately eight times higher than that of SunGold kiwifruit (Chao, 2016).

### **2.4.2. Kiwifruit proteins and enzymes**

Fresh kiwifruit contains approx. 1 % crude protein and only 0.3 % soluble proteins. Soluble proteins consist of proteolytic enzyme actinidin (30 kDa); kiwellin (28 kDa) with

unknown function and its proteolysis product KiTH; and thaumatin-like protein (24 kDa) (Boland, 2013; Ciardiello *et al.*, 2008; Gavrović-Jankulović *et al.*, 2002). Another 17 kDa protein called kirola has recently been described (D'avino *et al.*, 2011). Actinidin and thaumatin-like protein have been identified by some researchers as major allergens in kiwifruit (Boland, 2013; Bublin, 2013; Bublin *et al.*, 2008).

### **2.4.3. Actinidin**

The name actinidin (EC3.4.22.14), extracted from *Actinidia*, was first proposed by Arcus (1959). For green kiwifruit (*Actinidia deliciosa*), actinidin accounts up to 40 % of the soluble proteins. Actinidin is a single polypeptide chain globular protein which is folded into a two-domain structure, L-domain and R-domain. Actinidin is synthesised as a proenzyme (or zymogen, inactive form) about 15 kDa larger than the mature enzyme, with a C-terminal extension of 25 amino acid residues and an N-terminal extension of 57 amino acid residues (Praekelt, McKee, & Smith, 1988). Therefore, it is likely that the proenzyme is activated either on secretion or on sequestration to a location in the cell (Paul *et al.*, 1995). However, the activation mechanism of actinidin in kiwifruit is not known. It is postulated that actinidin has a protective role in ripe fruit against the attack of pathogens, due to its wide specificity towards various substrates and a broad range of polymorphic forms, including inactive forms (Nieuwenhuizen *et al.*, 2012).

Actinidin has the ability to catalyse the hydrolysis of peptide bonds of proteins and also simple esters and amides (Boland, 2013). Specificity studies of actinidin catalytic activity have been carried out by ester hydrolysis and competitive inhibition (Boland &

Hardman, 1972), or by using B chain of insulin as substrate (McDowall, 1973). Actinidin has different optimal pH range for different activities and substrates. For esterolysis activity, the optimal pH is from 5 to 7, with benzoyl-L-arginine ethyl ester as a substrate (Boland & Hardman, 1972). When food proteins, such as gelatin are used as a substrate, actinidin remains active in pH 3-6 (Arcus, 1959). For actin and myosin in meat proteins, the optimal pH range has been reported to be 3-4.5 and 3-8, respectively (Nishiyama, 2007).

Overall, actinidin was chosen as the study target, because of the agreement of its optimal pH (5-7) with normal meat pH (5.5), less intensive tenderising ability, and high tenderisation specificity towards myofibrillar proteins and collagen.

#### **2.4.4. Applications of actinidin in food processing**

Actinidin has been proposed and studied for a range of commercial applications, including milk clotting (Zhang, Sun, Liu, Li, & Jiang, 2017), cheese making (Katsaros, Tavantzis, & Taoukis, 2010) and meat tenderisation.

The use of actinidin or kiwifruit-based solutions in meat tenderisation has been confirmed by some studies (Christensen *et al.*, 2009; Lewis & Luh, 1988; Liu, Xiong, & Rentfrow, 2011; Toohey *et al.*, 2011). Lewis and Luh (1988) reported that actinidin with an activity of 400 U/mL resulted in an equivalent sensory tenderness of beef (*semitendinosus*) to that produced by a papain-containing meat tenderiser (18 U/mL), and both of the actinidin and papain tenderised steaks were more tender than untreated meat. Marination and injection with actinidin increased both the instrumental and sensory

tenderness of porcine *M. biceps femoris*, and affected connective tissue and myofibrillar particle size (Christensen *et al.*, 2009). The effects of a kiwifruit solution on tenderness and colour stability of injected bovine muscles (*M. semimembranosus*) have been reported by Toohey *et al.* (2011). The tenderness decrease of freeze (-29 °C)-thaw (4 °C) abused pork loin (*Longissimus lumborum*) muscle due to the injection of kiwifruit juice compared with no-injection control has also been reported by Liu *et al.* (2011), with substantial degradation of myosin shown in the SDS-PAGE.

However, the effects of actinidin on tenderness and *in vitro* digestibility of beef brisket (deep pectoral or superficial pectoral) have not been reported.

#### **2.4.5. The effect of kiwifruit on digestion**

Consumption of kiwifruit has been known to assist gastric digestion, due to the action of actinidin hydrolysing food proteins. Rutherford-Markwick and Moughan (2005) claimed that compared to using pepsin and pancreatin alone, actinidin in concert with pepsin and other digestive enzymes can lead to a different array of peptides in both gastric and small intestinal digestion. Some of the peptides potentially have biological activity.

Results of both *in vitro* (Kaur *et al.*, 2010a, 2010b) and *in vivo* (rat and pig) (Rutherford *et al.*, 2011) studies showed that the consumption of green kiwifruit along with protein-rich foods increases the rate and extent of protein digestion, especially gastric digestion (although the gastric environment has previously been considered too severe for the enzyme to be active). This is useful particularly for elderly with compromised digestibility. The protein sources studied included meat protein (beef and

chicken), fish proteins and dairy proteins (Na-caseinate, yoghurt and cottage cheese) (Kaur *et al.*, 2010a, 2010b). Actinidin has been reported to improve the stomach emptying rate of beef muscle protein in rats and pigs (Rutherford *et al.*, 2011), and therefore potentially reduce feelings of over-fullness. There are some digestive enhancers and bowel health supplements derived from green kiwifruit that are commercially available, such as Phloe™ and Kiwi Crush™ (Vital Food Processors Ltd., NZ) (Kaur & Boland, 2013).

## **2.5. Factors affecting kiwifruit enzyme activity**

Enzyme-tenderised meat usually has a tendency to be over-tenderised, displaying mushy texture, undesired appearance and palatability (Bekhit *et al.*, 2014). This is likely to be due to the high intensity hydrolysing ability of commonly used enzymes, and sometimes a relatively long storage time. Although actinidin has a moderate proteolytic activity compared with traditional proteases papain and bromelain, factors and methods still need to be considered to inactivate the residual enzyme to avoid over-tenderisation of meat.

### **2.5.1. Temperature**

It is well known that enzymes are proteins and could be denatured irreversibly (i.e. conformational changes entailing a loss of biological activity) at temperatures higher than those to which they are normally exposed to in their natural environment. The enzyme activity of actinidin has been reported to be highest at 45 °C and lowest at around 5 °C

(Ha *et al.*, 2013). Higher temperature (more than 45 °C) is presumed to adequately inactivate actinidin. The thermal inactivation kinetics of actinidin in kiwifruit juice at 40-55 °C have been studied by Katsaros, Katapodis, and Taoukis (2009b). But there is no information available in the literature on the thermal inactivation of actinidin after it is applied to meat.

### **2.5.2. pH of enzyme solution**

The pH of solution could influence the activity and alter the structure of enzymes. pH can impact the ionisation state of acidic (with carboxyl functional groups) or basic amino acid residues (with amine functional groups). The alterations of ionisation state change the ionic bonds and result in alterations of the tertiary structure of the protein, which can lead to a change of protein function or inactivation of the enzyme. Research has shown that papain and bromelain thoroughly hydrolysed all myofibrillar proteins (including actin and myosin heavy chain) at pH 2-8. In contrast, actinidin non-selectively hydrolysed all myofibrillar proteins at pH 3-4, however in the range of pH 5.5-8, it selectively degraded myosin heavy chain rather than actin (Nishiyama, 2001). This implied the potential use of pH to control the meat tenderisation level caused by actinidin. However, in order to inactivate the exogenous enzymes to avoid over-tenderisation, usually an extremely high or low pH is required, which could also adversely affect the flavour, colour and texture of meat (Andrés-Bello, Barreto-Palacios, García-Segovia, Mir-Bel, & Martínez-Monzó, 2013). These changes caused by pH are complex and dependent on the pH values and muscle varieties.

### **2.5.3. High pressure**

It has been reported that high hydrostatic pressure (HHP) processing has the ability to inactivate enzymes and microorganisms, while preserving the flavour, colour and nutritional contents of foods (Katsaros *et al.*, 2009b). However, a recent study has reported that HHP at 600 MPa for 10 min modified the colour and texture of meat and made it as cooked (Kaur *et al.*, 2016). The mechanism of protein denaturation by HHP is referred to as the *Le Chatelier* principle, which presumes that application of pressure shifts an equilibrium to the state that has the smallest volume, so any reaction accompanied by volume decrease is accelerated by increased pressures (Cano, Hernandez, & Ancos, 1997). High pressure alters the enzyme hydrogen bonds and 3-D structure. The effect of HHP and temperature on enzyme inactivation of papain and ficin has been studied (Katsaros, Katapodis, & Taoukis, 2009a), and results showed a significant resistance of both enzymes against pressure and temperature. Adequate inactivation required pressure and temperature higher than 750 MPa and 70 °C at the same time (Katsaros *et al.*, 2009a). Actinidin appears to be more significantly sensitive to high pressure in concert with thermal processing. But some antagonistic effects of HHP with thermal processing (40, 45 and 50 °C) on actinidin have been reported by Katsaros *et al.* (2009b).

### **2.5.4. Salt solutions**

Salt ions (Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>) generate different influences on proteins. Salt ions can



increase or decrease protein solubility, depending on the amount of salt addition and the type of protein. At the optimal pH of 6, actinidin is covered by large negative charges on the surface, the enzyme activity and structure of which is considered to be influenced by the high concentration of salt (Morimoto, Furuta, Hashimoto, & Inouye, 2006). Research showed that 0.5 M salt (NaCl, KCl and LiCl) solution led to minimal actinidin activity due to the increase in the electrostatic interaction between the substrate and enzyme, however higher concentrations (0.5-3 M) of salt (NaCl and KCl) re-activated enzyme due to the conformational changes of tryptophan residues which was related with reactivation (Morimoto *et al.*, 2006).

### **2.5.5. Inhibitors**

The application of inhibitors to reduce or inactivate the plant-derived enzymes has been studied with some promising results. Inhibitors of actinidin (**Table 4**), papain, bromelain, ficin and zingibain are summarised by Bekhit *et al.* (2014). The inhibiting effects of both NaCl and KCl on actinidin activity have been discussed in section 2.5.5. Funaki, Yano, Arai, and Abe (1995) have reported that oryzacystatin from rice seed can inhibit actinidin to make gelatin jellies using fresh kiwifruit, or could be used to help to solve problems associated with actinidin as a food ingredient for protein-based foods, such as meat. It has also been reported by Sugiyama, Ohtsuki, Sato, and Kawabata (1996) that the enzyme activity of all 6 kiwifruit proteases (including actinidin) can be inhibited by metal ions (e.g. 2 mM Zn<sup>2+</sup> and Cu<sup>2+</sup>) to various extents.

**Table 4.** Actinidin inhibitors

Inhibitors	Inactivation Extent	Reference
KCl	Partial	Morimoto <i>et al.</i> (2006)
NaCl	Partial	Auerswald <i>et al.</i> (1996)
Cystatin (Chicken egg white)	Complete	Bjoerk and Ylinenjaervi (1990)
Oryzacystatin (Rice seed)	Complete	Funaki <i>et al.</i> (1995)
Zn <sup>2+</sup> , Cu <sup>2+</sup>	Partial	Sugiyama <i>et al.</i> (1996) Lindahl, Abrahamson, and Björk (1992)
Hg <sup>2+</sup> , Cd <sup>2+</sup>	Complete	Sugiyama <i>et al.</i> (1996)

Overall, the use of high pressure processing to inactivate actinidin will lead to an increase in the total production cost but it is not worth exploring because of its adverse effects on sensory and textural attributes of meat as described above. The adjustment of pH may lead to complex changes (Andrés-Bello *et al.*, 2013). Salt solutions could inhibit enzyme activity to a certain extent but it may not be possible to achieve complete enzyme inactivation (Morimoto *et al.*, 2006). Moreover, the addition of salt solutions will affect the flavour and potentially have adverse health effects. Most of the inhibitors are metal ions that are not permitted for use in edible food products – most are toxic. Compared to these treatments, the use of heat treatment (under *sous vide* conditions) for enzyme inactivation seems safer and more economic.

## **2.6. Evaluation of enzyme-tenderised meat**

### **2.6.1. Enzyme activity measurements**

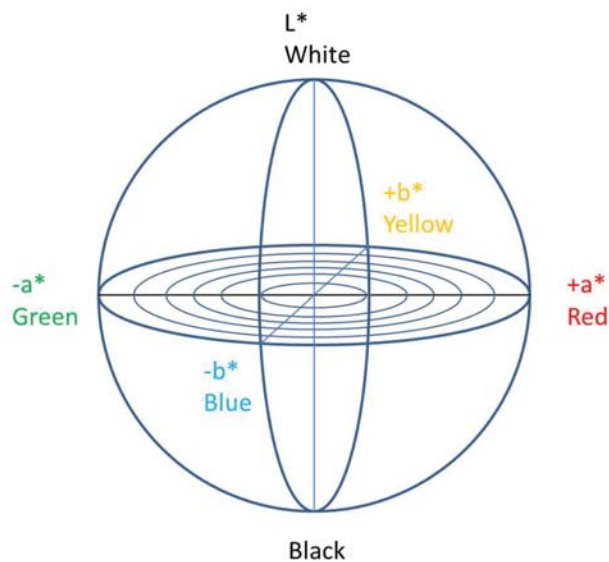
There are various methods that have been used to measure the proteolytic activity, such as spectrophotometric, chromatographic, fluorimetric, colourimetric, radiometric, solid phase and voltammetric methods (Baş & Hakkı Boyacı, 2010; Sarath, De La Motte, & Wagner, 1989). Various methods have been reported using different substrates, such as casein, gelatin, haemoglobin and synthetic amino acid substrates. Synthetic substrates containing amino acid (e.g. CBZ-Lys-p-nitrophenol) and caseins in different forms (e.g. native, denatured or labelled) are most commonly used to determine esterase and proteolytic activity of various enzymes (Boland, 1973; Kholif *et al.*, 2011; Ladd & Snow, 1993; Wretlind & Wadström, 1977). Elastase activity is quantified using labelled elastin (Wretlind & Wadström, 1977) while collagenase activity is determined by collagen discs as substrate (Smyth & Arbuthnott, 1974). All the above methods do not reflect the actual activity of the enzyme when it is applied to meat, since the more complex protein structure of meat needs to be considered (Bekhit *et al.*, 2014). In this project, the method of Boland (1973) was chosen to measure the specific enzyme activity of kiwifruit enzyme, since the kinetics of the enzyme actinidin have been systematically studied in his research.

### **2.6.2. Colour measurements and the CIELAB system**

Meat colour is dependent on the balance between metmyoglobin reduction and

oxymyoglobin oxidation. Commission Internationale de l'Eclairage (CIE) values (known as  $L^*a^*b^*$ ) are typically used for meat products (Warriss, 2001).

The CIELAB system has a sphere shape that closely describes the visual uniformity by equalling the distance in the system with that received by human visualisation. Any set of  $L^*a^*b^*$  values can exactly describe the colour as a point in the 3-D colour sphere. The  $L^*$  value represents lightness;  $a^*$  and  $b^*$  are chromaticity coordinates, which separately measure red-greenness and yellow-blueness (**Figure 3**) (Warriss, 2001). CIELAB has several specific illuminants, and illuminant D-65 is equivalent to natural light and contains part of the ultraviolet. The human visual colour is also influenced by the angle of view. Usually,  $2^\circ$  and  $10^\circ$  angles are used in the CIELAB system since the angle of view field slightly affects the colour.



**Figure 3.** The CIELAB colour space showing the  $L^*$ ,  $a^*$  and  $b^*$  coordinates (Warriss, 2001)

The papain-treated meat available in the market usually shows darker colour than untreated meat, indicating a preponderance of myoglobin rather than oxymyoglobin or metmyoglobin (Hutchins, Liu, & Watts, 1967). Meat injected with the kiwifruit-based solution has been reported to be less dark and less red than non-injected meat (Toohey *et al.*, 2011). However, the information on the effects of actinidin on the colour of cooked meat is lacking in the literature.

### **2.6.3. Texture analysis**

The common method to measure tenderness is the Warner-Bratzler shear force test (WBSF), which is originally associated with the American workers K.F. Warner and L.J. Bratzler in the early 1930s. Cylindrical meat samples with about 100 mm<sup>2</sup> cross-section area are removed by a cork-borer device with the grain of the muscle from the meat steaks. Then the samples are sheared by an inverted V-shaped blade, which mimics the gap between teeth. The force required to push the blade and cut through the meat sample is used to calculate the shearing force of the meat (Bratzler, 1949).

A modified WBSF has been developed by Shackelford, Wheeler, and Koohmaraie (1999) with a flat blade instead of a V-shaped blade, which is known as slice shear force (SSF). Cooked meat samples were prepared as slices (1 cm thickness × 5 cm length) cut parallel to the grain direction and are sheared perpendicularly the direction of muscle fibres by the blade. This method has potential benefits such as higher accuracy and easier and quicker application than the original WBSF (Warriss, 2001). The shear force of raw beef steaks ranges from 30-70 N (WBSF) and approx. 230 N (SSF) (Calkins & Sullivan,

2007). The average shear force of cooked beef steaks is 35 N (WBSF) and 160 N (SSF), respectively (Shackelford *et al.*, 1999). If the yield force is over 100 N (WBSF), the meat piece would be considered rather tough (Warriss, 2001).

It has been reported that the kiwifruit juice-injected cooked beef (*M. semimembranosus*, topside, 1-day post-mortem) has a shear force of 36.5 N (WBSF) while the water-injected control is 46.9 N (Toohey *et al.*, 2011). Information on the tenderness of raw or cooked actinidin-treated beef brisket is not available in the current literature.

#### **2.6.4. Sensory assessment**

Although the instrumental assessment could approximately measure particular characteristics of the eating quality, no machine could assess the eating quality and palatability, which are contributed to by interacting characteristics. Consumers perceive the quality of meat from its tenderness, juiciness, flavour and odour at the same time, rather than isolated attributes (Warriss, 2001).

There are several sorts of panel and sensory tests. A trained panel typically consists of eight to ten trained individuals tasting prepared samples under controlled conditions of environment, sample preparation and assessment. Consumer panels, compared with trained panels, usually have less controlled conditions and need a large number of individuals (more than 100). The selected members of the public generate more variations due to their own preparation and cooking methods and are highly influenced by third parties such as the family members. The “Hall” test, in which the samples are prepared

by researchers and offered to consumers in a public place, is a form developed to overcome some of those variables in traditional consumer panels (Warriss, 2001).

Taste panel methods mainly include discrimination tests, category scaling, ranking tests and sensory profiling. More methods are available in the literature (Nute, 1996; Nute, 1999; Piggott, Simpson, & Williams, 1998). Normally, the sensory evaluation of meat products includes several attributes such as tenderness, hardness, flavour (metal flavour and warm-over flavour), juiciness, threadiness and crumbliness (Christensen *et al.*, 2009).

The effects of freezing and chilling on sensory evaluation of beef *M. longissimus dorsi* has been studied by Lagerstedt *et al.* (2008) by a semi-trained sensory panel and a consumer panel. Research of Christensen *et al.* (2009) showed that injection of kiwifruit enzyme resulted in a decrease in WBSF, an increase in sensory tenderness and had no impact on juiciness and flavour of cooked pork *M. biceps*. It has also been reported that actinidin solution-incubated bovine *semitendinosus* (*ST*) steaks were more tender than untreated sample evaluated by sensory panel (Lewis & Luh, 1988).

In this study, a semi-trained panel was chosen to focus on the difference of tenderness, flavour and juiciness, which were the most important attributes of consuming meat products.

### **2.6.5. Protein digestibility assessment**

There are two methods used to access the digestion of foods: *in vivo* and *in vitro* systems.

*In vivo* systems usually use human or animals (e.g. rats and pigs) to provide the most

accurate results of food digestibility due to the comprehensive digestion systems and their interactions. However, this method is restricted due to ethical constraints and huge experimental cost, and is time-consuming. Moreover, due to high costs, only limited numbers of individuals could be observed. Therefore, a large variability of mastication and digestion could possibly be achieved, which leads to potential interference in analysing results (Astruc, 2014).

*In vitro* systems, compared with *in vivo* systems, are the most effective method to collect data and can be used in the laboratory without specific constraints. They can achieve and separately simulate the different stages of digestion by using artificial systems or settings (Astruc, 2014). There are artificial masticators (Nieto, Díaz, Bañón, & Garrido, 2010; Salles *et al.*, 2007) and separated artificial gastric and small intestinal digestion systems (Kaur *et al.*, 2010a, 2010b). The applications of different *in vitro* digestion systems on simulating human digestions have been summarised by Hur, Lim, Decker, and McClements (2011). However, some phenomena of compensation and adaptations of digestion in response to external stimuli are hard to reproduce in *in vitro* systems (Astruc, 2014).

The gastro-small intestinal digestion (*in vitro*) of bovine meat proteins with the addition of actinidin has been described by Kaur *et al.* (2010a) and Kaur *et al.* (2010b), as mentioned in section 2.4.5. It was presumed that during the simulated gastric digestion, actinidin in concert with pepsin may lead to a different array of peptides (potentially bioactive peptides) compared to the action of pepsin in the absence of actinidin (Kaur *et al.*, 2010a, 2010b; Rutherford-Markwick & Moughan, 2005). Studies of (Kaur *et al.*,



2010b) reported that kiwifruit extract had more positive effects on beef protein digestibility during simulated gastric digestion than in intestinal digestion. However, no information on the *in vitro* gastric protein digestibility of enzyme-tenderised *sous vide* cooked meat cuts is available in the literature.

## **2.7. Importance and purpose of this study**

To systematically study the effects of kiwifruit enzyme treatment on the tenderisation and digestibility of a low-value cut, beef brisket, the study was divided into two parts.

Part I examined the inactivation kinetics of kiwifruit enzyme by either high pressure or thermal treatment. The objective of this part was to find a the most suitable inactivation method to control the enzyme activity in order to avoid over-tenderisation of beef brisket muscles.

Part II was focused on the application of the kiwifruit enzyme (actinidin) on beef brisket steaks and further consisted of two stages. In the first stage, trials of various methods of enzyme application (marinating and injection), temperatures and time of *sous vide* cooking, enzyme concentrations, injection amounts (% weight increase compared with fresh meat weight), numbers of injection sites, size of needles and tumbling or without tumbling were tested to determine the optimal enzyme treatment conditions.

The second stage included the assessment of the final kiwifruit enzyme-pre-treated and *sous vide* cooked beef brisket steaks. It was hypothesised that enzyme treatment in combination with *sous vide* cooking would achieve desirable tenderness in significantly shorter cooking times, along with improving the rate and extent of protein digestibility

under gastric conditions. The actinidin-tenderised *sous vide* cooked steaks were compared with a control water-injected *sous vide* cooked brisket sample for basic attributes, including physical appearance, pH, colour, cook loss (%), and collagen solubility (% soluble collagen in the cook loss). The effects of actinidin on meat tenderness (instrumental and sensory), microstructure (transmission electron microscopy) and *in vitro* protein digestibility (using protein solubility, SDS-PAGE and ninhydrin-reactive free amino N release) under simulated gastric conditions were evaluated.

This study could help to solve the over-tenderisation problems from kiwifruit enzyme (actinidin) or to be referred to in the use of other cysteine proteases.

# Chapter 3. Materials and Methods

## 3.1. Materials

Whole bovine brisket muscles (deep and superficial pectoral) were excised (hot bone) from both sides of 19-month old Dairy Beef (Hereford sires with unknown dairy dams, mix of Friesian and cross breed). The pre-rigor muscles were stored at 15 °C until rigor. The whole piece of brisket was vacuum packed immediately, labelled and frozen at -18 °C (blast freezer). The whole procedure was carried out at the ANZCO plant at Eltham (NZ). Before each processing and measurement, the frozen meat was thawed at 4 °C for 16 h (overnight). In total, beef briskets from 8 carcasses were used in this study.

The green fresh kiwifruit (*Actinidia deliciosa* cv. Hayward) used in this study were purchased from a local supermarket and kept at 4 °C until extraction was carried out. Food grade commercial green kiwifruit enzyme (actinidin) extract (CEE, Actazin™) powder was kindly supplied by Anagenix Ltd. (Auckland, NZ).

All the chemicals and reagents used in the study were of analytical grade.

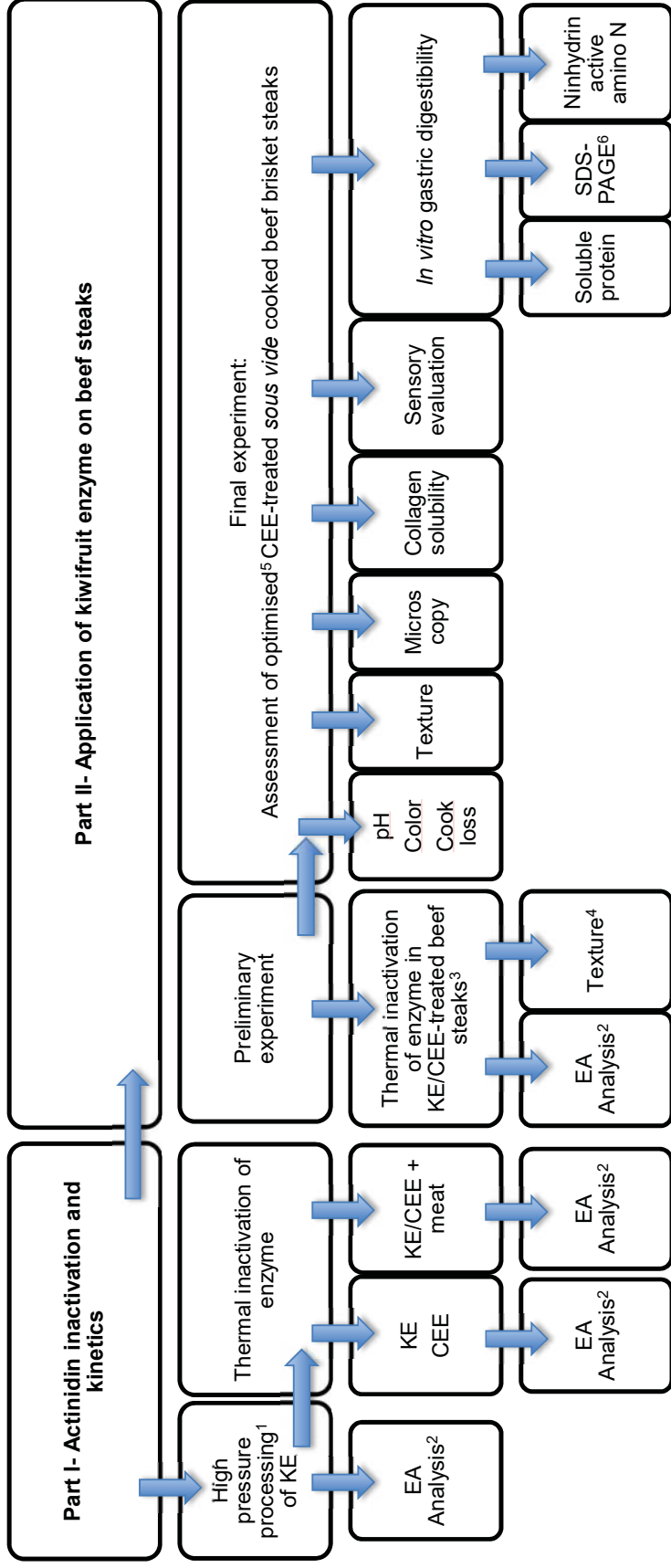
## 3.2. Methods

The study design is given in **Figure 4**. The study was divided into two parts:

Part I. To study the inactivation kinetics of actinidin (KE and CEE) by either high pressure or thermal treatment.

Part II. Application of the actinidin (CEE) on beef brisket steaks and characterisation

of the steaks for textural attributes and protein digestibility *in vitro*.



**Figure 4.** Study design

KE is kiwifruit extract. CEE is commercial kiwifruit enzyme extract. EA is enzyme activity. (1) 20-600 MPa, 5-30 min. (2) Measure the enzyme activity to confirm the complete inactivation of actinidin (EA = 0). (3) Find the balance between enzyme concentration, injection amount, cooking temperature ( $\geq 60$  °C due to microbial reason) and cooking time to achieve lowest processing cost, suitable tenderness and the most appealing appearance. (4) Measure the shear force to confirm the significant decrease of hardness. (5) The optimised parameters included using CEE at a concentration of 3 mg/mL, total injection amount of 5 %, post-injection vacuum tumbling at 4 °C for 15 min and cooking at 70 °C for 30 min. (6) Measure protein breakdown.

### **3.2.1. Part I- Kiwifruit enzyme inactivation**

#### **3.2.1.1. Fresh kiwifruit extract (KE) preparation**

Green kiwifruit were peeled, pureed and filtered through a muslin cloth as described by Kaur *et al.* (2010a). A small amount (18 mL/100 g filtrate) of ice-cold sodium phosphate buffer (0.05 M, pH 6.0) was added to the pulp followed by mixing and centrifugation (Thermo Scientific, USA) at  $13100 \times g$ , at 0 °C for 10 min. The supernatant was collected as the kiwifruit extract (KE) and was immediately stored at 4 °C until further use. Previous experiments have shown that the enzyme activity of KE remained stable at 4 °C for 6 h, therefore fresh KE was used within 6 h of storage at 4 °C.

#### **3.2.1.2. Commercial kiwifruit enzyme (CEE) extract preparation**

The commercial kiwifruit enzyme powder was dissolved in ice-cold phosphate buffer (0.05 M, pH 6.0) to make into a concentration at 50 mg/mL. This concentration was chosen because a higher concentration ( $> 50$  mg/mL) resulted in the formation of a gel, which was not suitable for the injection processing. To better understand the correlation between protein content and enzyme activity of CEE, the protein content was measured as described in section 3.2.1.3.

#### **3.2.1.3. Protein content estimation of CEE**

The protein content of the CEE powder was determined by Hartree-Lowry Assay (Hartree, 1972). This method is based on the principle that under alkaline conditions the

divalent copper ion and radical groups of cysteine, tryptophan and tyrosine react with Folin reagent to produce molybdenum/tungsten blue. Three reagents were prepared before the assay. Reagent A was prepared by dissolving 2 g of potassium sodium tartrate and 100 g of sodium carbonate in 500 mL of sodium hydroxide (1 N), and diluting with Milli-Q water (Thermo Scientific, USA) to 1 L. Reagent B consisted of 2 g of potassium sodium tartrate and 1 g of copper sulphate diluted in 90 mL of Milli-Q water and 10 mL of sodium hydroxide (1 N). Reagent C was 1 vol Folin-Ciocalteu diluted with 15 vol Milli-Q water.

CEE powder was dissolved in phosphate buffer (0.05 M, pH 6.0) at the ratio of 1:50. A series (0.06 to 0.3 mg/mL) of standard bovine serum albumin fraction V (BSA, Invitrogen Corporation, Auckland, NZ) solutions were prepared in sodium phosphate buffer (0.05 M). One (1) mL of each standard solution, phosphate buffer (used as blank) or CEE solution was added into 0.9 mL of reagent A in separate test tubes and mixed well. The tubes were kept in a water bath at 50 °C for 10 min and then cooled to room temperature (20 °C), before adding 0.1 mL of reagent B in each tube. The tubes were then mixed well and incubated at room temperature for 10 min. Three (3) mL of reagent C was added to each test tube, mixed well and incubated at 50 °C for 10 min, then cooled to room temperature. The final volume in each test tube was 5 mL. Then 1 mL of solution was taken from each test tube in a 2 mL cuvette and measured for the absorbance at 650 nm, using a spectrophotometer (Shimadzu, Japan).

The standard curve ( $R^2 = 0.994$ ) was plotted with absorbance on the y-axis and BSA content (0.06 to 0.3 mg/mL) on the x-axis. All the chemicals and reagents used in this

study were purchased from Sigma-Aldrich Pty Ltd (USA), except where specified.

#### **3.2.1.4. Measurement of enzyme (actinidin) activity**

The enzyme activity (EA) was measured by the method of Boland (Boland, 1973; Kaur *et al.*, 2010a). The actinidin substrate (1.2 g/L) consisted of 6 mg of substrate N- $\alpha$ -carbobenzoxy-L-lysine P-nitrophenyl ester hydrochloride (Z-L-Lys-ONp hydrochloride, CBZ, Sigma-Aldrich Pty Ltd., USA) dissolved in 5 mL of Milli-Q water. The enzyme activator, DL-Dithiothreitol (DTT, Sigma-Aldrich Pty Ltd., USA) was dissolved in Milli-Q water at a concentration of 15.3 g/L. One hundred (100)  $\mu$ L of DTT and 100  $\mu$ L of sample (diluted with buffer solution if the enzyme activity was too fast) were mixed well 3 min before the measurement, to let the enzyme in the sample become fully activated. A 4-mL cuvette with 2.85 mL of phosphate buffer (0.05 M) was put in a Shimadzu 160A UV-Visible Recording Spectrophotometer (Tokyo, Japan). One hundred (100)  $\mu$ L of CBZ was directly added to the buffer in the cuvette. After 20 s spontaneous hydrolysis of Z-L-Lys-ONp, fifty (50)  $\mu$ L of mixture (DTT and sample) were added. The changing absorbance was measured at 348 nm and recorded every 2 s over 100 s. Each sample was measured three times. Enzyme activity (U/mL) was calculated using the initial absorbance changing rate (max hydrolysis rate) with  $\Delta\epsilon = 5400 \text{ M}^{-1}\text{cm}^{-1}$  (Boland & Hardman, 1972). One unit is defined as the amount of enzyme producing 1  $\mu$ mol of product per minute (Boyes *et al.*, 1997). The enzyme activity was expressed as enzyme activity (Units)/kiwifruit (mL or g).



### **3.2.1.5. High pressure treatment of KE**

Freshly prepared KE was used for this experiment. 10 mL of KE was added into a 10 mL polypropylene tube before placing in the high pressure processing machine S-FL-085-9-W/FPG5740 (Stansted Fluid Power Ltd., Harlow, UK). As mentioned in section 2.5.3, high pressure at 600 MPa for 10 min could alter the surface colour of meat as being cooked. Treatments of short time at high pressure and long time at low pressure were tested. The samples were treated at different pressures- 20 MPa, 50 MPa, 100 MPa, 200 MPa, 400 MPa, and 600 MPa for 30 min, 20 min, 15 min, 10 min, 10 min and 5 min, respectively at 25 °C. The pressure was built up at the rate of 10 MPa/s and reviewed every 15 s. After processing, the samples were immediately stored at 4 °C for enzyme activity analysis as described in section 3.2.1.4. A KE sample without high-pressure treatment was treated as the control.

### **3.2.1.6. Thermal inactivation of actinidin in KE and CEE**

Aliquots (3 mL) of KE were added into 15-mL plastic test tubes and heated in water baths at various temperatures ranging from 35 °C to 65 °C for 1 min-24 h (**Table 5**) until complete enzyme (actinidin) inactivation. The heated samples were cooled in an ice bath. After cooling, they were centrifuged at  $13100 \times g$ , at 0 °C for 10 min and the supernatants were stored at 4 °C until analysed for residual enzyme activity as described in section 3.2.1.4. For each temperature-time combination, at least 3 replications were performed.

**Table 5.** Temperatures and times used to study thermal inactivation of actinidin in kiwifruit extract (KE)

Temperature (°C)	Time (min)												
65	1	3											
60	1	3											
55	1	3	5	10									
50	1	3	5	10	20								
45	1	3	5	10	20	30	60						
40	1	3	5	10	20	30	60	120	240	540			
35	1	3	5	10	20	30	60	120	240	540	840	1440	

Replacing the KE, CEE solution (50 mg/mL) was treated using the same processing conditions except that it was only heated for the inactivation temperature-time combinations obtained from the results of KE inactivation, to confirm and compare the thermal enzyme inactivation profiles for KE and CEE.

### **3.2.1.7. Thermal inactivation of actinidin in KE or CEE combined with homogenised meat**

Thawed (4 °C for 16 h) beef brisket was homogenised (in an ice bath) using a blender (Breville Mini Wizz, NZ) for 10 s. The KE was then added to the homogenised meat at a ratio of 5: 100 (KE in mL: homogenised meat in g) and mixed well. Approx. 10 g of mixtures were placed in plastic test tubes (50 mL capacity) and heated in water baths at 60 °C, 65 °C and 70 °C for 0.5-24 h (**Table 6**) until complete enzyme inactivation was achieved. Heating was followed by cooling in an ice bath. The cooled samples were centrifuged at 13100 × g, at 0 °C for 10 min and the supernatants were stored at 4 °C until

analysed for residual enzyme activity as described in section 3.2.1.4. For each temperature-time combination, at least 3 replications were performed.

**Table 6.** Temperatures and times used to study thermal inactivation of actinidin in KE-meat mixtures

Temperature (°C)		Time (h)							
70		0.5	1	2	3				
65		-	-	-	3	6	12	18	24
60		-	-	-	3	6	12	18	24

- No sample was tested for this time point.

Replacing the KE, CEE solution (50 mg/mL) was treated using the same processing conditions except that it was only heated for the inactivation temperature-time combinations obtained from the results of KE-meat mixtures inactivation, to confirm and compare the thermal enzyme inactivation profiles for KE-meat mixtures and CEE-meat mixtures.

### 3.2.1.8. Inactivation kinetics and thermodynamic analysis

Enzyme inactivation kinetics is normally described as a first-order reaction (Eq. 1) (Gouzi, Depagne, & Coradin, 2011; Sadana, 1988):

$$\frac{EA}{EA_0} = \exp(-k_T \cdot t) \quad (1)$$

where  $EA$  is the enzyme activity at time  $t$ , U/mL;  $EA_0$  is the initial enzyme activity of KE without processing ( $t = 0$ ), U/mL;  $t$  is the processing time, min; and  $k_T$  is the inactivation rate constant,  $\text{min}^{-1}$ . To calculate the inactivation rate constant ( $k_T$ -values), this equation

could be rearranged as the following equation (Eq. 2):

$$\ln\left(\frac{EA}{EA_0}\right) = -k_T \cdot t \quad (2)$$

where the  $k_T$ , standard deviation (SD) and correlation coefficient ( $R^2$ ) can be estimated by linear regression analysis.

The half-life value ( $t_{1/2}$ ) of inactivation is expressed as follows (Eq. 3),

$$t_{\frac{1}{2}} = \frac{\ln 2}{k_T} \quad (3)$$

Arrhenius' equation (Eq. 4),

$$k_T = Ae^{-\frac{E_a}{RT}} \quad (4)$$

where  $T$  is the absolute temperature, K;  $A$  is the pre-exponential factor;  $E_a$  is the activation energy for the reaction, J/mol; and  $R$  is the universal gas constant, 8.314 J/(mol·K), gives the relationship of the rate constant ( $k_T$ ) of a chemical reaction on the absolute temperature, a pre-exponential factor ( $A$ ) and other constants (i.e.  $E_a$  and  $R$ ) of the reaction. The effects of temperature on the inactivation rate is described adequately by the rearranged Arrhenius' equation (Eq. 5),

$$\ln k_T = \frac{-E_a}{R} \left(\frac{1}{T}\right) + \ln A \quad (5)$$

The values of activation energy  $E_a$  can be used to determine thermodynamic parameters, i.e. entropy ( $\Delta S$ ), enthalpy ( $\Delta H$ ) and Gibbs free energy ( $\Delta G$ ) for the reaction. A linear dependence according to Eq. 5 is indicative of a single rate-limiting process for enzyme inactivation.

## **3.2.2. Part II- Application of kiwifruit enzyme on beef steaks**

### **3.2.2.1. Preliminary experimentation**

#### *3.2.2.1.1. Thermal inactivation of enzyme extract-treated beef brisket steaks*

Thawed beef briskets were cut into fixed size (length 7 cm × width 5 cm × thickness 3 cm, approximately 100 g/piece). The beef steaks were manually injected (or marinated) using with enzyme extract (KE or CEE) at different concentrations (only for CEE)- 0.5 mg/mL, 1 mg/mL, 2.5 mg/mL, 3 mg/mL, 3.75 mg/mL, 5 mg/mL, 10 mg/mL and 50 mg/mL. Different extract usage amounts ranging from 5 %, 7.5 %, 10 % to 12.5 % (% weight increase after injection/original meat weight) were tested. Injections were done perpendicularly to the direction of muscle fibre at various sites about 1 cm apart using a 5 mL syringe and needle, followed by tumbling of 30 min under vacuum conditions at 5 rpm, and at 4 °C. Meat samples were then vacuum packaged (Multivac, Germany), and immersed in the water bath at various temperatures for different times. Control meat steaks were injected with water instead of enzyme extract and were prepared in the same way.

The residual enzyme activity (after homogenisation) and texture of the samples were measured, respectively (as described in sections 3.2.1.4 and 3.2.2.1.2, respectively), after cooling to room temperature (20 °C) in an ice bath.

#### *3.2.2.1.2 Texture measurements*

Any visible fat was removed from the processed or unprocessed muscle samples.

Samples were then cut into rectangular cross-sections of about 4 cm × 1 cm × 1 cm (length × width × height). The maximum slice shear force (SSF) was measured as the hardness, with a texture analyser TA.XT.plus (Stable Micro Systems, UK) by modifying the method of Shackelford *et al.* (1999). Calibration of the analyser was done before each batch of tests. The tested meat sample, a minimum of 6 pieces from the same processed sample (6 measurements), was placed under a flat blade and sheared perpendicularly to the direction of muscle fibres. Samples were sheared using a 50 kg load cell at 1 mm/s pre-test speed and 1.6 mm/sec test speed with trigger force of 0.05 N.

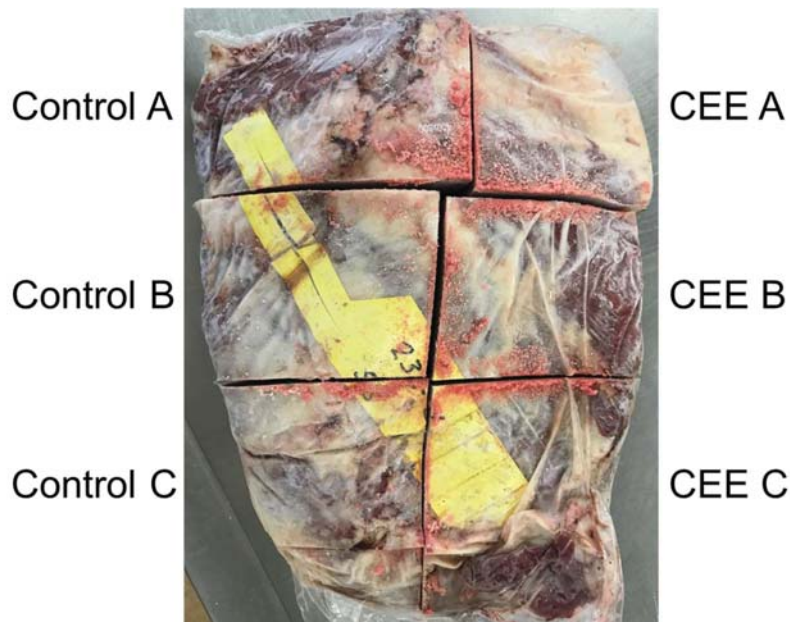
### **3.2.2.2 Assessment of the final actinidin (CEE)-tenderised *sous vide* cooked beef brisket steaks**

#### *3.2.2.2.1. Beef brisket*

The whole piece of frozen beef brisket (including deep and superficial pectoral) was cut into 6 pieces as shown in **Figure 5**. To eliminate the internal variables among different pieces of brisket, one half was treated with water as control and the other half was treated with CEE solution (actinidin). Each half was allocated as three replicates A, B and C. Each piece was trimmed and cut into a steak with fixed weight and size (approx. 200 g/steak, 10 cm length × 7 cm width × 3 cm height) after the removal of any surface fat and connective tissue. It was presumed that steaks from one half had similar attributes to those of the steaks from the counterpart opposite half, and that they were comparable.

Two briskets (a pair from the same carcass) were used in the collagen solubility analysis. Eight briskets (4 pairs from 4 carcasses) were used in the sensory analysis. One

brisket was used for color, pH, cook loss, texture and microstructure analysis, while the counterpart brisket (from same carcass) was used for analysis of *in vitro* protein digestibility.



**Figure 5.** Whole piece of beef brisket showing the allocation of control and treated samples

The left half was treated with water as control sample, the right half was treated with CEE solution (actinidin). A, B and C represent the replicates for control or treated samples.

#### 3.2.2.2.2. *Treatment with CEE and sous vide cooking*

CEE-treated meat steaks (CEE A, B and C, as shown in **Figure 5**) were injected with CEE at a concentration of 3 mg/mL (as selected during preliminary experimentation, based on visual observations and texture measurements) at a ratio of 5:100 (CEE: meat, w/w), followed by 15 min of tumbling (Sunhow Tumbler, China) under vacuum conditions at 4 °C (as determined by preliminary experimentation). Steaks were then

vacuum packaged, labeled and cooked under *sous vide* conditions at 70 °C for 30 min, followed by immediate cooling to room temperature (20 °C). Control steaks (Control A, B and C, as shown in **Figure 5**) were given the same treatment but replacing CEE solution with Milli-Q water.

#### 3.2.2.2.3. *Colour measurements*

The colour of meat samples (obtained from section 3.2.2.2.2) was measured by a Minolta Chroma Meter CR-200 (Chemiplas NZ Ltd., AU) equipped with a standard illuminant D65 using a 10 ° position of the standard observer at  $L^*a^*b$  colour space. Each sample was thick enough to be opaque (10-15 mm) and measured on a minimum of 6 different locations at the surface of each sample (Hunt *et al.*, 1991; Hunt *et al.*, 2012).

#### 3.2.2.2.4. *pH measurements*

Ten (10) g of the meat samples (obtained from section 3.2.2.2.2) were homogenised (Heidolph FIAx 600, Germany) with an addition of 100 mL of Milli-Q water. After 30 s blending on high speed, pH was measured using a pH meter (Orion 3 Star, Thermo Electron Corporation) with constant stirring (Seggern, Calkins, Johnson, Brickler, & Gwartney, 2005). The pH electrode was rinsed with RO water between the measurements of each sample and periodically rinsed with acetone to remove any residual fat.

#### 3.2.2.2.5. *Cook loss measurements*

Meat samples obtained from section 3.2.2.2.2 were measured for the cook loss. Cook loss was determined as the difference in weight of meat before and after *sous vide* cooking (Chiavaro, Rinaldi, Vittadini, & Barbanti, 2009), expressed in percentage of the weight



before cooking (Eq. 6).

$$\% \text{ Cook loss} = \left( \frac{W_A - W_B}{W_A} \right) \times 100 \quad (6)$$

where  $W_A$  is the weight of meat after cooking, g; and  $W_B$ , is the weight of meat before cooking, g.

#### 3.2.2.2.6. *Texture measurements*

Slice shear force (SSF) of the processed meat samples obtained from section 3.2.2.2.2 were studied (as described in section 3.2.2.1.2) for effects of 2-days frozen storage. Same samples were stored at -18 °C for 2 days and thawed overnight, then were measured for the SSF.

#### 3.2.2.2.7. *Microstructure analysis*

Pre-treated samples were processed and observed according to the protocol of Livney, Ruimy, Ye, Zhu, and Singh (2017). Two replications were done for each sample.

***Pre-treatment.*** Pieces of muscle (1 × 0.3 × 0.3 cm) were cut (using a carbon steel surgical blade) from the control and CEE-treated muscle samples processed as described in section 3.2.2.2.2, both before (raw) and after cooking (cooked). The pieces were immersed in 2.5 % glutaraldehyde prepared in sodium cacodylate buffer (SCB, 0.1 M, pH 5.6) at 4 °C to fix overnight. Each fixed sample was washed by SCB (0.1 M) three times (10 min per time, pipetting out the old solution and pipetting in fresh one), post-fixed in 1 % Osmium Tetroxide for one hour, then washed with the buffer three times again in the same day. After washing, samples were dehydrated through a graded acetone series (25 %, 50 %, 75 %, 95 %, 100 %) for 10-15 min, each, then followed by two

changes of 100 % acetone (1 h per change). Then samples were put into a mixture of fresh resin (Procore 812, ProSciTech Australia) and acetone (at a ratio of 50: 50) and stirred overnight. On the second day, the sample was washed and the mixture was replaced by fresh 100 % resin and stirred for 8 h. This step was repeated twice, firstly in 100 % resin for overnight and then in 100 % resin for 8 h. The fixed and dehydrated samples were embedded in moulds with fresh resin and cured in a 60 °C oven for 48 h.

**Transmission Electron Microscopy (TEM).** The pre-treated muscle block was cut into 100 nm sections using a diamond knife (Diatome, Switzerland). The cut slice samples were stretched with chloroform vapour and fixed on a grid using a Quick Coat G pen (Daido Sangyo, Japan). Then the samples on grids were stained with saturated uranyl acetate (in 50 % ethanol) for 4 min, washed with 50 % ethanol and Milli-Q water. Afterwards, samples were stained with lead citrate (Venable & Coggeshall, 1965) for another 4 min, followed by a washing with Milli-Q water. The processed samples were used for ultrastructure observations and viewed with a microscope FEI Tecnai G<sup>2</sup> Spirit BioTWIN (Czech Republic).

#### 3.2.2.2.8. Soluble collagen in cook loss

Collagen solubility (% soluble collagen in the cook loss) is expressed in Eq. 7. The total collagen content of raw solid and soluble collagen in the liquid samples was measured by modified methods of Kolar (1990) and AOAC (1993). Three replications were done for each sample.

$$\% \text{ Collagen solubility} = \left( \frac{\text{Soluble collagen in cook loss, g}}{\text{Total collagen of raw meat, g}} \right) \times 100 \quad (7)$$

**Total collagen content.** Three brisket steaks obtained from section 3.2.2.2.1 were used as the raw control to determine the total collagen content. Each raw brisket steak was injected with 5 % Milli-Q water (w/w), tumbled at 5 rpm, at 4 °C for 15 min, and then homogenised for 1 min using a food processor. Four (4) g of homogenised meat was weighed in an Erlenmeyer flask and immersed in 30 mL of H<sub>2</sub>SO<sub>4</sub> (3.5 M). Covered by foil, the flask was placed in a drying oven (Contherm Scientific Ltd., NZ) at 105 ± 1 °C for 16 h (or overnight) for hydrolysis. The hot hydrolysate was quantitatively transferred to a 500 mL volumetric flask and diluted to volume with Milli-Q water. Part (50-75 mL) of the solution was filtered through qualitative filter paper (acid-washed, 12.5 cm diameter, flow speed 700 mL/min) into an Erlenmeyer flask. The filtrate was stable at 4 °C for at least 2 weeks. Five (5) mL of filtrate was diluted with Milli-Q water to 100 mL (the hydroxyproline concentration of final dilution is in range 0.5-2.4 µg/mL). Two (2) mL of final dilution or water (blank) was pipetted into a test tube, and 1 mL oxidant solution (including 1.41 g chloramine-T reagent and 100 mL pH 6.0 buffer solution) was added, mixed well and left at room temperature for 20 ± 2 min. The buffer solution was made by dissolving 90 g sodium acetate trihydrate, fifteen (15) g sodium hydroxide and 30 g citric acid monohydrate into 290 mL 1-propanol, then diluted to 1 L with Milli-Q water. One (1) mL of colour reagent (dissolving 10 g 4-(Dimethylamino)benzaldehyde in 65 mL 2-propanol and 35 mL perchloric acid) was added into each test tube and mixed thoroughly. Tubes were capped tightly and heated in the 60 ± 0.5 °C water bath for 15 min. After cooling, the absorbance of solutions was measured at 558 nm with a spectrophotometer (Shimadzu, Japan). The hydroxyproline and collagen content of raw meat was calculated

as described in Eq. 8 and Eq. 9.

$$\text{Hydroxyproline (H)}, \frac{mg}{g} = \frac{h \times 50}{m \times V_1} \quad (8)$$

$$\text{Collagen (C)}, \frac{mg. collagen}{g. meat} = 8 \times H = \frac{h \times 400}{m \times V_1} \quad (9)$$

where  $h$  is the hydroxyproline of final filtrate,  $\mu\text{g/mL}$  filtrate;  $m$  is the weight of solid test sample, g; and  $V_1$  is the volume of the first filtrate used for dilution to 100 mL, mL. Collagenous connective tissue contains 12.5 % hydroxyproline when the nitrogen-to-protein factor is 6.25 (Feiner, 2006).

***Soluble collagen in the cook loss.*** Cooking liquid samples obtained from section 3.2.2.2.2 (control and CEE-treated brisket steaks after cooking) were measured for the heat-soluble collagen in the cooking liquid. The cook loss liquid was collected, cooled, filtered (63  $\mu\text{m}$ ) and centrifuged at  $5069 \times g$ , at 4 °C for 15 min. 5mL of the supernatant was hydrolysed, diluted with Milli-Q water (first to 100 mL, then to 50 mL) and measured for hydroxypropylation concentration as described for total collagen. The hydroxyproline concentration in the cook loss was expressed as Eq. 10 and Eq. 11.

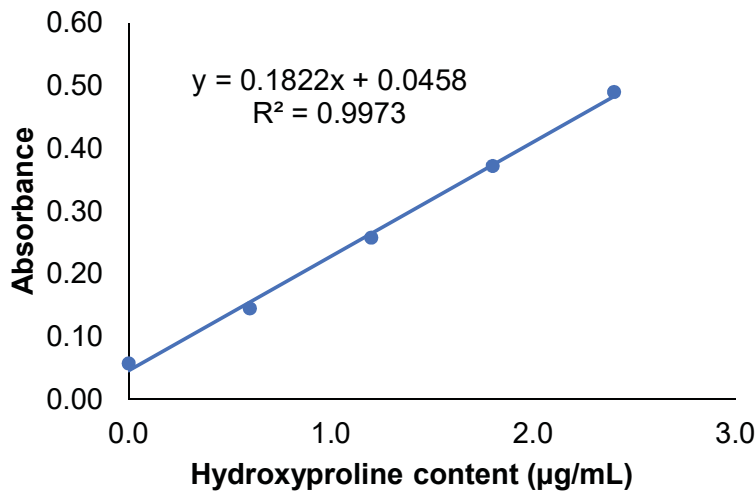
$$\text{Hydroxyproline (H)}, \frac{mg}{ml} = \frac{h \times 5}{V_S \times V_2} \quad (10)$$

$$\text{Collagen (C)}, \frac{mg. collagen}{mL. cook loss} = 8 \times H = \frac{h \times 40}{V_S \times V_2} \quad (11)$$

where  $V_S$  is the volume of test cook loss sample, mL; and  $V_2$  is the volume of the first filtrate used for dilution to 50 mL, mL. Collagenous connective tissue contains 12.5 % hydroxyproline when the nitrogen-to-protein factor is 6.25.

***Standard curve.*** Sixty (60) mg of hydroxyproline was dissolved in Milli-Q water and

diluted with Milli-Q water to 100 mL. Five (5) mL solution was pipetted and diluted with Milli-Q water to 500 mL with water. Standard solutions containing 0, 0.6, 1.2, 1.8, and 2.4  $\mu\text{g}$  hydroxyproline/mL were prepared on the day of use. Two (2) mL standard solution was added into test tubes, followed by colour development and measurement as described above. The standard curve (**Figure 6**) was plotted with absorbance on the y-axis and hydroxyproline content on the x-axis.



**Figure 6.** Standard curve for hydroxyproline content

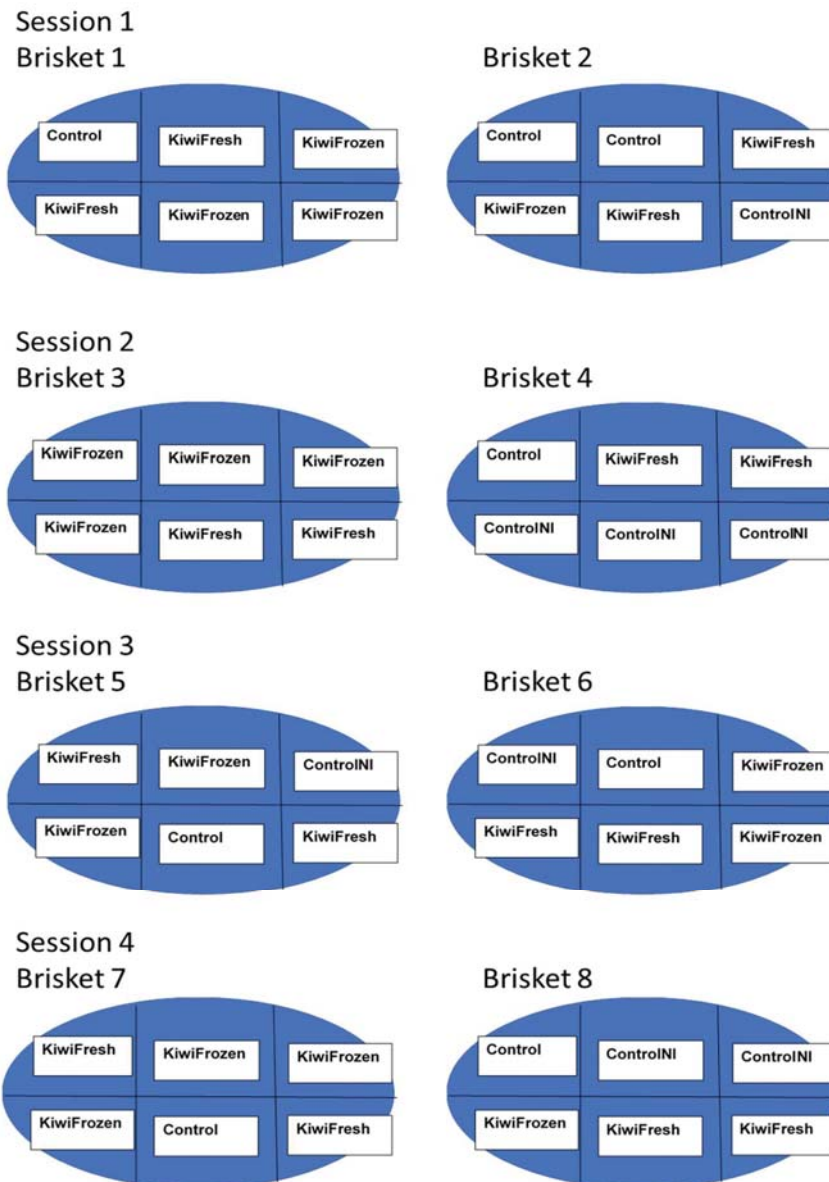
#### 3.2.2.2.9. Sensory analysis of fresh and post-treatment stored brisket

The objective of this sensory study was to understand the effect of kiwifruit enzyme on the perceived tenderness of beef brisket and the effect of frozen storage on the sensory profile of tenderised meat. Questions focused on the possibility that panellists could detect differences in the tenderness, juiciness and flavour among the cooked control and cooked actinidin-tenderised beef brisket steaks. The whole sensory evaluation was carried

out according a modified method from the research of Craigie, Wagstaff, Cullingworth, Ross, and Maltin (2014).

**Treatment allocation.** Eight pieces (4 pairs, from 4 carcasses) of whole beef brisket (same brisket as other tests) were used for the sensory analysis. It was presumed that brisket pairs from the same animal had similar texture and were comparable. Each brisket was cut into 6 steaks (approx. 200 g/steak, 10 cm length × 7 cm width × 3 cm height). Four treatments were randomly applied to the 48 steaks designed by CycDesign 6.0 (VSN International Ltd., UK), as shown in **Figure 7**. The four treatments included ‘ControlNI’ (sample not injected with water or CEE solution but cooked and had no post-treatment storage), ‘Control’ (sample injected with water instead of CEE solution followed by cooking with no post-treatment storage), ‘KiwiFresh’ (sample injected with CEE solution followed by cooking with no post-treatment storage) and ‘KiwiFrozen’ (sample injected with CEE solution followed by cooking and frozen storage at -18 °C for 3 weeks). Four sessions and in total 40 panellists (10 panellists/session) were involved in this sensory test, to decrease the effect of human variance.

**Storage study.** To study the effect of post-processing frozen storage on the sensory profile of beef brisket steaks, the allocated ‘KiwiFrozen’ meat was treated with CEE following the protocol described in section 3.2.2.2.2, and immediately stored at -18 °C for 3 weeks. On the day before the sensory test, the ‘KiwiFrozen’ meat was removed from the freezer and thawed at 4°C overnight (16 h). ‘Control’, ‘ControlNI’ and ‘KiwiFresh’ meats were prepared following the same protocol (as described in section 3.2.2.2.2) one day before the sensory test and stored at 4 °C overnight (16 h).



**Figure 7.** Randomised allocation of 4 treatments (Control, ControlNI, KiwiFresh and KiwiFrozen) on different parts of brisket pieces

‘Control’, sample injected with water instead of CEE solution followed by cooking but with no post-treatment storage; ‘Control NI’, sample not injected with water or CEE solution but cooked and had no post-treatment storage; ‘KiwiFresh’, sample injected with CEE solution followed by cooking with no post-treatment storage; ‘KiwiFrozen’, sample injected with CEE solution followed by cooking and frozen storage at -18 °C for 3 weeks.

**Reheating.** On the day of the sensory test, all samples were placed in a 60 °C water bath to be warmed for 55 min. This time was selected based on preliminary experimentation. It could guarantee the core temperature reaching 60 °C and minimise the growth of harmful microbes, according to the ‘Food Control Plan-Serve Safe (March 2017)’ published by Ministry for Primary Industries (MPI, 2017) of New Zealand. The samples were then removed from the water bath, sectioned into 1.27 × 2.54 × 2.54 cm serving samples and served to the panellists.

**Panellist Recruitment.** An Ethics Application was approved by Massey University and AgResearch, and this test was notified as low risk (Appendix A) before the sensory evaluation. The panellists were notified of the safety and risk through the first email invitation and at the beginning of the survey. An agreement about attending the sensory evaluation was signed by the panellists before the test.

Forty panellists were recruited from scientists and support staff based at AgResearch (Ruakura Research Centre, Hamilton, NZ). An email invitation was sent to the semi-trained panellist: they had done sensory work on meat with the team before, and some background information of them were recorded by the team, such as, they had all consumed *sous vide* cooked meat, they had no diet requirements that were unable to be accommodated by the team. Special requirements such as Halal and non-nut allergen meat were achieved. Most of the panellists liked their meat medium well done and they all ate beef on a regular basis (at least once fortnightly). The panellists were aged from 30 to 70 years old and were composed of New Zealanders (mainly), Americans, Africans, Asians and Europeans.

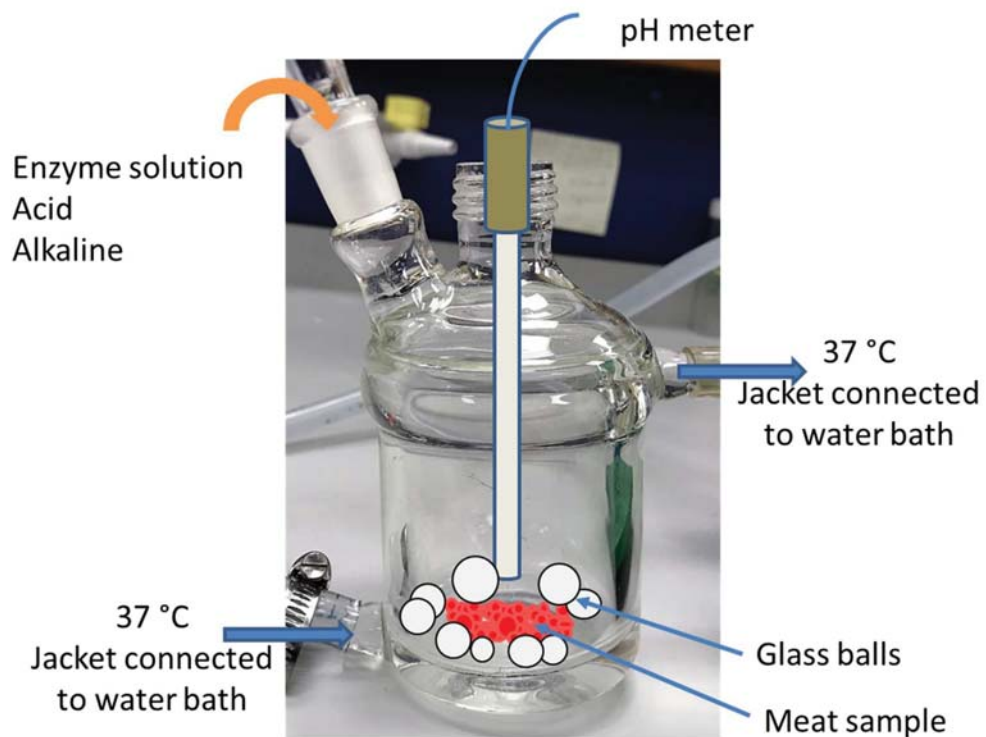


**Sensory session.** In each session, each panellist was provided with two samples labelled “A” and “B”. They were asked to taste both samples and then asked if these two samples were different. If they indicated no difference then they were asked to score the tenderness, juiciness and flavour of the samples. If they indicated that the samples were different, then they were asked to score each sample separately on its tenderness, juiciness and flavour. Tenderness, juiciness and flavour were scored according to a 100-point structure scale (0 is extremely tough or dry or bland, and 100 is extremely tender or juicy or strong). The process above was repeated 3 times during the same session, so that each panellist tasted six samples a total (the allocation of serving samples was randomised). There was a rest period between each set with crackers and water provided as palate cleansers.

#### 3.2.2.2.10. *In vitro* gastric digestion

Samples obtained from section 3.2.2.2 were used in this experiment. Both control and CEE-treated *sous vide* cooked meats were finely chopped into small pieces (approximately 2 mm) and digested under simulated gastric conditions in a jacketed glass reactor at 37 °C (**Figure 8**), by modified methods of Kaur *et al.* (2016) and Minekus *et al.* (2014). Five (5) g of the chopped samples were mixed with 5 mL simulated salivary fluid (SF) containing  $\alpha$ -amylase (75 U/mL) from *Aspergillus oryzae* (Sigma-Aldrich Pty Ltd., USA) for 2 minutes, and the pH was maintained at  $7.0 \pm 0.1$ . After that, twenty (20) mL of simulated gastric fluid (SGF) containing pepsin from porcine gastric mucosa (enzyme to substrate ratio of 1: 100, Sigma-Aldrich Pty Ltd., USA) was added to the

digestion mixture and the pH was adjusted to  $3.0 \pm 0.1$  to simulate the human gastric digestion environment. The SF and SGF were made of enzymes ( $\alpha$ -amylase or pepsin), corresponding electrolyte stock solutions,  $\text{CaCl}_2$  and water (Minekus *et al.*, 2014). Some glass balls (3-5mm) and a stirring bar were added into the reactor for sample maceration. Aliquots were taken at 2, 30 and 60 minutes of gastric digestion. In order to stop digestive enzyme activity, the digested samples were immediately immersed in an ice bath and stored frozen at  $-18^\circ\text{C}$  for further analysis. Another set of control and CEE-treated samples were incubated using the same procedure described above but without the addition of digestive enzymes to serve as controls. The digestion was performed in triplicate.



**Figure 8.** Glass digestion reactor

### 3.2.2.2.11. Soluble nitrogen measurements

The digested samples (obtained from section 3.2.2.2.10) were thawed at 4 °C and centrifuged at 10000 × g, at 4 °C for 20 min, followed by filtration through a 0.45 µm syringe filter (13 mm diameter, PVDF). The clear supernatant was measured for soluble nitrogen (%) by the Kjeldahl method (AOAC, 1990). The test was performed in triplicate.

**Digestion.** Five (5) g of each sample supernatant was weighed and placed in the digestion tube with the following addition of 2 Kjeltabs (each tablet containing 0.0035 g Se and 3.5 g K<sub>2</sub>SO<sub>4</sub>, Thermo Fisher Scientific Pty Ltd., AU) and 17 mL of concentrated H<sub>2</sub>SO<sub>4</sub> (95 % - 98 %). The total nitrogen content of processed meat was determined by weighing 0.6 g of control meat before digestion. A blank digestion without sample but containing all the other reagents was carried out at the same time. The water aspirator was placed on the digester unit (2006 Digester, FOSS TECATOR) and fully turned on. Samples were digested at 420 °C for around 30 min until the liquid in the tubes was clear. Tubes were removed from the heating unit and left in the exhaust manifold to be cooled. Seventy (70) mL of distilled water was added to each tube and mixed gently.

**Distillation and Titration.** A conical flask with 25 mL of 4 % boric acid and a tube with sample were placed in the pre-warmed distilling unit (Kjeltec™ 2100, FOSS). After the distillation, the solution in the conical flask was titrated with HCl (0.01 M) until the colour changed from pink to grey.

**Calculation.** The nitrogen percentage of the sample was calculated by Eq. 12,

$$\% \text{ Nitrogen} = \frac{A \times B \times 14 \times 100}{1000 \times C} \quad (12)$$

where  $A$  is the amount of used HCl, mL;  $B$  is molarity of HCl;  $C$  is weight of used sample, g. As the nitrogen in the supernatant is considered as soluble nitrogen, the soluble nitrogen percentage of the process sample after simulated gastric digestion was determined as the percentage of soluble nitrogen of the total nitrogen (Eq. 13),

$$\% \text{ Soluble nitrogen} = \frac{D \times C}{E \times F} \times 100 \quad (13)$$

where  $C$  is the weight of used sample, g;  $D$  is nitrogen percentage of digested sample;  $E$  is total nitrogen percentage of processed meat before digestion;  $F$  is the weight of sample, g.

#### 3.2.2.2.12. *Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

The supernatants of digested samples collected from section 3.2.2.2.11 were used to carry out Tricine SDS-PAGE. The Tricine sample buffer, Precision Plus protein™ dual Xtra prestained protein standards, 10-20 % Criterion™ Tris-HCl precast gels, 10 x Tris/Tricine/SDS running buffer and Coomassie Brilliant Blue R-250 staining solution were purchased from Bio-Rad Laboratories Pty Ltd. (NZ). The supernatant was diluted to 0.05 % soluble protein concentration with Tricine sample buffer containing 2 % β-mercaptoethanol, followed by heat treatment at 100 °C for 5 min. Samples were stored at -18 °C until electrophoresis was performed.

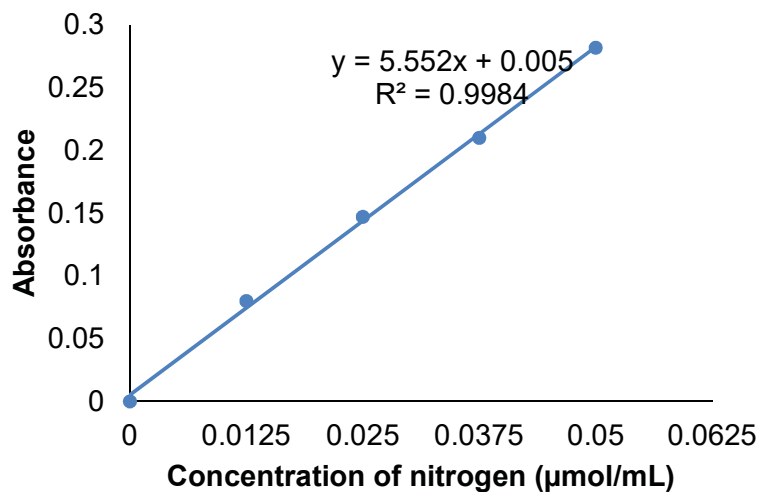
Samples (25 μL of samples or 10 μL of the Precision Plus Protein™ Dual Xtra Prestained Protein Standards) were injected in the 10-20 % Criterion™ Tris-HCl Precast Gels. Tricine-SDS-PAGE was run at 125 V for around 2 h until the blue label was almost

running out of the edge of gel. Then the gel was fixed in fixation solution (40 % methanol and 10 % acetic acid in Milli-Q water) for 30 min, dyed with bio-safe Coomassie Brilliant Blue R-250 staining solution for 30 min and rinsed with Milli-Q water. The gels were kept in the Milli-Q water at 4 °C until observation. Gels were scanned by a gel scanning densitometer (Gel Doc XR+, Bio-Rad Laboratories Pty Ltd., NZ) and bands were quantified by using Image Lab™ software version 6.0.0 (Bio-Rad Laboratories Pty Ltd., NZ) (Kaur *et al.*, 2016). Three replications were performed on each sample.

#### 3.2.2.2.13. Ninhydrin-reactive amino N release during digestion

The ninhydrin-reactive amino N (%) of digested samples (obtained from section 3.2.2.2.10) was analysed using 2 % ninhydrin reagent (Sigma-Aldrich Pty Ltd., USA) following the procedure of Sigma-Aldrich (2003). All the chemicals and reagents used in the study were of analytical grade. Five standard solutions were made by dissolving glycine in 0.05 % glacial acetic acid (Sigma-Aldrich Pty Ltd., USA) into a series of concentrations at 0, 0.125, 0.025, 0.375 and 0.05 µmol/mL. Two (2) mL of standard solution was mixed gently with 1 mL of ninhydrin reagent and then placed in the boiling water bath for 10 min. After cooling to room temperature, five (5) mL of 95 % ethanol was added to each standard mixture, mixed gently and the samples were measured for absorbance at 570 nm with spectrophotometer (Thermo Spectronic, USA). The standard solution with glycine concentration of 0 µmol/mL was treated as the reference. The standard curve (**Figure 9**) was plotted with absorbance on the y-axis and nitrogen content on the x-axis.

To measure the ninhydrin-reactive amino N concentration of the digests, 2 mL of the standard solution described above was replaced with the digested sample. Amino N concentration was determined by comparison of the A 570 readings to the standard curve. Samples were diluted with 95 % ethanol, if the absorbance was over 1.0. Three replications were performed in each sample. The results were recorded as means of three measurements.



**Figure 9.** Standard curve for glycine N concentration

### 3.2.3. Statistical analysis

The data reported are means of at least three measurements. Most of the experiments were carried out in three replications, except the high pressure processing experiment (section 4.1.2), and preliminary experimentation (section 4.3). The data were subjected to analysis of variance (ANOVA) and Tukey's test to analyse the significance of

differences ( $p < 0.05$ ) using Minitab version 17.3.1 Statistical Software (Minitab Inc., State College, PA). Error bars shown in the figures represent standard deviation (SD), which was also shown in the tables (except the table for sensory test results which indicate standard error, SED). The data from the sensory test were analysed using R version 3.3.2 with packages “lme4” and “lsmeans” (R Core Team, 2016).

## **Chapter 4. Results**

### **4.1. Inactivation of kiwifruit enzyme with or without meat**

#### **4.1.1. pH, protein content and enzyme activity of KE and CEE**

The pH of fresh kiwifruit extract (KE) and commercial kiwifruit enzyme extract (CEE) solutions (50 mg/mL) was 3.4 and 3.5, respectively. The measured enzyme activity (EA) of freshly prepared KE was 45.6 U/mL extract or 21.2 U/g fresh pulp. As fresh green kiwifruit contains 0.3 % soluble protein (Boland, 2013) and 83 % water on average (Drummond, 2013), the soluble protein content and total EA of dried green kiwifruit can be expressed as 1.8 % and 124.6 U/g dry weight, respectively. CEE powder contained 7.6 % soluble protein and its EA was 102 U/g powder. These variations may be due to the difference in the methods used for extract preparation.

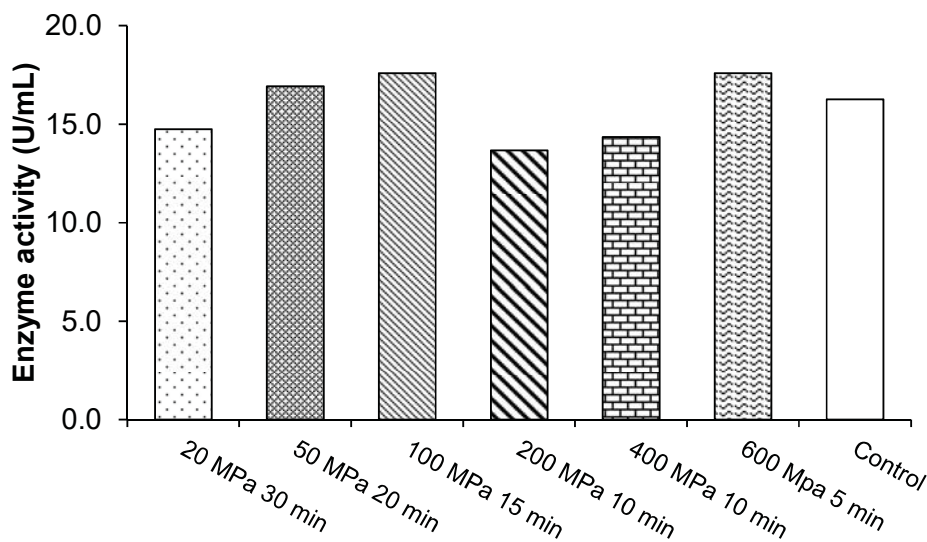
Some enzyme activity loss was observed in KE and CEE after centrifugation. The loss was about 10 % of the activity before centrifugation, indicating that the actual enzyme activity (i.e. total enzyme activity of supernatant and sediment) may be higher than the measured results (i.e. enzyme activity of supernatant).

#### **4.1.2. Effects of high pressure on actinidin (KE) inactivation**

No obvious difference was observed on the EA of the samples treated at different



pressures as shown in **Figure 10**, which meant that the pressure treatment ranging from 20 to 600 MPa for times less than 30 min had no observable effect on the actinidin activity. These results were different from those reported by Katsaros *et al.* (2009b), who demonstrated that the treatment at 600 MPa for 5 min at 25 °C could achieve around 20 % decrease in EA of kiwifruit juice.



**Figure 10.** The effects of different high pressures and processing times on the actinidin in kiwifruit extract (KE)

Control is untreated fresh KE. Results shown are means of three measurements of enzyme activity per treatment. Treatments were not carried out in triplicates.

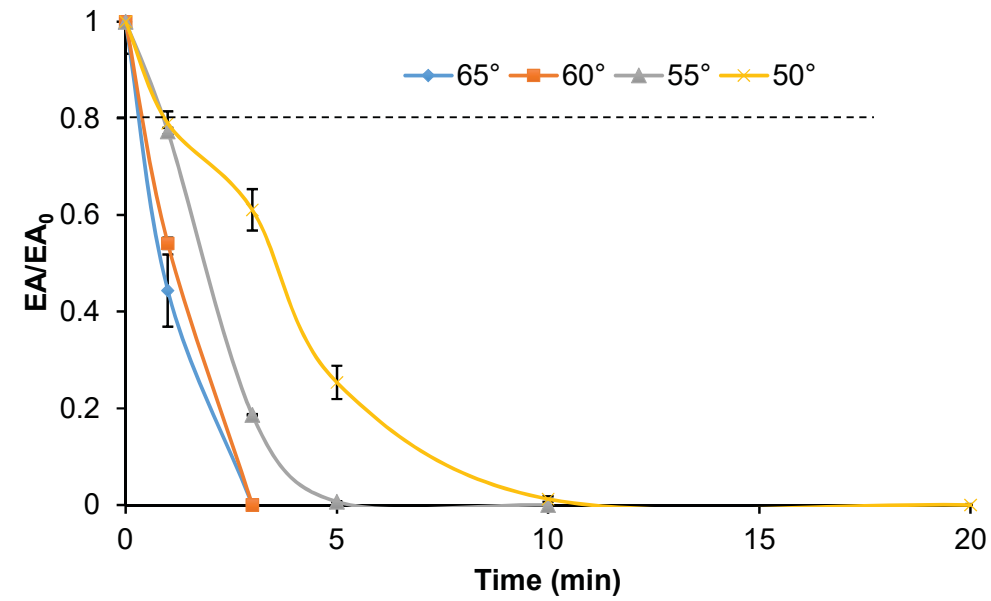
It has been reported that the treatment of meat at 600 MPa for 10 min resulted in modification of the meat colour and texture, similar to cooked meat (Kaur *et al.*, 2016). Considering the minimal effect of high pressure processing on enzyme inactivation, and the adverse impact of high pressure on meat appearance (colour and texture), the use of high pressure for kiwifruit enzyme inactivation was not used in further study.

### **4.1.3. Thermal inactivation of actinidin**

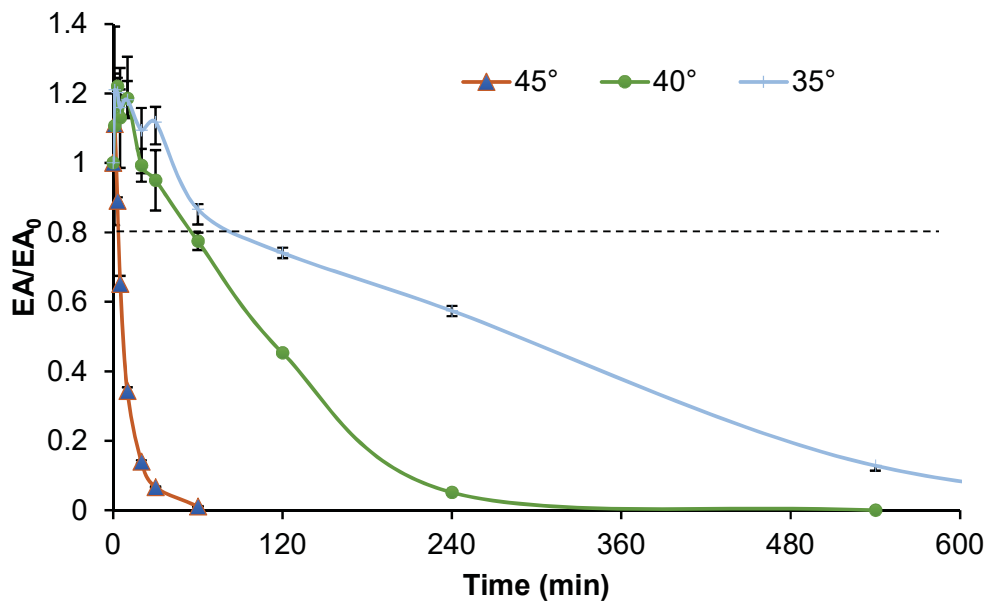
#### **4.1.3.1. Studies on KE**

No significant change in the pH of KE was observed after heating.

The thermal inactivation kinetics of enzyme in KE, expressed as  $EA/EA_0$ , are shown in **Figure 11**. Heating at moderate temperatures, ranging from 35 °C to 65 °C, had significant effects on the enzyme (actinidin) activity, and could effectively decrease the enzyme activity. For temperatures in the range of 50-65 °C, the EA dropped quickly and the enzyme was effectively inactivated within 10 min of heating (**Figure 11 a**). At temperatures in the range of 35-45 °C, an increase in EA (10-20 % compared with the control-untreated sample, depending on the temperature) was observed during the first few minutes of heating, followed by a drop (**Figure 11 b**). This increasing trend in EA lasted for longer times at lower temperatures (35-40 °C) compared with the higher temperatures (45 °C). Higher temperatures are known to denature proteins and inactivate enzymes. However mild temperatures in this experiment may have changed the structure of the protein and converted inactive (proenzyme) forms of actinidin to the active form.



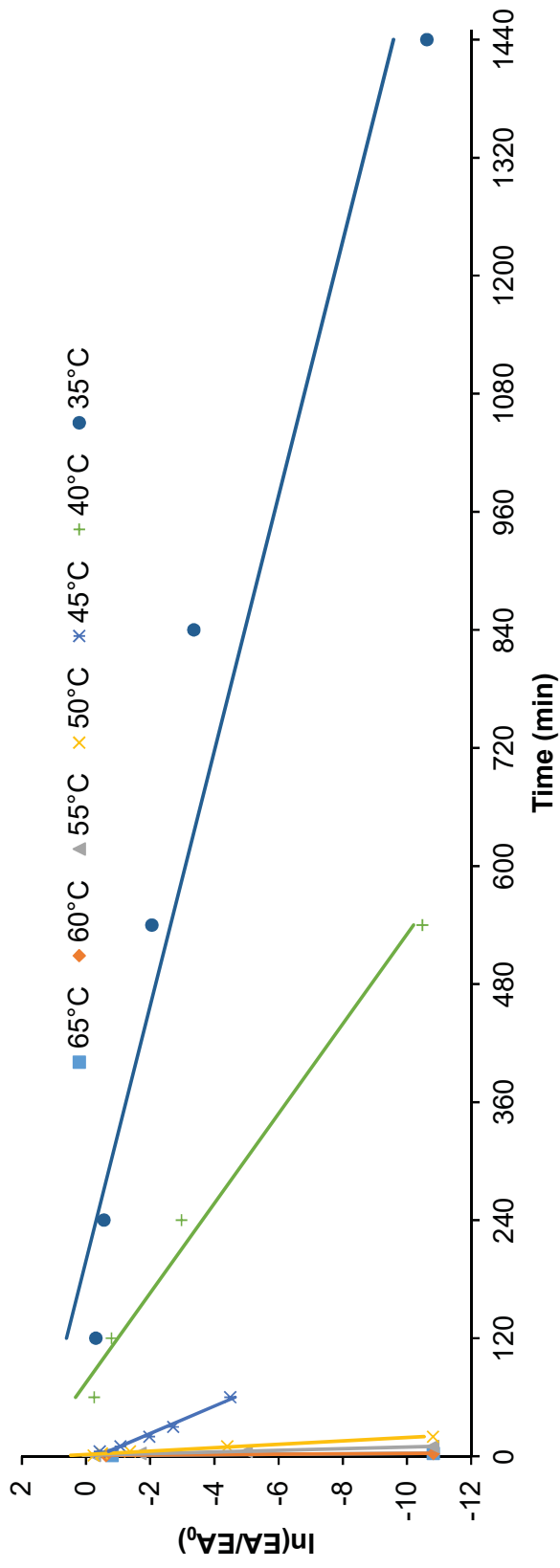
(a)



(b)

**Figure 11.** Thermal inactivation kinetics for actinidin in kiwifruit extract (KE)

(a) The effect of heating at 50-65 °C. (b) The effect of heating at 35-45 °C.  $EA$  (U/mL), the enzyme activity at time  $t$ .  $EA_0$ , the original enzyme activity of KE without heat treatment.  $EA/EA_0 = 1$  when  $t = 0$ . Values shown in (a-b) are means ( $n = 3$ )  $\pm$  SD (error bar).



(c)

**Figure 11.** Thermal inactivation kinetics for actinidin in kiwifruit extract (KE)

(c) The decrease of enzyme activity followed an exponential regression (first order kinetics,  $R^2 > 0.93$ ). Data points used in (c) are those with a value of  $EA/EA_0 < 0.8$  (dotted line shown in a-b) as the confounded reactions of both activation and inactivation may be significant at data points with values  $> 0.8$ .

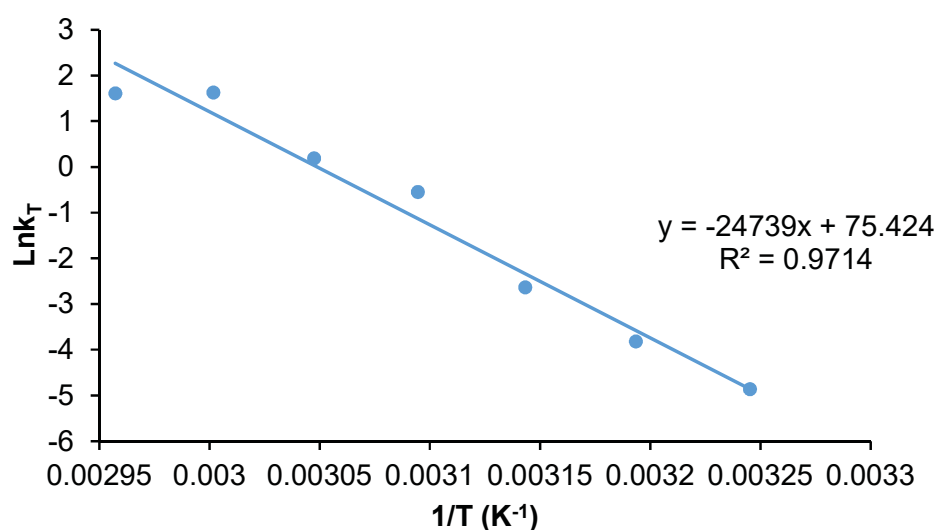
As shown in **Figure 11. (a-b)**, kiwifruit enzyme was thoroughly inactivated at 65 °C for 3 min, 60 °C for 3 min, 55 °C for 10 min, 50 °C for 20 min, 45 °C for 1 h, 40 °C for 9 h, and 35 °C for 24 h. The thermal inactivation followed first order kinetics (Eq. 1) and could be described by exponential curves, where  $R^2 > 0.93$ . To see it clearly, results were rearranged (Eq. 2) and plotted by  $\ln(EA/EA_0)$  against time as shown in **Figure 11 (c)**. Data of  $EA/EA_0$  higher than 0.8 (dotted line shown in **Figure 11 a-b**) were not considered in the calculation of inactivation rate constants ( $k_T$ , Eq. 2), as at early stages of heating, activation of actinidin may happen at the same time as inactivation (especially at 35 °C and 40 °C), and this may lead to a compound curve of both activation and inactivation. This assumption can be confirmed with more early data points and should be explored further.

As shown in **Table 7**, the inactivation rate constant ( $k_T$ ) increased ~620-fold in the range of 35-65 °C, indicating the high sensitivity of KE proteolytic activity to a temperature in this range. The half-life value ( $t_{1/2}$ ) of inactivation, expressed as Eq. 3, showed that the higher the temperature, the shorter the half-life value. The effect of temperature on the inactivation rate constant, described adequately by the rearranged Arrhenius' equation (Eq. 5), is shown in **Figure 12**. The activation energy ( $E_a$ ) is  $206 \pm 16$  kJ/mol ( $R^2 = 0.971$ ), which is likely to be related to the breaking down of covalent and hydrogen bonds of actinidin (Hammes, 1982). Further thermodynamic parameters ( $\Delta S$ ,  $\Delta H$  and  $\Delta G$ ) were not analysed as limited time points data were obtained from 60 °C and 65 °C (only two time-points data, respectively) and values of thermodynamic parameters were not different within experimental error.

**Table 7.** Inactivation rate constant ( $k_T$ ) and half-life time ( $t_{1/2}$ ) for the thermal inactivation of actinidin in fresh kiwifruit extract (KE) at different temperatures

Temperature (°C)	$k_T$ (min <sup>-1</sup> )*	$t_{1/2}$ (min)	R <sup>2</sup>
65	5.003±0	0.14	-
60	5.103±0	0.14	-
55	1.207±0.082	0.57	0.991
50	0.580±0.037	1.20	0.988
45	0.072±0.006	9.67	0.982
40	0.022±0.002	31.59	0.987
35	0.008±0.001	89.78	0.932

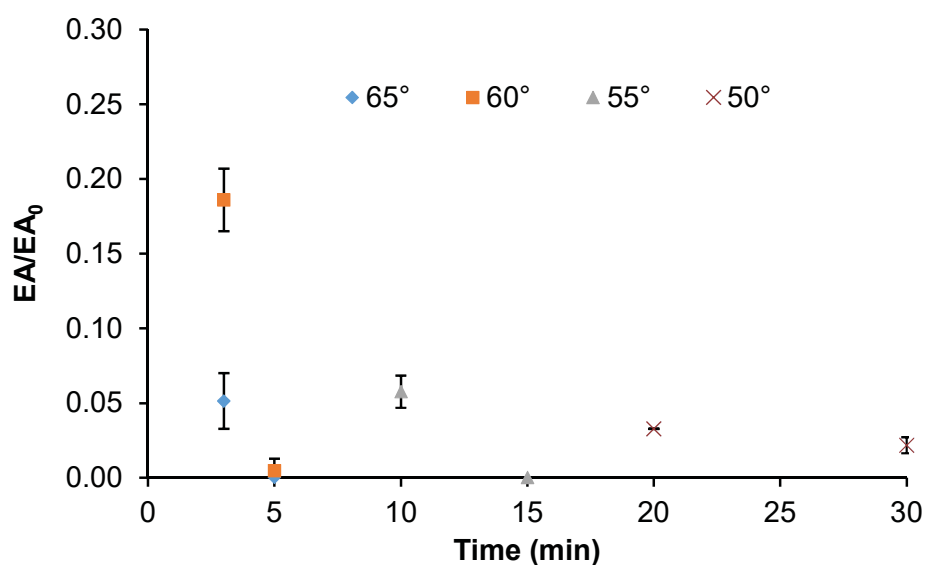
\*Mean ( $n = 3$ ) ± SD



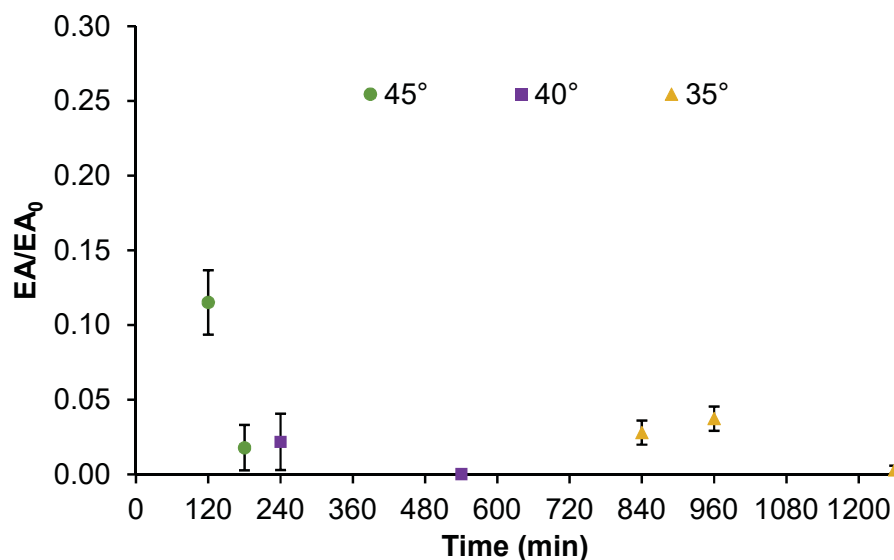
**Figure 12.** Relationship between inactivation constant ( $k_T$ ) and temperature ( $T$ ) for kiwifruit extract (KE)

#### 4.1.3.2. Studies on CEE

As observed for KE, heating did not lead to any significant effects in the pH of CEE solution.



(a)



(b)

**Figure 13.** Thermal inactivation of commercial kiwifruit enzyme extract (CEE)

(a) The effect of heating at 50-65 °C, and (b) The effect of heating at 35-45 °C.  $EA$  (U/mL), the enzyme activity at time  $t$ .  $EA_0$ , the original enzyme activity of CEE without heat treatment.  $EA/EA_0 = 1$  when  $t = 0$ . Values are means ( $n = 3$ )  $\pm$  SD (error bar).

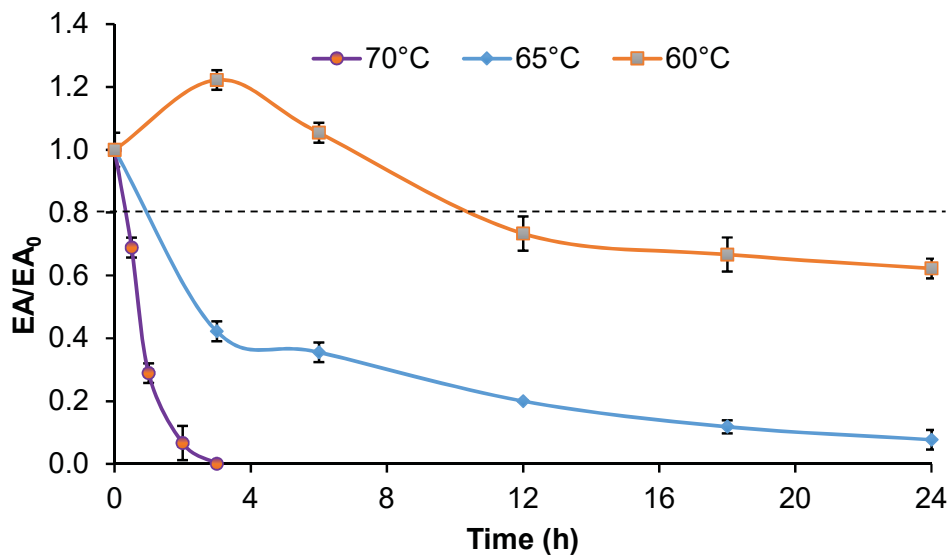
The thermal inactivation time-temperature combinations tested for KE were applied and repeated on CEE. However, the CEE could not be totally inactivated at the same time-

temperature processing points as KE (**Figure 13 a-b**). The enzyme activity of CEE solution (4.75 U/mL) was substantially lower than KE (45.6 U/mL), and it was expected that low activity sample should be easy to be inactivated. However, results showed that longer heating times were needed to inactivate the enzyme in CEE than in KE. This may be due to a protective effect of the complex composition of CEE derived from kiwifruit, which contains large amounts of pectin and sugar (as described in section 2.4.1). The pulp present in CEE might lead to an increase in viscosity of the solution, thereby leading to a decrease in heat transfer (Kays, 2012) and an increase in resistance towards heating.

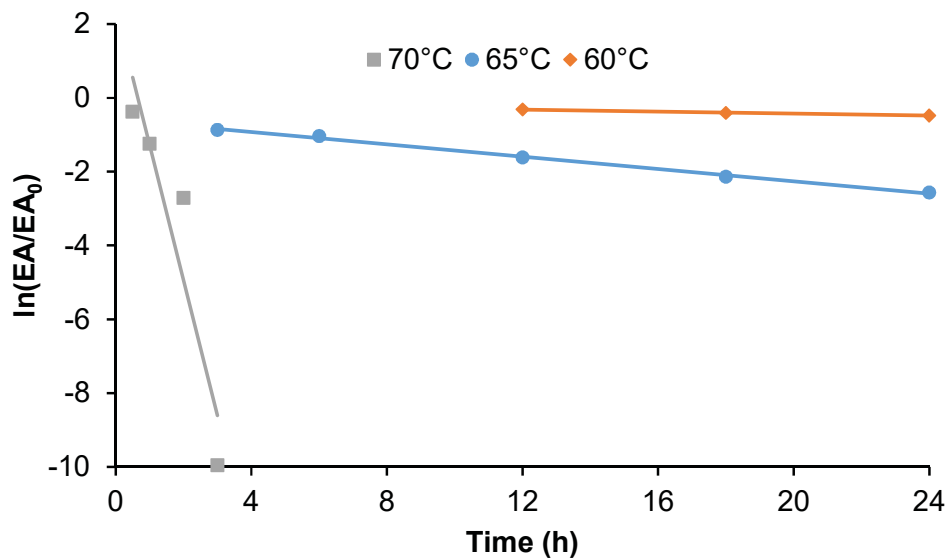
#### **4.1.3.3. Studies on KE-meat mixtures**

Compared with fresh KE alone, a longer heating time was required for enzyme inactivation when the KE was mixed with meat. As shown in **Figure 14 (a)**, actinidin in homogenised meat mixture was thoroughly inactivated after heating at 65 and 70 °C for 24 h and 3 h, respectively. However, when it was heated at 60 °C, the enzyme activity increased during the first 6 h of heating, and then slowly decreased with ongoing heating. After 24 h of heating, 50 % of enzyme activity (compared with unheated mixture) was still observed in the mixture. The reason for the initial increase in enzyme activity was not known but may be due to the same reason of KE activation (as described in section 4.1.3.1) that mild temperature activated the proenzyme form of actinidin.





(a)



(b)

**Figure 14.** Thermal inactivation kinetics of actinidin in KE-meat mixtures

(a) The effect of heating at 60-70 °C, and (b) The decrease of enzyme activity followed the exponential regression (first order kinetics,  $R^2 > 0.86$ ).  $EA$  (U/mL), the enzyme activity at time  $t$ .  $EA_0$ , the original enzyme activity of KE-meat mixture without heat treatment.  $EA/EA_0 = 1$  when  $t = 0$ . Values shown in (a) are means ( $n = 3$ )  $\pm$  SD (error bar). Data points used in (b) are those with values of  $EA/EA_0 < 0.8$  (dotted line shown in a), as complex reactions of both activation and inactivation may happen at data points with values  $> 0.8$ .

The pH of homogenised meat increased from  $5.5 \pm 0.1$  (raw) to  $5.8 \pm 0.1$  after cooking. The pH of KE-meat mixture was  $5.4 \pm 0.1$ , similar to that of raw meat but different from KE (pH = 3.4), indicating the buffering capacity of meat towards KE. After cooking, the pH of mixtures increased to  $5.7 \pm 0.1$ , which was mainly due to the pH change of meat. It has been reported by Chao (2016) that under incubation at 25 °C, the actinidin activity in KE had a poor stability at pH 6 than that at pH 3.5. This indicated that the increase in actinidin's heat resistance upon the addition of meat was not due to the increase in pH from 3.4 to 5.4. However, the heat stability ( $> 25$  °C) of actinidin at different pH values was not tested in this study.

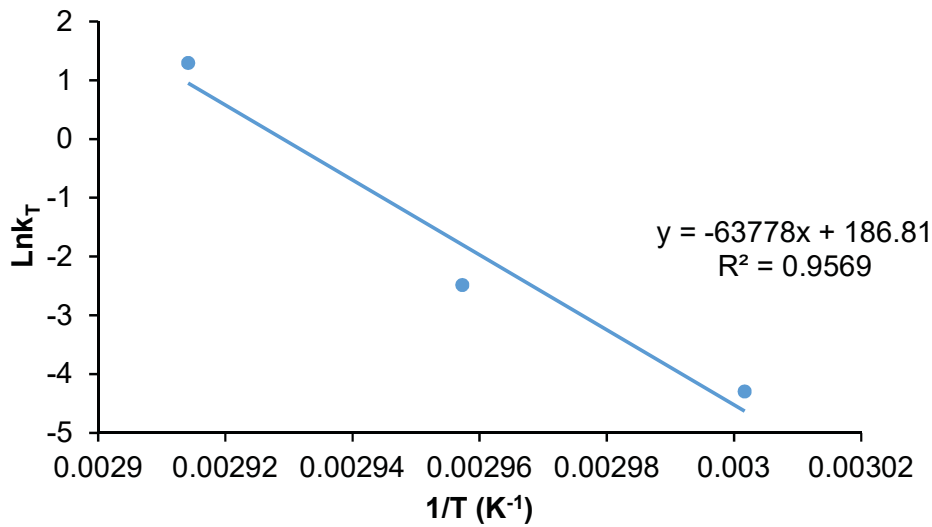
The presence of meat proteins was presumed to provide protection to the enzyme present in KE due to an increase in viscosity (Kays, 2012). Another possible reason could be that at higher temperatures, actinidin hydrolysed itself in the absence of other proteins; and when other proteins from meat were present, the protection of actinidin itself was achieved. Unlike the case with KE alone, the enzyme inactivation of KE-meat mixtures could not be well described by exponential regression (**Figure 14 b**,  $R^2 > 0.86$ ).

As shown in **Table 8**, the inactivation rate constant ( $k_T$ ) increased ~260-fold and the half-life time decreased ~270-fold when the temperature increased from 60 to 70 °C, indicating the high sensitivity of actinidin in KE-meat mixtures to the temperature in this range.

**Table 8.** Inactivation rate constant ( $k_T$ ) and half-life time ( $t_{1/2}$ ) for the thermal inactivation of actinidin in KE-meat mixtures at different temperatures

Temperature (°C)	$k_T$ (min <sup>-1</sup> )*	$t_{1/2}$ (h)	R <sup>2</sup>
70	3.662±1.019	0.19	0.866
65	0.084±0.003	8.28	0.997
60	0.014±0.001	50.62	0.992

\*Values are means ( $n = 3$ ) ± SD.



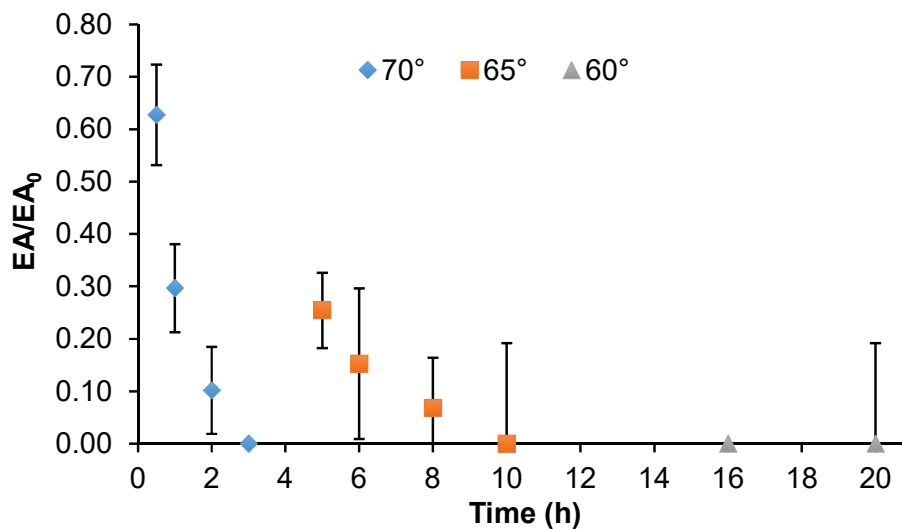
**Figure 15.** Relationship between inactivation constant ( $k_T$ ) and temperature ( $T$ ) for KE-meat mixtures

The effect of temperature on the inactivation rate constant was plotted according to the modified Arrhenius equation (**Figure 15**). The calculated activation energy value ( $E_a$ ) was  $550 \pm 124$  kJ/mol ( $R^2 = 0.951$ ), which was much higher than for that of KE alone. This indicated that more energy was required to inactivate the actinidin protected by meat.

#### 4.1.3.4. Studies on CEE-meat mixture

The pH of CEE-meat mixtures increased from  $5.5 \pm 0.1$  (raw) to  $5.8 \pm 0.1$  after

cooking, showing a similar trend as KE-meat mixtures due to the buffering capacity of meat.



**Figure 16.** Thermal inactivation for actinidin in CEE-meat mixture

$EA$  (U/mL), the enzyme activity at time  $t$ .  $EA_0$ , the original enzyme activity of CEE-meat mixture without heat treatment.  $EA/EA_0 = 1$  when  $t = 0$ . Values are means ( $n = 3$ )  $\pm$  SD (error bar).

Compared with CEE solution alone, a longer heating time was required for enzyme inactivation when the CEE was mixed with meat. However, the inactivation times were shorter than those for KE-meat mixtures despite CEE alone showing longer enzyme inactivation times than KE alone. As shown in **Figure 16**, actinidin in homogenised meat mixture was thoroughly inactivated after heating at 65 and 70 °C for 10 and 3 h, respectively. It could also be inactivated in less than 16 h at 60 °C, which could not be used to inactivate the actinidin in KE-mixture within 24 h. The reason is unknown and needs to be further explored. A possibility is that the protection of CEE caused by pulp viscosity was diminished when meat was present. Meanwhile, meat proteins combined

with actinidin, leading to more exposure of actinidin to the heat treatment.

#### **4.1.4. Conclusions**

High pressure processing alone was found to be unsuitable for the inactivation of kiwifruit enzyme in KE as it did not lead to any decrease in enzyme activity. Thermal treatment led to complete inactivation of the enzyme, depending on the heating temperature-time combinations. Therefore, heat treatment was selected as a method of enzyme inactivation in the following parts of the study. Actinidin in KE and CEE was observed to be highly sensitive to temperature, even when mixed with meat. Although the temperatures ranging from 60 to 70 °C will lead to the cooking of the meat, this temperature range is used in industrial and food service *sous vide* cooking. *Sous vide* cooking, a method of cooking in which food is vacuum-sealed in a plastic pouch and then placed in a water bath to achieve even cooking and to retain moisture, is normally carried out at 55-80 °C for 6-48 h in the meat industry (Baldwin, 2012). However, according to the 'Food Control Plan-Serve Safe (March 2017)' published by Ministry for Primary Industries (MPI, 2017) of New Zealand, cooking temperature should be above 60 °C to minimise the growth of harmful microbes present in the meat. It was anticipated that the enzymatic pre-treatment could reduce cooking time, particularly for tough meat briskets. The concept of *sous vide* cooking was used in the following part of this study.

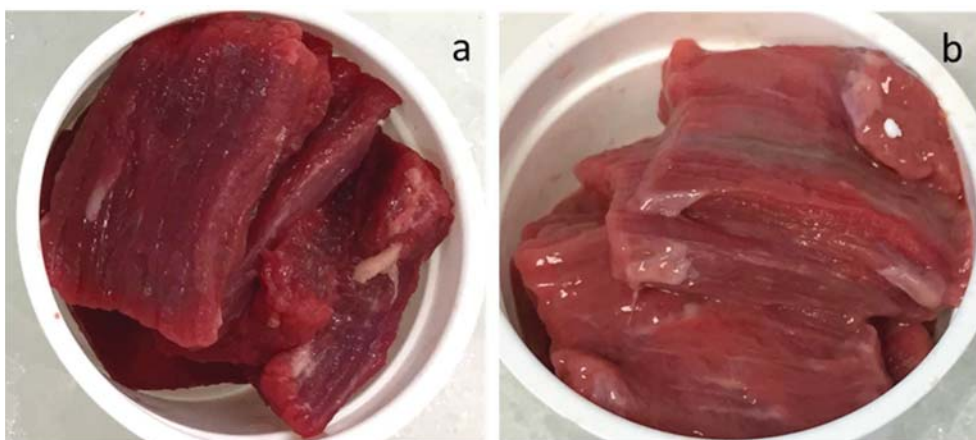
## **4.2. Application of KE and CEE on brisket steaks and their effects on meat tenderisation**

The goal of this study was to achieve optimum tenderisation and complete actinidin inactivation by heating for the minimum possible time at the minimum possible temperature, as that would lead to fewer changes in meat texture and reduction of cooking times, which are currently used in the industry for *sous vide* cooking of meat.

A trial of KE-marination combined with 30 min vacuum tumbling was applied on brisket steaks and led to non-uniform tenderisation with mushy over-tenderised surface and slightly tenderised core. Compared with marinating, injection processing led to more uniform changes in the texture, however, the texture was still over-tenderised. The standardisation of the enzyme concentration and injection amounts were needed to achieve optimum tenderisation. This is discussed in more detail in the following sections. The effect of tumbling on tenderness improvement was also tested and it was found that 15 or 30 min of tumbling did not improve the tenderness compared with untumbled brisket steaks, but improved the injection distribution.

### **4.2.1. Injection of 12 % KE followed by a two-step cooking procedure under *sous vide* conditions (at 55 °C for 1 h and 65 °C for 5 h)**

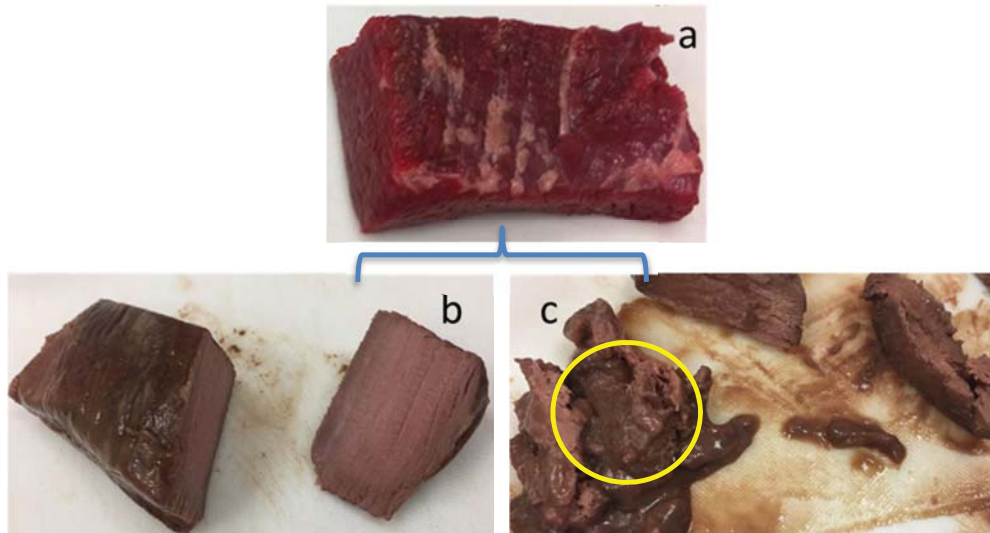
The raw meat injected with 12 % KE (amounts referring to the studies of Ashie *et al.* (2002) and Christensen *et al.* (2009)) had an undesirable over-hydrolysed, slippery and pale surface compared with untreated control (**Figure 17**).



**Figure 17.** Appearance of (a) untreated raw control and (b) KE-treated raw brisket steaks

Some preliminary experiments were done on beef using a two-step post-injection heating procedure. A low temperature and a short time heating (55 °C for 1 h, under *sous vide* conditions) were used as an enzyme incubation step (based on thermal enzyme kinetics of KE alone and KE-meat mixtures), to enhance the tenderisation effect of KE. This was followed by a moderately high temperature and a long-time heating (65 °C for 5 h, under *sous vide* conditions) to inactivate the enzyme in order to avoid any further tenderisation. However, as shown in **Figure 18**, brisket steaks were over-tenderised and became mushy, which was not expected. Results of slice shear force demonstrated the tenderising ability of KE, that KE-treated meat showed approx. 50 % reduction in shear force compared with the untreated control (decreasing from 101 N to 50 N). The undesirable texture of treated steaks showed that KE injection amount (12%) was significantly higher than that required to achieve optimum tenderisation. Also, results of enzyme activity assays showed that the enzyme in KE-treated beef steak was not totally

inactivated by heat treatment ( $EA \neq 0$ ), which meant further tenderisation due to residual active actinidin will keep on going.



**Figure 18.** Appearance of (a) untreated raw meat, (b) untreated cooked control (no water injection) and (c) KE-treated brisket steaks after heat processing of 55 °C for 1 h followed by 65 °C for 5 h

The mushy texture is circled.

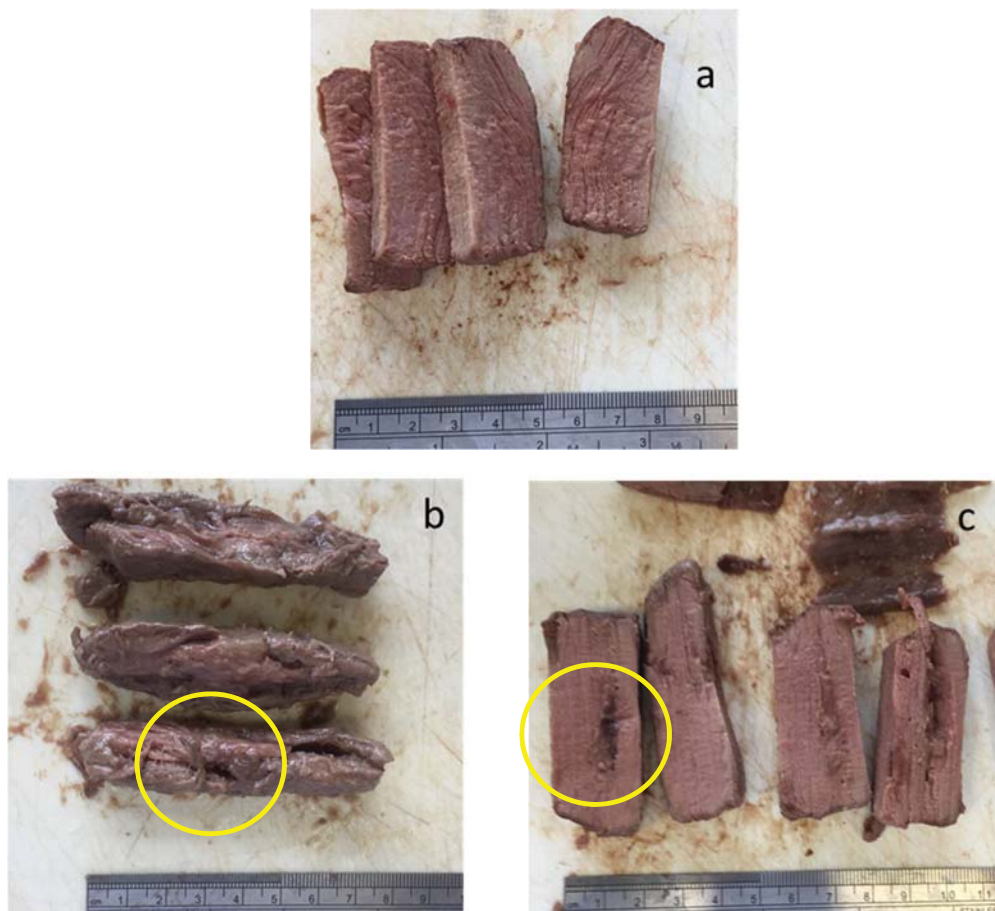
It is important to mention here that the heating temperatures-times used in these experiments were based on the enzyme kinetics studies on both KE alone and KE-homogeneous meat mixtures (reported in the previous section). And it was assumed that the enzyme will be inactivated at lower temperatures/times when the extract was injected into the meat steaks compared to mixing with homogenised meat.

#### **4.2.2. Injection of 5 % KE or CEE (50 mg/mL) followed by cooking under *sous vide* conditions at 70 °C for 1 h**



To decrease the degree of enzyme tenderisation (as reported in the last section), a temperature of 70 °C and heating for 1h (based on the thermal inactivation kinetics) were used and the enzyme incubation time was rejected to directly inactivate kiwifruit enzyme. It was assumed that some tenderisation would be achieved during heating before the enzyme was completely inactivated. The CEE solution (50 mg/mL), which had lower enzyme activity, was also introduced to compare with the tenderisation effects of KE. Meanwhile, the injection amounts were reduced from 12 % to 5 %.

According to the thermal inactivation kinetics shown in **Figure 14** and **Figure 16**, a minimum of 3 h of heating was required at 70 °C for KE-homogenised meat and CEE-homogenised meat mixtures, respectively. However, as the enzyme-meat mixtures showed considerably higher inactivation temperature-times than enzyme alone due to the presence of meat, as described in sections 4.1.3.3 and 4.1.3.4, it was assumed that inactivation times would be shorter for the enzymes injected into meat than the enzyme-meat mixtures. Therefore, heating at 70 °C for 1 h was tested.

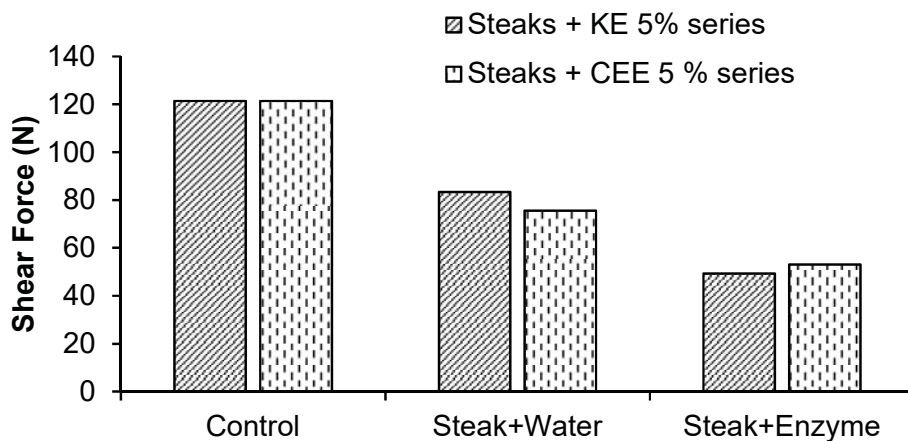


**Figure 19.** Appearance of (a) control meat injected with 5 % water, (b) meat injected with 5 % kiwifruit extract (KE) and (c) meat injected with 5 % commercial kiwifruit enzyme (CEE)

All treatments followed heating under *sous vide* conditions at 70 °C for 1 h. The mushy texture around injection sites is circled.

Less intensive mushy texture (**Figure 19**) was observed for both KE and CEE-injected steaks. As shown in **Figure 20**, the water injection increased tenderness over that of the un-injected control, and the shear force was reduced by more than half with the enzyme injections. Enzyme injected samples showed greater enzyme hydrolysis (observed as mushy points) around the injection sites, possibly due to higher concentration (enzyme activity) of the extracts or higher loading amounts per injection

site. Less reduction of shear force was observed for CEE-injected meat than the KE-injected counterpart, which could be explained by the lower enzyme activity of CEE (5 U/mL compared to 45 U/mL for KE).



**Figure 20.** Shear force (N) of control, water and enzyme-injected (5% water, KE or CEE) meat steaks after heat processing at 70 °C for 1 h

Control is untreated that has not been injected with any solution but cooked; Steak+Water is the sample injected with 5 % water followed by heating under *sous vide* conditions. Results shown are means of six measurements. Each treatment was carried out in duplicate.

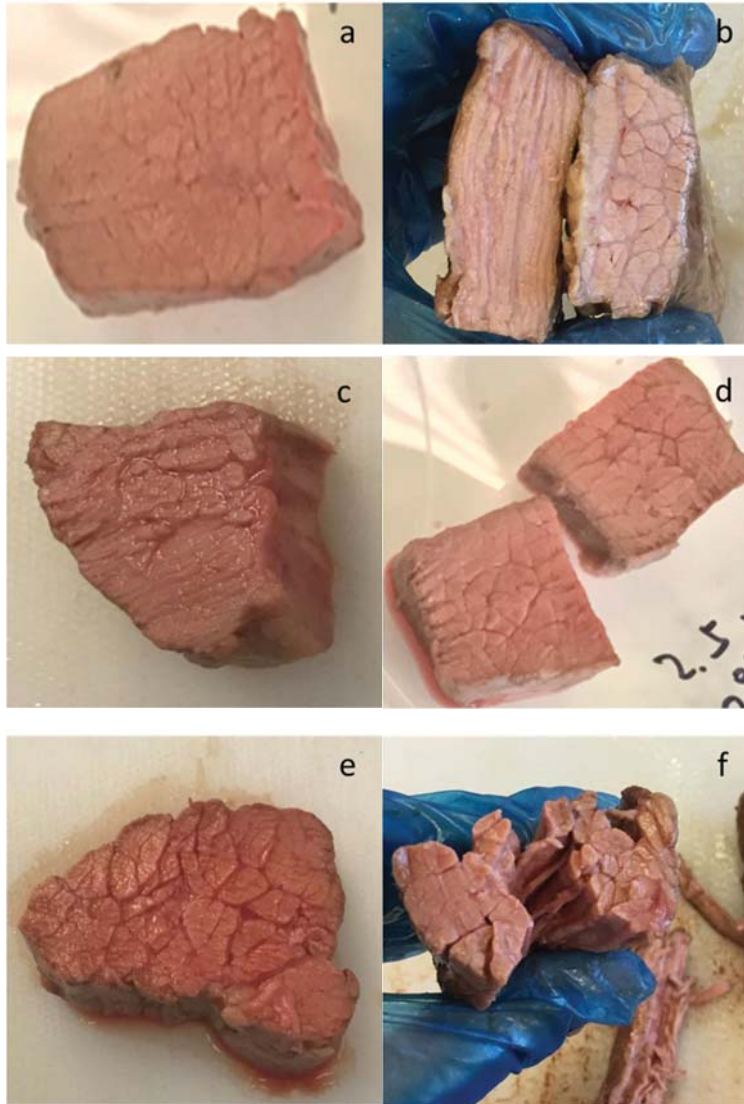
Results of enzyme activity analysis showed that the enzyme in both KE and CEE injected meat steaks was completely inactivated by heating at 70 °C for 1 h, which indicated that brisket steak would not be tenderised further by enzyme during post-cooking storage. The lesser inactivation time also demonstrated that the protection provided by meat steaks towards thermal inactivation of the enzyme was not as strong as by homogenised meat.

### 4.2.3. Standardisation of CEE solution concentration for injection

In order to minimise the over-tenderisation around injection sites (as seen in **Figure 19**) and achieve homogeneously tenderised meat texture, lower concentrations of CEE along with lower injection amounts at each injection point (but increased numbers of injection points to keep the overall injection amount same) and a finer needle (18G) were tested on brisket steaks, followed by the similar heat processing at 70 °C for 30 min (less cooking time was tested again).

A series of CEE solutions with different concentrations (0.5, 1, 2.5, 5 and 10 mg/mL) were injected into raw brisket steaks with injection amount of 0.2 mL at each point. A meat steak sample injected with water was treated as the control, as water also improved the tenderisation.

As shown in **Figure 21**, steaks injected with 0.5 and 1 mg/mL CEE were similar to the control, visibly tough in texture but had elasticity. There were a few soft points existing on the steaks injected with 2.5 mg/mL but overall the texture seemed better (improved) than other concentrations. At concentrations higher than 5 mg/mL, muscle fibres of steaks could be easily torn apart. At 10 mg/mL, muscle fibres were totally separated accompanied by mushy texture. The enzyme activity in each sample was completely inhibited after heating at 70 °C for 30 min. Based on visual observation, the concentration of 3 mg/mL was chosen to be used in the final injection processing.



**Figure 21.** Appearance of brisket steaks treated by 5 % (weight increase) water (a) and 5 % CEE at a concentration of 0.5 mg/mL (b), 1 mg/mL (c), 2.5 mg/mL (d), 5 mg/ml (e) and 10 mg/mL (f) followed by heat processing at 70 °C for 30 min

#### **4.2.4. Conclusions**

Direct injection of freshly prepared KE resulted in over-tenderisation and could not tenderise the meat optimally. Dilution or mixing with other solution would be necessary for KE to be used as the tenderiser. The injection was observed to be more useful than

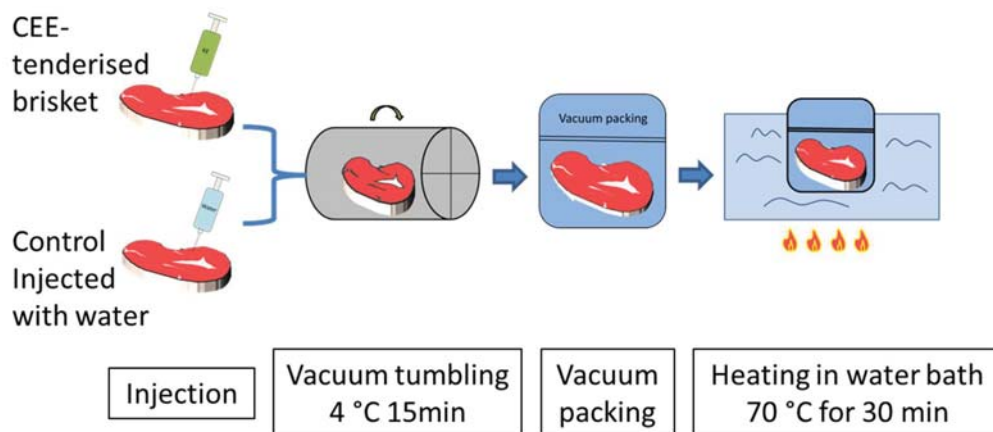
marinating, since the latter caused more effects on the surface.

The concentration of CEE affected the tenderisation level and determined the extent of mushy texture. The lower the concentration of CEE was, the less mushy the meat texture. Low enzyme concentration would not be sufficient to tenderise meat. A fine needle (18G) and a decrease of injection amount (0.2 mL per injection site) improved the tenderisation without over-tenderising the meat. Vacuum tumbling of meat post-injection also helped to improve the distribution of injected enzyme. The parameters chosen for the final experiments (described in next section) included using CEE at a concentration of 3 mg/mL, total injection amount of 5 %, post-injection vacuum tumbling at 4 °C for 15 min and cooking at 70 °C for 30 min.

### **4.3. Assessment of the final actinidin (CEE)-tenderised *sous vide* cooked brisket steaks**

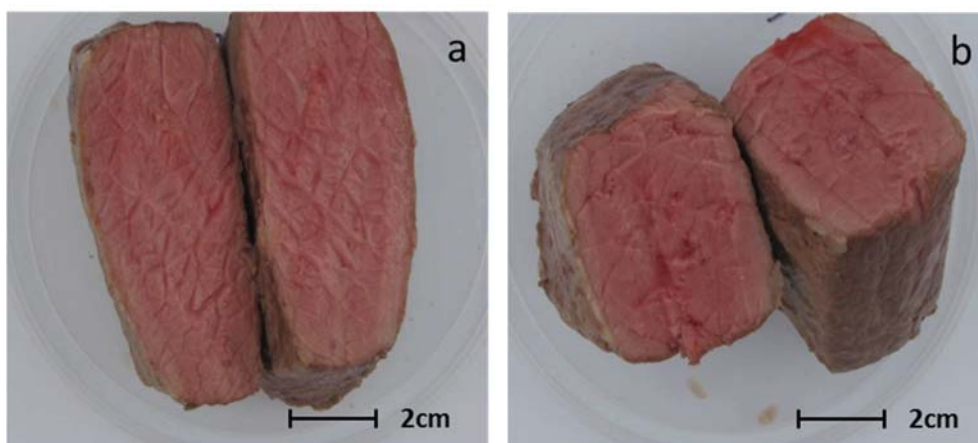
#### **4.3.1. Treatment plan and appearance of the meat steaks after treatment**

For the final experiments, brisket steaks were prepared by injecting CEE solution at a concentration of 3 mg/mL and 5 % total injection amount (w/w), tumbled for 15 min at 4 °C, vacuum packaged, cooked at 70 °C for 30 min (EA = 0) followed by immediate cooling to room temperature (**Figure 22**). Part of the samples were stored at 4 °C until further analysis, while some samples were stored at –18 °C for 3 weeks after the above-mentioned treatment (referring to section 3.2.2.2.9 for details) to observe any changes in texture and sensory properties of meat during storage and reheating (at 60 °C).



**Figure 22.** Schematic diagram showing the treatment plan for brisket steaks

Cut surfaces (middle of steaks) of control (water injected) and CEE-treated samples are shown in **Figure 23**. A few more, but acceptable, hydrolysis pockets around injection sites could still be observed in the CEE-treated sample, which could be eliminated during scale-up of this technology through the use of better-automated injection systems.



**Figure 23.** Appearance of (a) control brisket steak injected with 5 % water (w/w) and (b) brisket steaks injected with 5 % CEE amount (w/w) at 3 mg/mL, followed by heat processing at 70 °C for 30 min



### 4.3.2. pH, colour and cook loss

The pH and colour of CEE-treated and control *sous vide* cooked brisket steaks are shown in **Table 9**. There was no evidence of a significant change ( $p > 0.05$ ) in the mean pH value and  $L^*a^*b^*$  values after enzymatic pre-treatment (CEE), which meant that the actinidin had no effect on the pH and colour of meat steaks after cooking.

Mean cook loss of the CEE-treated and control brisket steaks was  $18.6 \pm 0.9$  % and  $23.7 \pm 3.3$  % respectively ( $p > 0.05$ ), which indicated that the CEE treatment didn't significantly affect the water holding capacity of meat muscle (juiciness). Although each reported value was based on triplicate analysis, the cook loss of each piece of brisket varied considerably. To eliminate the inherent variables of meat and to achieve more accurate results, a bigger sample size ( $n > 3$ ) is recommended for future work.

**Table 9.** pH, colour and cook loss (%) of control (injected with water) and CEE-treated, fresh and stored brisket steaks after cooking

Samples	pH <sup>1</sup>	Colour			Cook loss %
		$L^*$	$a^*$	$b^*$	
Control	5.39±0.06 <sup>a</sup>	51.52±3.83 <sup>a</sup>	26.55±5.46 <sup>a</sup>	11.56±1.75 <sup>a</sup>	23.7±3.3 <sup>a</sup>
CEE-treated meat	5.44±0.18 <sup>a</sup>	46.51±2.02 <sup>a</sup>	30.46±2.72 <sup>a</sup>	11.15±1.71 <sup>a</sup>	18.6±0.9 <sup>a</sup>
p-value	0.652	0.115	0.329	0.788	0.416

<sup>1</sup>Tukey pairwise comparisons. Different letters in the same column represent a significant difference ( $p < 0.05$ ). Results are expressed as means ( $n = 3$ ) ± SD.



### 4.3.3. Slice shear force

The slice shear force (**Table 10**) of control and CEE-treated meat steaks were  $105 \pm 15$  N and  $68 \pm 4$  N, respectively and were significantly different ( $p < 0.05$ ). The shear force after enzyme treatment decreased to 67 % of that of the control. After 2 days' frozen storage, there was also a significant difference of tenderness ( $p < 0.05$ ) among the control and CEE-treated meat steaks. However, there was no evidence of tenderness difference among control and 2-days frozen control, and CEE-treated and 2-days frozen CEE-treated steaks, which meant that frozen storage did not cause any change in sensory texture.

**Table 10.** Slice shear force (N) of control and CEE- treated brisket steaks following frozen (-18 °C) storage for 2 days

Samples	Shear Force* (N)
Control	105±15 <sup>a</sup>
Control (2 days frozen at -18 °C)	123±20 <sup>a</sup>
CEE-treated meat	68±4 <sup>b</sup>
CEE-treated meat (2 days frozen at -18 °C)	62±4 <sup>b</sup>
p-value	0.002

\*Tukey pairwise comparisons. Different letters in the same column represent a significant difference ( $p < 0.05$ ). Results are expressed as means ( $n = 3$ )  $\pm$  SD.

### 4.3.4. Sensory evaluation

The data analysis used mixed effects model, which including fixed effects (i.e. treatments) and random effects (i.e. locations within brisket and panellists within session).

As shown in **Table 11**, since  $p < 0.001$ , there was evidence that at least one treatment

combination differed from another treatment. There was no evidence of a difference in the tenderness scores for the two controls (injected and not injected with water,  $p = 0.532$ ). There was evidence that 'KiwiFresh' and 'KiwiFrozen' were scored more tender than both the controls ( $p < 0.05$ ). 'KiwiFrozen' was scored significantly more tender than 'KiwiFresh' (39.11 for the fresh versus 52.61 for the frozen sample,  $p = 0.002$ ), which was different from the results obtained from the instrumental test (section 4.3.3, measurements after 2-days frozen storage) that showed insignificant differences in tenderness between these two samples. The samples tested for instrumental shear force measurements were not reheated at 60 °C, indicating the results from both sections could not be compared directly: it was assumed that the reheating will provide the same level of toughness change in both the samples ('KiwiFresh' and 'KiwiFrozen'). Since only triplicates were tested for instrumental tenderness, and considering the big variations among meat pieces, sensory evaluation results obtained from a bigger sample size (60 samples for each treatment) were more likely to pick up differences. The tenderness increase caused by freezing may be due to the formation of ice crystals during freezing, damaging the microstructure and altering physical quality parameters of the meat (Leygonie *et al.*, 2012).

**Table 11.** Sensory scores of tenderness, juiciness and flavour<sup>1</sup> of control (no injection), control (water injection) and CEE-treated brisket steaks following frozen (-18 °C) storage for 3 weeks

	<b>Tenderness</b>	<b>Juiciness</b>	<b>Flavour</b>
<b>ControlNI</b>	18.35 <sup>a2</sup>	33.6 <sup>a</sup>	33.03 <sup>a</sup>
<b>Control</b>	22.16 <sup>a</sup>	37.79 <sup>ab</sup>	35.1 <sup>ab</sup>
<b>KiwiFresh</b>	39.11 <sup>b</sup>	42.64 <sup>bc</sup>	39.74 <sup>bc</sup>
<b>KiwiFrozen (3 weeks)</b>	52.61 <sup>c</sup>	42.33 <sup>bc</sup>	41.9 <sup>c</sup>
<b>P-value</b>	<0.001	0.069	0.02
<b>SED</b>	5.16	3.6	3.02

<sup>1</sup>0-100, 0 is too tough/dry/bland, and 100 is too tender/juicy/strong for tenderness, juiciness, and flavor, respectively.

<sup>2</sup>Tukey pairwise comparisons. Different letters in the same column represent a significant difference ( $p < 0.05$ ). Results are expressed as means ( $n = 3$ )  $\pm$  SD.

There was weak evidence of a difference in mean juiciness ( $p = 0.069$ ). ‘ControlNI’ had the lowest mean juiciness, going towards too dry. Both CEE-treated samples had more juice, which was not significantly different from the ‘Control’ but significantly higher than ‘ControlNI’. No significant difference of juiciness was observed between fresh and frozen CEE-treated samples. Both CEE-treated samples had means of 42.64 and 42.33, respectively, indicating potential “just-right” juiciness.

There was evidence of a difference in mean flavour ( $p = 0.02$ ). Injection of water does not change the flavour ( $p = 0.559$ ). Both CEE-treated meats had a stronger flavour than ‘ControlNI’ ( $p < 0.05$ ). There was evidence that ‘KiwiFrozen’ has a stronger flavour than ‘Control’ ( $p = 0.031$ ). Overall, the injection of kiwifruit enzyme enhanced the flavour of meat, and the scores were around the “just right” line (middle point of the 100-points

scale), meaning that the addition of kiwifruit enzyme does not have adverse impacts on the meat flavour.

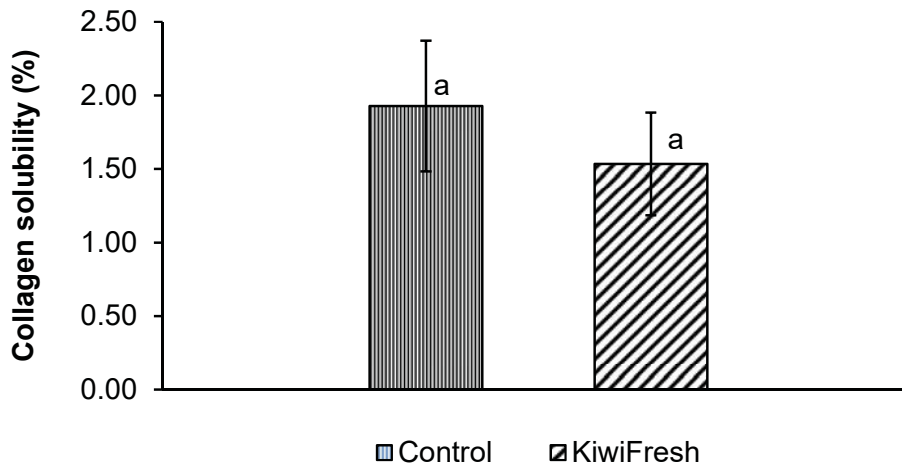
In conclusion, the injection of CEE solution made the meat more tender with the “just right” tenderness. The increase in juiciness may be due to the addition of water, but the CEE-treated meat was indeed juicier than normal untreated meat. The flavour of meat was enhanced by the addition of CEE (actinidin). The 3-weeks frozen storage increased the tenderness, but didn't affect the juiciness and flavour.

#### **4.3.5. Soluble collagen in cook loss**

Total collagen content of brisket muscle was observed to be  $10.60 \pm 1.95$  mg/g muscle, which was around 1.06 % of the fresh weight. The collagen solubility in cook loss (% soluble collagen in cook loss/total collagen) of both control and CEE-treated steaks was  $1.93 \pm 0.44$  % and  $1.53 \pm 0.35$  %, respectively, indicating no evidence of a difference in the collagen solubility among different treatments ( $p = 0.394$ , **Figure 24**).

It was reported by Christensen *et al.* (2009) that injection with 10 g/L actinidin powder increased the percentage of heat-soluble collagen, but a lower injection concentration (4 g/L) resulted in an insignificant difference in soluble collagen compared with the control. Although the activity of the actinidin-containing kiwifruit powder used in that study is not known, it could be presumed that a lower addition of kiwifruit enzyme may not significantly affect the collagen solubility, which could be used to explain my results showing no effect from actinidin treatment on the collagen solubility in the cook loss. Moreover, the soluble collagen in the cook loss measured in the current study was

different from the total heat-soluble collagen content measured by Christensen *et al.* (2009). In order to investigate the effect of CEE on connective tissue, measurements of the total heat-soluble collagen content are needed to be done in a future study.

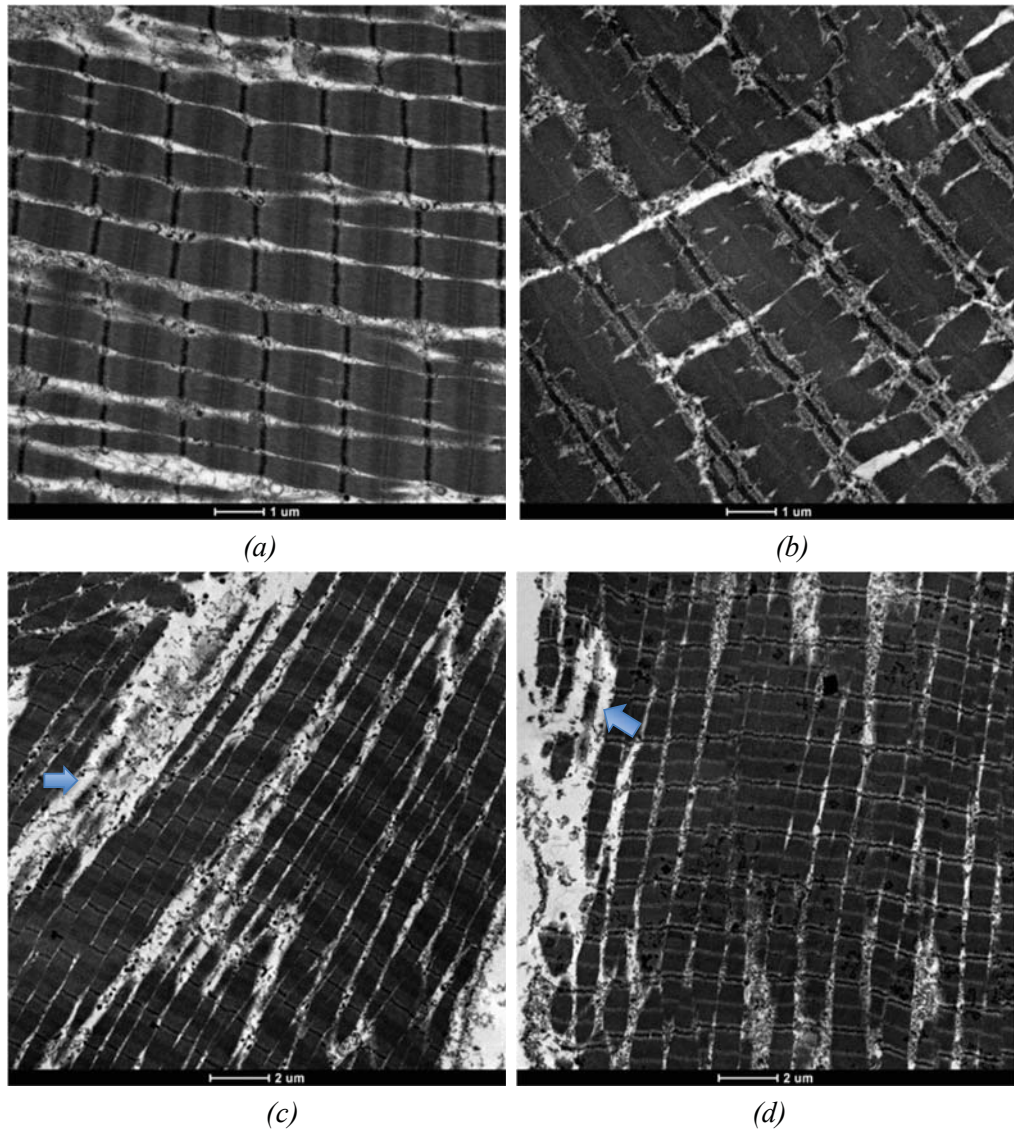


**Figure 24.** Comparison of collagen solubility (% soluble collagen in cook loss/ total collagen) for cooked control (water injected) and CEE-treated brisket steaks ( $p = 0.394$ ) Values are means ( $n = 3$ )  $\pm$  SD (error bar).

#### 4.3.6. Microstructure-TEM

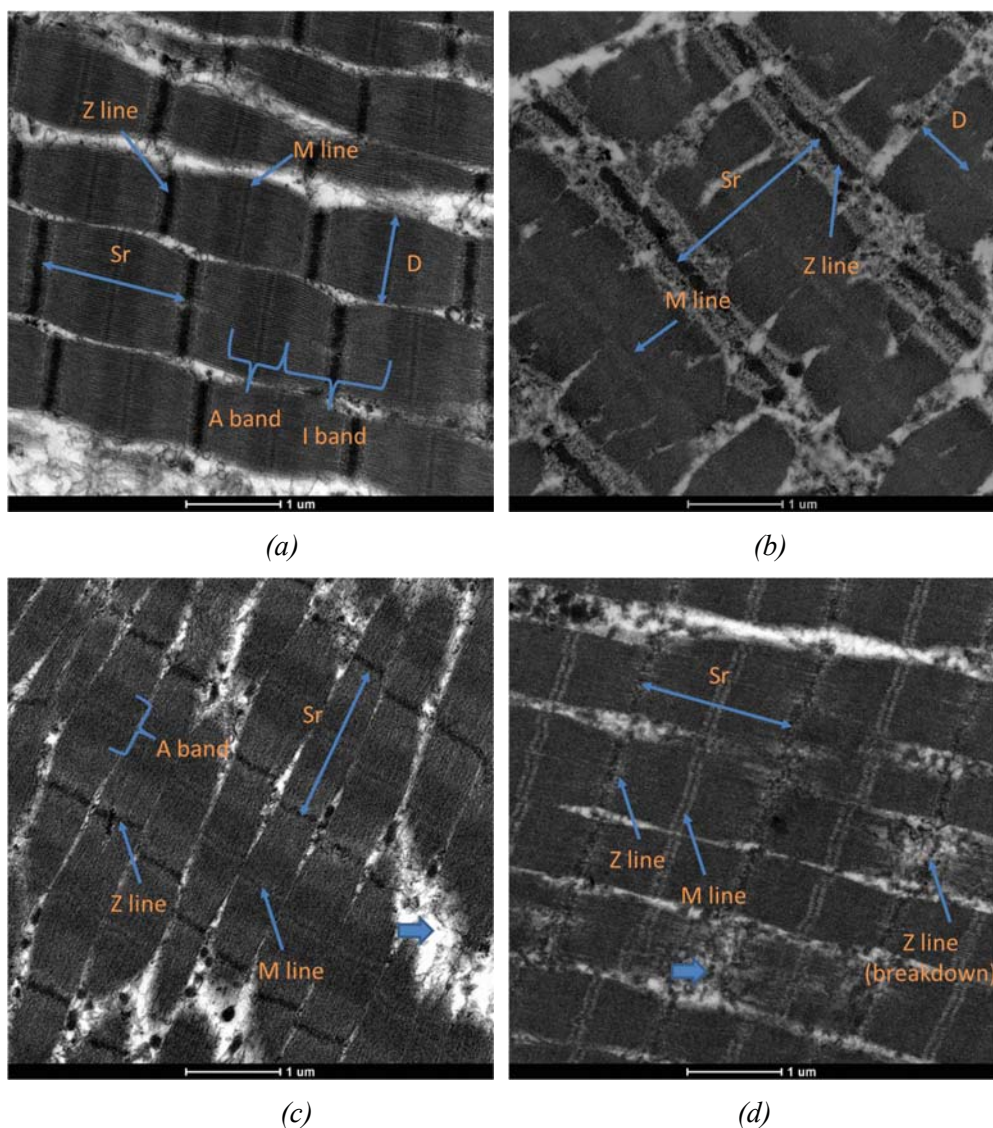
When comparing the raw control (water injected) and cooked control (water injected) (**Figure 25 & Figure 26**), cooking generated some structural changes in the beef muscle such as the shrinkage of muscle myofibrils, which was in agreement with the study results of Kaur, Maudens, Haisman, Boland, and Singh (2014). This change could possibly be due to the thermal coagulation of proteins and water loss during cooking (Kaur *et al.*, 2014). The diameter of meat myofibrils of raw control and cooked control varied from 0.75-1.15  $\mu\text{m}$  to 0.50-0.60  $\mu\text{m}$ , respectively (**Figure 26 a-b**). However, the mean

sarcomere (Sr) length increased from 1.60  $\mu\text{m}$  for raw to 1.95  $\mu\text{m}$  after cooking. Changes around Z line and M line were also observed after cooking (**Figure 26 b**).



**Figure 25.** Transmission Electron Microscope (TEM) micrographs of control (a-b) and CEE-treated brisket (c-d) myofibrils before (a-c) and after (b-d) *sous vide* cooking  
Thick arrows show the hydrolysed myofibrils





**Figure 26.** Transmission Electron Microscope (TEM) micrographs of water injected control (a-b) and CEE-treated brisket (c-d) myofibrils before (a-c) and after (b-d) *sous vide* cooking, at higher magnification, showing sarcomere structural detail. Thick arrows show hydrolysed myofibrils. Sr, sarcomere and D, diameter.

It appeared that the injection of CEE (actinidin) caused considerable structural changes in myofibrils, starting the action from the edges of myofibrils and proceeding towards the centre (**Figure 25 c**), and potentially causing more structural damages along the Z line (**Figure 26 c**). These reactions did not go through the myofibrils uniformly,

which could be due to the less uniform distribution of the injected enzyme. Those unaffected myofibrils shown in the micrograph may be the ones which were not exposed to the kiwifruit enzyme. The sarcomeres of CEE-treated raw brisket myofibrils (ranged from 1.65-1.95  $\mu\text{m}$ ) were longer than raw control (about 1.59-1.63  $\mu\text{m}$ ). An extension of the A band was also observed in CEE-treated raw meat. These structural changes demonstrated the action of actinidin from CEE on myofibrillar proteins (Christensen *et al.*, 2009; Ha *et al.*, 2012).

After cooking, the length of the sarcomere in the CEE-treated sample (**Figure 26 d**) was maintained at 1.60  $\mu\text{m}$  (similar to the raw control), which suggested less shrinkage of the myofibrils due to lower water loss or less muscle contraction during cooking. Myosin, as the main contractile protein, has been found to be broken down by the cysteine protease, papain, and to release HMM-S1 (Xiong, 1997). It has also been reported by Liu *et al.* (2011) that kiwifruit juice resulted in significant loss of myosin heavy chain, indicating the effect of actinidin on myosin degradation. The protein degradation by cysteine proteases has been discussed in section 2.3.1. This might help to explain the less intensive sarcomere contraction observed in CEE-treated muscle after cooking. Breakdown around some Z lines was also observed (**Figure 26 d**), which was similar to the changes observed in CEE-treated raw meat. Moreover, the Z line could be distinguished but it was fragmented.

Overall, the observations above indicated weakening of the microstructure, particularly around the Z line, after actinidin (CEE) treatment and agreed well with its positive effects on muscle tenderness, observed during instrumental texture and sensory



analyses.

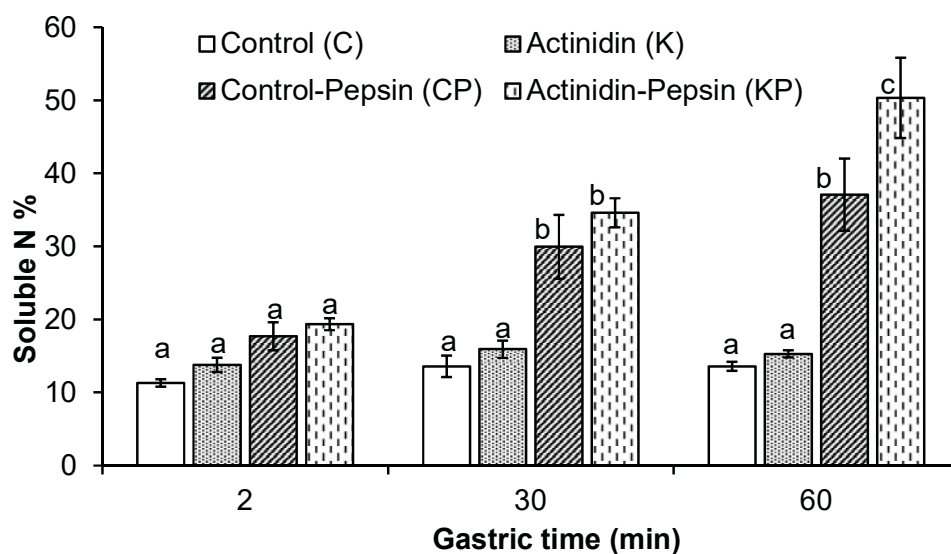
#### **4.3.7. *In vitro* gastric digestibility**

It has been reported by Kaur *et al.* (2010b) that kiwifruit extract showed more positive effects on beef protein digestibility during the simulated gastric digestion than in subsequent small-intestinal digestion. Meat samples used in this study were digested only under simulated gastric conditions.

##### **4.3.7.1. Protein solubility**

The soluble nitrogen of digested control and CEE-treated (actinidin) meat after cooking, with (CP, KP) or without (C, K) the addition of pepsin, respectively, are shown in **Figure 27**. No significant difference ( $p < 0.05$ ) was observed among the 'C' and 'K' during the whole processing, indicating no effect from actinidin (inactivated) without pepsin addition, on the protein hydrolysis.

There was a significant difference ( $p < 0.05$ ) in soluble nitrogen among the 'CP' and 'KP' digested for 60 min under simulated gastric conditions, which meant that more protein was solubilised during digestion after CEE-pretreatment compared with the control. However, this difference was not significant after digestion of 2 min or 30 min, which indicated that any effect caused by actinidin (inactivated) addition was slow.



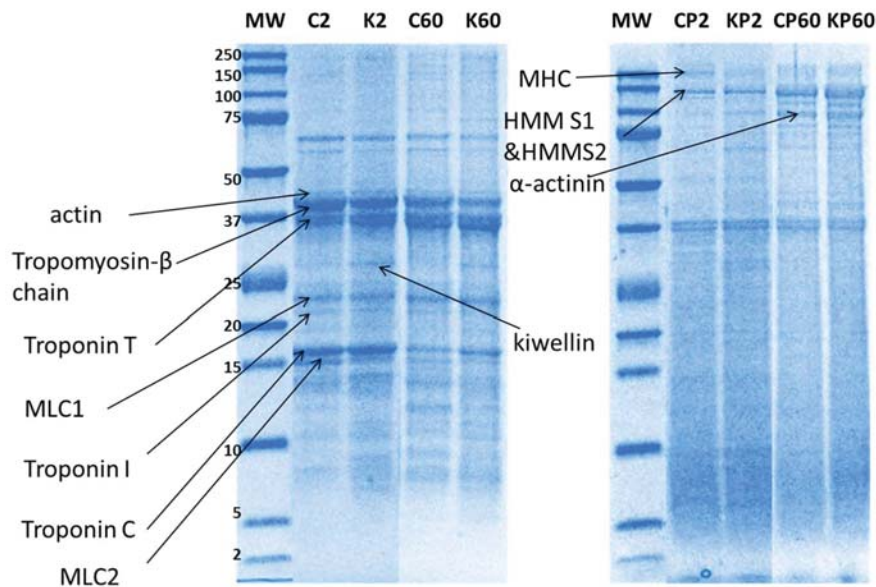
**Figure 27.** Soluble nitrogen (%) of cooked control (water injected) and CEE-treated (actinidin) meat digests after 2, 30 and 60 min of *in vitro* gastric digestion, both with (CP, KP) or without (C, K) the addition of pepsin

Means ( $n = 3$ ) that do not share a letter are significantly (using Tukey method and 95 % confidence) different.

#### 4.3.7.2. SDS-PAGE

The SDS-PAGE showed that control brisket muscles after incubation (digestion) for 2 min under simulated gastric conditions, but without pepsin (C2) were composed of many proteins and peptides with molecular weights (MW) ranging from 250 kDa to 15 kDa (**Figure 28**). The main components were myosin, troponin, tropomyosin and actin. The bands observed in the gel were identified by MW, respectively, as myosin-heavy chain (MHC, 250 kDa); heavy meromyosin subfragment-1 (HMM S1, 140 kDa) and heavy meromyosin subfragment-2 (HMM S2, 130 kDa);  $\alpha$ -actinin (95 kDa); actin (43 kDa); tropomyosin- $\beta$  chain (39 kDa); troponin T (35 kDa); myosin-light chain (MLC1, 23 kDa); troponin I (21 kDa); troponin C (18 kDa); and myosin-light chain (MLC2, 16

kDa) (Kaur *et al.*, 2010a; Xiong, 1997).



**Figure 28.** Tricine SDS-PAGE of meat digested under simulated gastric conditions MW: Molecular weight (kDa); C2, cooked control (injected with water) brisket muscle after *in vitro* gastric digestion for 2 min in the absence of pepsin; K2, cooked CEE-treated brisket muscle after *in vitro* gastric digestion for 2 min in the absence of pepsin; C60, cooked control brisket muscle after *in vitro* gastric digestion for 60 min in the absence of pepsin; K60, cooked CEE-treated brisket muscle after *in vitro* gastric digestion for 60 min in the absence of pepsin; CP2, cooked control brisket muscles after *in vitro* gastric digestion for 2 min in the presence of pepsin; KP2, cooked CEE-treated brisket muscles after *in vitro* gastric digestion for 2 min in the presence of pepsin; CP60, cooked control brisket muscles after *in vitro* gastric digestion for 60 min in the presence of pepsin; KP60, cooked CEE-treated brisket muscles after *in vitro* gastric digestion for 60 min in the presence of pepsin.

The SDS-PAGE pattern of the CEE-treated meat incubated in the absence of pepsin for 2 min (K2) was different from the control counterpart (C2). There was an appearance of peptides with MW < 15 kDa, indicating more protein hydrolysis caused by actinidin from CEE. Also, a new band with MW 28 kDa, which was absent in the

control meat digest, was identified as kiwellin (Boland, 2013) appeared in the CEE-treated digest.

When the cooked control meat was digested with pepsin under gastric conditions for 2 min (CP2), it showed significant digestion indicated by the disappearance or decrease in intensity of bands corresponding to parent meat proteins and appearance of low molecular weight peptides (MW < 10 kDa). The same was observed for the kiwifruit enzyme treated cooked meat digest (KP2).

Several high-MW bands, corresponding to myosin heavy chain (MHC, 250 kDa), and its hydrolysis products- heavy meromyosin subfragment-1 and heavy meromyosin subfragment-2 (HMM S1 & HMM S2, 140 & 130 kDa), appeared on the pattern of control and CEE-treated samples after digestion with pepsin for 2 min (CP2 and KP2, respectively). These may be hydrolysed peptides from large protein components, such as myosin. Moreover, the intensity of the band corresponding to MHC was considerably lower in the CEE-treated digest (KP2) than the control (CP2), showing enhanced hydrolysis in the former.

No positive effect of actinidin could be seen after 60 min of digestion, instead, the intensity of all the bands was observed to be higher in the CEE-treated meat than the counterpart control sample. The reason could be more solubilisation of parent meat proteins in the former as shown in **Figure 28**. Moreover, it appeared that the protein hydrolysing ability of pepsin was too strong, so that the effects of actinidin could not be clearly seen.

These results were mostly in agreement with the study of Kaur *et al.* (2010a) on meat

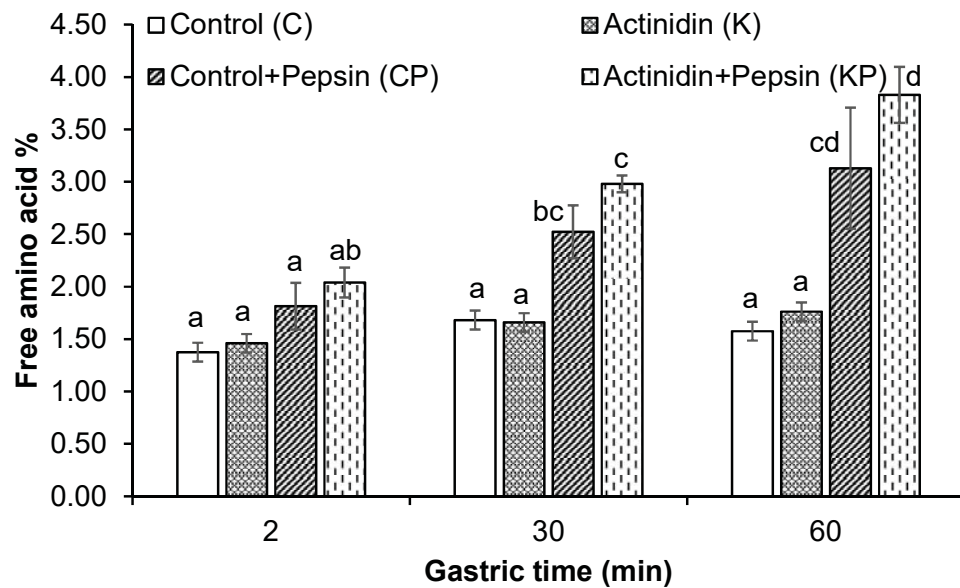
protein digestion after the addition of kiwifruit extract, except that the difference between 'CP60' and 'KP60' was not as visible as reported in that study. The potential reason could be due to the lower dosage (3 mg/mL, 5 % injection amount) of kiwifruit enzyme used in the present study.

Overall, the addition of kiwifruit enzyme (pre-treatment) enhanced the rate of brisket muscle protein breakdown during simulated gastric digestion, both with or without pepsin. These results agreed with those reported by Kaur and Boland (2013), who observed similar effects of kiwifruit extract on the digestibility of meat proteins.

#### **4.3.7.3. Ninhydrin reactive amino acid**

As shown in **Figure 29**, both water-injected control (C) and CEE-treated cooked meats incubated in gastric juice in the absence of pepsin (K) did not show any significant differences in the free amino N release during the simulated gastric digestion. The control (CP) and CEE-treated meat (KP) digested in the presence of pepsin for 2 min did not show any significant difference in amino N compared with 'C' and 'K'.

An increase in amino N release was observed over the digestion course for both 'CP' and 'KP' samples. The increases among the 'KP' samples after 2, 30 and 60 min of digestion were significant. However the increase was not statistically significant among the 'CP' samples digested for 30 and 60 min, indicating no significant change in ninhydrin-reactive amino N for this sample between these digestion times.



**Figure 29.** Ninhydrin reactive amino nitrogen (%) of cooked control (water injected) and CEE-treated meat digests after 2, 30 and 60 min of *in vitro* gastric digestion, both with (CP, KP) or without (C, K) the addition of pepsin

Means ( $n = 3$ ) that do not share a letter are significantly (using Tukey method and 95 % confidence) different.

There was a weak evidence ( $p = 0.054$ ) that the highest amino N (%) was observed from the ‘KP’ samples digested for 60 min, higher than that of ‘CP60’, which potentially supports a positive effect of actinidin on gastric digestion. However, the difference between ‘KP30’ and ‘CP30’ was not statistically significant.

Overall, CEE treatment resulted in higher ninhydrin-reactive amino N (%) in cooked meat digest after 60 min of digestion than in the cooked control sample. This was in agreement with the results of SDS-PAGE that showed more breakdown of muscle proteins in the CEE-treated meat digests, which also resulted in the observed enhanced protein solubility in that CEE-treated sample during gastric digestion. These results suggested that enzymatic (CEE) pre-treatment before *sous vide* cooking of brisket steaks

resulted in an improved rate of beef protein digestion after cooking.

## Chapter 5. Discussion

### 5.1. Thermal inactivation of KE and CEE

This study was planned to investigate thermal inactivation of actinidin in fresh (KE) or commercially available (CEE) kiwifruit extract when heated alone or applied on meat. It provided an overall picture of actinidin thermal inactivation with or without meat, which will contribute to the manipulation of actinidin activity and the understanding of its usage in meat processing.

Thermal inactivation kinetics of actinidin proteolytic activity have been studied and described as the first order reaction kinetics by Katsaros *et al.* (2009b), but results of that study were different from those in the current study. They obtained a higher rate of loss of enzyme activity at similar temperatures. This might be due to the differences in thermal processing parameters, such as sample amount and concentration, containers and difference of water bath device used, or may be ascribed to inherent differences in the kiwifruit, such as the cultivar, ripeness, growing environment or post-harvest storage (Boland, 2013; Chao, 2016).

A significant increase in enzyme activity was observed when KE was heated at low temperatures (35-45 °C) for less than 30 min, which could be ascribed to the mild heat treatment changing the structure of kiwifruit enzyme from proenzyme to an active form. Richardson *et al.* (2004) observed an increase in actinidin activity accompanied with an increase in actinidin steady-state mRNA in kiwifruit during the fruit development when



the temperature was raised. No reports on an increase in enzyme activity upon heating were found in the literature and therefore, additional experiments are required to be done to understand the reason for this increase.

The increase in thermal inactivation temperatures/times of actinidin in extract-meat mixtures compared to extracts alone, showed the possibility of binding between enzyme protein and meat proteins, creating a strong resistance to thermal treatment and providing protection to the actinidin. The increase in viscosity when meat was present, was also assumed to be another possible reason. An alternative possibility was that, at higher temperatures, actinidin hydrolysed itself in the absence of other proteins; and when other proteins from meat were present, the protection of actinidin itself was achieved.

This could also be used to explain the results that actinidin (CEE) in brisket steaks can be inactivated at 70 °C for 30 min, which cannot be used to totally denature the actinidin in homogenised meat. The potential reason could be that the binding of actinidin with meat proteins in steaks was not as thorough as in homogenised meat, or the contact surfaces of actinidin and meat proteins were limited so that their interactions were not well achieved. No report on an increase in enzyme activity upon heating were found in the literature and therefore, additional experiments are required to be done to understand the reason for this increase.

In conclusion, the thermal inactivation kinetics of actinidin in both KE alone and KE-homogenised meat mixtures could be used as a good reference and a practical guideline for other researchers or industries to use actinidin in meat tenderisation and other applications.

## **5.2. Optimisation of method for actinidin application**

In this section, the effectiveness of heat treatment was confirmed on the inactivation of actinidin applied to brisket steaks, along with the study of its tenderisation effects. Injection processing led to more uniform changes in the texture than marinating. Concentrations of CEE solutions ranging from 0.5 mg to 50 mg/mL were applied to steaks and the extent of tenderisation was compared visually. Among different processing parameters, including variations in enzyme concentration, total injection amount and the injection amounts per injection site, the alteration of CEE concentrations had the biggest effect on meat texture. A concentration of 3 mg/mL and injection amount of 5 % (based on total weight increase, w/w) were selected as the optimal injection parameters. The problem of uneven distribution of injected enzyme in steaks was improved by vacuum tumbling for 15 min at 4 °C and use of a small injection volume (0.2 mL/injection site) with narrow injection gap (1 cm apart from each injection site) and fine injection needle (18G), which helped to counteract the problem of over-tenderisation around the injection sites. However, there is still scope for an improvement of the injection techniques in order to achieve more uniform meat texture after enzyme treatment.

## **5.3. Assessment of the final actinidin (CEE)-tenderised *sous vide* cooked brisket steaks**

This section provided a relatively complete picture of the attributes of the actinidin (CEE)-tenderised brisket steaks after *sous vide* cooking. Assessments of pH, colour and

collagen solubility showed insignificant effects from actinidin injection, which indicated the meat kept its basic attributes and did not have any adverse effects after the actinidin treatment.

An improvement in tenderness of brisket was demonstrated by both the instrumental test and sensory evaluation. The sensory evaluation also demonstrated that the actinidin enzymatic pre-treatment improved the juiciness and maintained the original flavour of brisket meat. Results from the TEM clearly demonstrated the effects of actinidin on the structural breakdown of meat myofibrils, particularly around Z line.

The CEE-tenderised meat also improved the rate of protein breakdown during simulated gastric digestion, as shown in SDS-PAGE, which agreed well with TEM results that meat structure was weakened by actinidin action. The weakened structure led to faster protein breakdown, particularly during the initial phase (2 min) of simulated digestion. However, there was no obvious difference in protein profiles of control and actinidin-treated cooked meats after 60 min of digestion. Overall, the actinidin enhanced the rate of muscle protein breakdown during simulated gastric digestion.

Results from ninhydrin-reactive amino N release demonstrated weak evidence ( $p = 0.054$ ) of a higher amino N release after simulated gastric digestion of 60 min, for the CEE-treated cooked meat. This was in agreement with SDS-PAGE and TEM results that showed a greater breakdown of muscle proteins in the CEE-treated meat digests.

Overall, the pre-treatment with actinidin made the *sous vide* cooked beef brisket muscles more tender through myofibrillar structure breakdown, which also improved the rate of meat protein digestion under simulated gastric conditions. These results are in

agreement with the research results of Kaur *et al.* (2010a) and Kaur and Boland (2013) who reported greater protein breakdown during simulated gastric digestion when kiwifruit extract was present. Research of Rutherford *et al.* (2011) also showed that dietary actinidin increased gastric degradability of beef muscle protein determined in the growing rat.

## **5.4. Recommendations for future work**

The results of the whole study could potentially be used in the application of actinidin for meat tenderisation, particularly for improving the texture of low-value tough meat cuts. This research could also be used as a base for further study, to further investigate the increase in actinidin's heat resistance when it is combined with meat proteins. The effects of actinidin on protein breakdown and peptide release during gastro-small intestinal digestion of meat (connective tissue/myofibrillar proteins) need to be investigated in detail using mass spectrometry.

The pre-treatment with kiwifruit enzyme considerably decreased the *sous vide* cooking time for beef brisket compared to the cooking times being used in the meat industry. This decrease may lead to a reduction in the overall processing costs, therefore cost analysis of the enzyme pre-treated *sous vide* cooking processing in comparison with the normal *sous vide* cooking is recommended. Moreover, the results of this study could potentially be used by the meat industry to add value to low-value meat cuts. However, the processing parameters need to be optimised during the scale-up process.



## References

- Aaslyng, M. (2002). Quality indicators for raw meat. In J. Kerry, J. Kerry, & D. Ledward (Eds.), *Meat Processing: Improving Quality* (pp. 157-174). Cambridge, UK: Woodhead Publishing Ltd.
- Adulyatham, P., & Owusu - Apenten, R. (2005). Stabilization and partial purification of a protease from ginger rhizome (*Zingiber officinale* Roscoe). *Journal of Food Science*, *70*, C231-C234.
- Andrés-Bello, A., Barreto-Palacios, V., García-Segovia, P., Mir-Bel, J., & Martínez-Monzó, J. (2013). Effect of pH on color and texture of food products. *Food Engineering Reviews*, *5*, 158-170.
- AOAC. (1990). Protein (Crude) Determination in Animal Feed: Copper Catalyst Kjeldahl Method (984.13). *AOAC Official Method*.
- AOAC. (1993). Hydroxyproline in Meat and Meat Products (990.26). *AOAC Official Method*.
- Arcus, A. (1959). Proteolytic enzyme of *Actinidia chinensis*. *Biochimica et Biophysica Acta*, *33*, 242-244.
- Ashie, I., Sorensen, T., & Nielsen, P. (2002). Effects of papain and a microbial enzyme on meat proteins and beef tenderness. *Journal of Food Science*, *67*, 2138-2142.
- Astruc, T. (2014). Muscle Structure and Digestive Enzyme Bioaccessibility to Intracellular Compartments. In M. Boland, H. Singh, & M. Golding (Eds.), *Food Structures, Digestion and Health* (pp. 193-222). San Diego: Academic Press.
- Auerswald, E. A., Nägler, D. K., Gross, S., Assfalg - Machleidt, I., Stubbs, M. T., Eckerskorn, C., . . . Fritz, H. (1996). Hybrids of chicken cystatin with human kininogen domain 2 sequences exhibit novel inhibition of calpain, improved inhibition of actinidin and impaired inhibition of papain, cathepsin L and cathepsin B. *European Journal of Biochemistry*, *235*, 534-542.
- Bailey, A. J., & Light, N. D. (1989). *Connective tissue in meat and meat products*. London, UK: Elsevier Applied Science.
- Baldwin, D. E. (2012). Sous vide cooking: A review. *International Journal of Gastronomy and Food Science*, *1*, 15-30.

- Barrett, A. J., Woessner, J. F., & Rawlings, N. D. (2012). *Handbook of proteolytic enzymes* (Vol. 1). London, UK: Elsevier Academic Press.
- Baş, D., & Hakkı Boyacı, İ. (2010). Rapid method for quantitative determination of proteolytic activity with cyclic voltammetry. *Electroanalysis*, *22*, 265-267.
- Bekhit, A., Han, J., Morton, J., & Sedcole, R. (2007). Effect of kiwifruit juice and water pre-rigor infusion on lamb quality. *Proceedings of the 53rd International Congress of Meat Science and Technology* (pp. 377-378). Beijing, China.
- Bekhit, A. A., Hopkins, D. L., Geesink, G., Bekhit, A. A., & Franks, P. (2014). Exogenous proteases for meat tenderization. *Critical Reviews in Food Science and Nutrition*, *54*, 1012-1031.
- Bhaskar, N., Sachindra, N., Modi, V., Sakhare, P., & Mahendrakar, N. (2006). Preparation of proteolytic activity rich ginger powder and evaluation of its tenderizing effect on spent - hen muscles. *Journal of Muscle Foods*, *17*, 174-184.
- Bjoerk, I., & Ylinenjaervi, K. (1990). Interaction between chicken cystatin and the cysteine proteinases actinidin, chymopapain A, and ficin. *Biochemistry*, *29*, 1770-1776.
- Boland, M. (1973). *Kinetics and mechanism of proteolytic enzyme catalysed reactions: a thesis presented for the degree of Doctor of Philosophy in Biochemistry*. Massey University.
- Boland, M. (2013). Kiwifruit proteins and enzymes: actinidin and other significant proteins. *Advances in Food And Nutrition Research*, *68*, 59-80.
- Boland, M., & Hardman, M. (1972). Kinetic studies on the thiol protease from *Actinidia chinensis*. *FEBS Letters*, *27*, 282-284.
- Bolumar, T., Enneking, M., Toepfl, S., & Heinz, V. (2013). New developments in shockwave technology intended for meat tenderization: Opportunities and challenges. A review. *Meat science*, *95*, 931-939.
- Boyes, S., Strübi, P., & Marsh, H. (1997). Actinidin levels in fruit of *Actinidia* species and some *Actinidia arguta* rootstock–scion combinations. *LWT-Food Science and Technology*, *30*, 379-389.
- Bratzler, L. J. (1949). Determining the tenderness of meat by use of the Warner-Bratzler method. *Proceedings of the 2nd Reciprocal Meat Conference of the*

*American Meat Science Association* (Vol. 2, pp. 117-121).

- Brewer, M., Zhu, L., Bidner, B., Meisinger, D., & McKeith, F. (2001). Measuring pork color: effects of bloom time, muscle, pH and relationship to instrumental parameters. *Meat Science*, *57*, 169-176.
- Bublin, M. (2013). Kiwifruit allergies. *Advances in Food and Nutrition Research*, *68*, 321-340.
- Bublin, M., Radauer, C., Knulst, A., Wagner, S., Scheiner, O., Mackie, A. R., . . . Breiteneder, H. (2008). Effects of gastrointestinal digestion and heating on the allergenicity of the kiwi allergens Act d 1, actinidin, and Act d 2, a thaumatin - like protein. *Molecular Nutrition & Food Research*, *52*, 1130-1139.
- Calkins, C. R., & Sullivan, G. (2007). Ranking of beef muscles for tenderness. *Universidad de Nebraska*, 1-5.
- Cano, M. P., Hernandez, A., & Ancos, B. d. (1997). High pressure and temperature effects on enzyme inactivation in strawberry and orange products. *Journal of Food Science*, *62*, 85-88.
- Chao, D. (2016). *Actinidin: the predominant protease in kiwifruit: a thesis presented in partial fulfilment of the requirements for the degree of Master of Philosophy in Food technology at Massey University, Manawatū, New Zealand*. Massey University.
- Chiavaro, E., Rinaldi, M., Vittadini, E., & Barbanti, D. (2009). Cooking of pork Longissimus dorsi at different temperature and relative humidity values: Effects on selected physico-chemical properties. *Journal of Food Engineering*, *93*, 158-165.
- Christensen, M., Tørngren, M. A., Gunvig, A., Rozlosnik, N., Lametsch, R., Karlsson, A. H., & Ertbjerg, P. (2009). Injection of marinade with actinidin increases tenderness of porcine M. biceps femoris and affects myofibrils and connective tissue. *Journal of the Science of Food and Agriculture*, *89*, 1607-1614.
- Ciardello, M. A., D'Avino, R., Amoresano, A., Tuppo, L., Carpentieri, A., Carratore, V., . . . Camardella, L. (2008). The peculiar structural features of kiwi fruit pectin methylesterase: Amino acid sequence, oligosaccharides structure, and modeling of the interaction with its natural proteinaceous inhibitor. *Proteins*:



- Structure, Function, and Bioinformatics*, 71, 195-206.
- Craigie, C., Wagstaff, T., Cullingworth, I., Ross, D., & Maltin, C. A. (2014). *The relationship between slice shear force and tenderness of beef assessed by an untrained consumer sensory panel*.
- D'avino, R., Bernardi, M., Wallner, M., Palazzo, P., Camardella, L., Tuppo, L., . . . Ciardiello, M. A. (2011). Kiwifruit Act d 11 is the first member of the ripening - related protein family identified as an allergen. *Allergy*, 66, 870-877.
- Dinh, N. T. T. (2006). Meat quality: understanding of meat tenderness and influence of fat content on meat flavor. *Science & Technology Development*, 9, 12.
- Dransfield, E. (1996). Calpains from thaw rigor muscle. *Meat Science*, 43, 311-320.
- Drummond, L. (2013). The Composition and Nutritional Value of Kiwifruit. *Advances in Food and Nutrition Research*, 68, 33-57.
- Du Toit, E., & Oguttu, J. W. (2013). Calpain and Calpastatin activity post mortem and meat tenderness: are the two related? *Journal of Animal and Veterinary Advances*, 12, 683-688.
- Etherington, D. J. (1987). Collagen and meat quality: Effects of conditioning and growth rate. In T. R. Dutson & A. J. Bailey (Eds.), *Advances in Meat Research* (pp. 351-360). New York: Van Nostrand Reinhold.
- Feiner, G. (2006). *Meat products handbook: Practical science and technology*. Cambridge, UK: Woodhead Publishing Ltd.
- Ferguson, A. (1990). Botanical nomenclature: *Actinidia chinensis*, *Actinidia deliciosa* and *Actinidia setosa*. In I. J. Warrington & G. C. Weston (Eds.), *Kiwifruit: science and management* (pp. 36-57). Auckland: Ray Richards in association with New Zealand Society for Horticultural Science.
- Foegeding, E. A., & Larick, D. (1986). Tenderization of beef with bacterial collagenase. *Meat Science*, 18, 201-214.
- Funaki, J., Yano, M., Arai, S., & Abe, K. (1995). Inhibiting the activity of actinidin by oryzacystatin for the application of fresh kiwifruit to gelatin - based foods. *Journal of Food Biochemistry*, 19, 355-365.
- Gavrović-Jankulović, M., Ćirković, T., Vučković, O., Atanasković-Marković, M., Petersen, A., Gojgić, G., . . . Jankov, R. M. (2002). Isolation and biochemical

- characterization of a thaumatin-like kiwi allergen. *Journal of Allergy and Clinical Immunology*, *110*, 805-810.
- Gerrard, D. E., & Grant, A. L. (2003). *Principles of animal growth and development*. USA: Kendall Hunt Publishing.
- Gouzi, H., Depagne, C., & Coradin, T. (2011). Kinetics and thermodynamics of the thermal inactivation of polyphenol oxidase in an aqueous extract from *Agaricus bisporus*. *Journal of Agricultural and Food Chemistry*, *60*, 500-506.
- Grzonka, Z., Kasprzykowski, F., & Wiczak, W. (2007). Cysteine proteases. In J. Polaina & A. P. MacCabe (Eds.), *Industrial enzymes* (pp. 181-195). New York, USA: Springer.
- Ha, M., Bekhit, A. E.-D. A., Carne, A., & Hopkins, D. L. (2012). Characterisation of commercial papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat proteins. *Food Chemistry*, *134*, 95-105.
- Ha, M., Bekhit, A. E. D., Carne, A., & Hopkins, D. L. (2013). Comparison of the proteolytic activities of new commercially available bacterial and fungal proteases toward meat proteins. *Journal of Food Science*, *78*, C170-C177.
- Hammes, G. (1982). *Enzyme catalysis and regulation*. New York, USA: Academic Press.
- Han, J., Morton, J., Bekhit, A., & Sedcole, J. (2009). Pre-rigor infusion with kiwifruit juice improves lamb tenderness. *Meat Science*, *82*, 324-330.
- Hartree, E. F. (1972). Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Analytical Biochemistry*, *48*, 422-427.
- Herring, H. K., Cassens, R. G., & Risky, E. J. (1965). Further Studies on Bovine Muscle Tenderness as Influenced by Carcass Position, Sarcomere Length, and Fiber Diameter. *Journal of Food Science*, *30*, 1049-1054.
- Huerta-Montauti, D., Miller, R., Pfeiffer, C. S., Pfeiffer, K., Nicholson, K., Osburn, W., & Savell, J. (2008). Identifying muscle and processing combinations suitable for use as beef for fajitas. *Meat Science*, *80*, 259-271.
- Hunt, M., Acton, J., Benedict, R., Calkins, C., Cornforth, D., Jeremiah, L., . . . Shivas, S. (1991). Amsa guidelines for meat color evaluation. *Proceedings of the 44th Annual Reciprocal Meat Conference* (pp. 3-17). Kansas State University, Manhattan, KS.

- Hunt, M., King, A., Barbut, S., Clause, J., Cornforth, D., Hanson, D., . . . Mohan, A. (2012). AMSA meat color measurement guidelines. *American Meat Science Association, Champaign, Illinois USA, 61820*, 1-135.
- Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). In vitro human digestion models for food applications. *Food Chemistry, 125*, 1-12.
- Hutchins, B. K., Liu, T. H. P., & Watts, B. M. (1967). Effect of Additives and Refrigeration on Reducing Activity, Metmyoglobin and Malonaldehyde Of Raw Ground Beef. *Journal of Food Science, 32*, 214-217.
- Hwang, I., Devine, C., & Hopkins, D. (2003). The biochemical and physical effects of electrical stimulation on beef and sheep meat tenderness. *Meat Science, 65*, 677-691.
- Jayasooriya, S. D., Torley, P., D'arcy, B. R., & Bhandari, B. R. (2007). Effect of high power ultrasound and ageing on the physical properties of bovine Semitendinosus and Longissimus muscles. *Meat Science, 75*, 628-639.
- Jung, S., de Lamballerie-Anton, M., & Ghoul, M. (2000). Modifications of ultrastructure and myofibrillar proteins of post-rigor beef treated by high pressure. *LWT-Food Science and Technology, 33*, 313-319.
- Katsaros, G., Katapodis, P., & Taoukis, P. (2009a). High hydrostatic pressure inactivation kinetics of the plant proteases ficin and papain. *Journal of Food Engineering, 91*, 42-48.
- Katsaros, G., Katapodis, P., & Taoukis, P. (2009b). Modeling the effect of temperature and high hydrostatic pressure on the proteolytic activity of kiwi fruit juice. *Journal of Food Engineering, 94*, 40-45.
- Katsaros, G. I., Tavantzis, G., & Taoukis, P. S. (2010). Production of novel dairy products using actinidin and high pressure as enzyme activity regulator. *Innovative Food Science & Emerging Technologies, 11*, 47-51.
- Kaur, L., Astruc, T., Vénien, A., Loison, O., Cui, J., Irastorza, M., & Boland, M. (2016). High pressure processing of meat: effects on ultrastructure and protein digestibility. *Food & function, 7*, 2389-2397.
- Kaur, L., & Boland, M. (2013). Influence of kiwifruit on protein digestion. *Advances in Food and Nutrition Research, 68*, 149-167.
- Kaur, L., Maudens, E., Haisman, D. R., Boland, M., & Singh, H. (2014). Microstructure

and protein digestibility of beef: The effect of cooking conditions as used in stews and curries. *LWT - Food Science and Technology*, 55, 612-620.

- Kaur, L., Rutherford, S. M., Moughan, P. J., Drummond, L., & Boland, M. (2010a). Actinidin enhances gastric protein digestion as assessed using an in vitro gastric digestion model. *Journal of Agricultural and Food Chemistry*, 58, 5068-5073.
- Kaur, L., Rutherford, S. M., Moughan, P. J., Drummond, L., & Boland, M. (2010b). Actinidin enhances protein digestion in the small intestine as assessed using an in vitro digestion model. *Journal of Agricultural and Food Chemistry*, 58, 5074-5080.
- Kays, W. M. (2012). *Convective heat and mass transfer*: Tata McGraw-Hill Education.
- Keith, F. K. M., Vol, D. L. D., Miles, R. S., Bechtel, P. J., & Carr, T. R. (1985). Chemical and Sensory Properties of Thirteen Major Beef Muscles. *Journal of Food Science*, 50, 869-872.
- Kholif, A., Mahran, G., El-Nawawy, M., Ismail, A. A., Salem, M., & Zaky, W. (2011). Evaluation of proteolytic activity of some dairy lactobacilli. *World Journal of Dairy & Food Sciences*, 6, 21-26.
- Killefer, J. (2004). Effect of enhancement of pork and beef on post mortem events *Proceedings of the 59th Reciprocal Meats Conference* (pp. 20-23). Lexington, Kentucky.
- Kolar, K. (1990). Colorimetric determination of hydroxyproline as measure of collagen content in meat and meat products: NMKL collaborative study. *J Assoc Off Anal Chem*, 73, 54-57.
- Kramer, D. E., & Whitaker, J. R. (1964). Ficus enzymes II. Properties of the proteolytic enzymes from the latex of *Ficus carica* variety Kadota. *Journal of Biological Chemistry*, 239, 2178-2183.
- Ladd, D. L., & Snow, R. A. (1993). Reagents for the preparation of chromophorically labeled polyethylene glycol-protein conjugates. *Analytical biochemistry*, 210, 258-261.
- Lagerstedt, Å., Enfält, L., Johansson, L., & Lundström, K. (2008). Effect of freezing on sensory quality, shear force and water loss in beef *M. longissimus dorsi*. *Meat Science*, 80, 457-461.

- Lantto, R., Kruus, K., Puolanne, E., Honkapää, K., Roininen, K., & Buchert, J. (2009). Enzymes in Meat Processing. In R. J. Whitehurst & M. van Oort (Eds.), *Enzymes in Food Technology* (pp. 264-291). Chichester, UK: Wiley-Blackwell.
- Lawrence, T. L. J., Fowler, V. R., & Novakofski, J. E. (2012). *Growth of farm animals*. Oxfordshire, UK: CABI.
- Lawrie, R. A. (1974). *Meat Science* (2nd ed.). Oxford, UK: Pergamon press.
- Leak, F. W., Kemp, J. D., Fox, J. D., & Langlois, B. E. (1987). Effects of Boning Time, Mechanical Tenderization and Partial Replacement of Sodium Chloride on the Quality and Microflora of Boneless Dry-Cured Ham. *Journal of Food Science*, *52*, 263-266.
- Lewis, D. A., & Luh, B. (1988). Application of actinidin from kiwifruit to meat tenderization and characterization of beef muscle protein hydrolysis. *Journal of Food Biochemistry*, *12*, 147-158.
- Leygonie, C., Britz, T. J., & Hoffman, L. C. (2012). Impact of freezing and thawing on the quality of meat: Review. *Meat Science*, *91*, 93-98.
- Lindahl, P., Abrahamson, M., & Björk, I. (1992). Interaction of recombinant human cystatin C with the cysteine proteinases papain and actinidin. *Biochemical Journal*, *281*, 49-55.
- Liu, C., Xiong, Y., & Rentfrow, G. (2011). Kiwifruit protease extract injection reduces toughness of pork loin muscle induced by freeze-thaw abuse. *LWT-Food Science and Technology*, *44*, 2026-2031.
- Livisay, S., Xiong, Y., & Moody, W. (1996). Proteolytic activity and calcium effect in dark-firm-dry and pale-soft-exudative meat. *LWT-Food Science and Technology*, *29*, 123-128.
- Livney, Y. D., Ruimy, E., Ye, A. M., Zhu, X., & Singh, H. (2017). A milkfat globule membrane-inspired approach for encapsulation of emulsion oil droplets. *Food Hydrocolloids*, *65*, 121-129.
- Mancini, R., & Hunt, M. (2005). Current research in meat color. *Meat Science*, *71*, 100-121.
- Maróstica, M. R., & Pastore, G. M. (2010). Some nutritional, technological and environmental advances in the use of enzymes in meat products. *Enzyme Research*, *2010*.

- Marsh, B. T., & Leet, N. G. (1966). Studies in meat tenderness. III. The effects of cold shortening on tenderness. *Journal of Food Science*, *31*, 450-459.
- McDowall, M. A. (1973). The action of proteinase A2 of *Actinidia chinensis* on the B-chain of oxidized insulin. *Biochimica et Biophysica Acta (BBA)-Enzymology*, *293*, 226-231.
- Melendo, J., Beltrán, J., Jaime, I., Sancho, R., & Roncalés, P. (1996). Limited proteolysis of myofibrillar proteins by bromelain decreases toughness of coarse dry sausage. *Food Chemistry*, *57*, 429-433.
- Mendiratta, S. K., Sharma, B. D., Narayan, R., & Mane, B. G. (2010). Effect Of Proteolytic Enzyme Treatments And Pressure Cooking On Quality Of Spent Sheep Meat Curry. *Journal of Muscle Foods*, *21*, 685-701.
- Miller, A., Strange, E., & Whiting, R. (1989). Improved Tenderness of Restructured Beef Steaks by a Microbial Collagenase Derived from *Vibrio B - 30*. *Journal Of Food Science*, *54*, 855-857.
- Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., . . . Dupont, D. (2014). A standardised static in vitro digestion method suitable for food—an international consensus. *Food & Function*, *5*, 1113-1124.
- Morimoto, K., Furuta, E., Hashimoto, H., & Inouye, K. (2006). Effects of high concentration of salts on the esterase activity and structure of a kiwifruit peptidase, actinidain. *Journal Of Biochemistry*, *139*, 1065-1071.
- MPI. (2017). *Food Control Plan- Serve safe*. Ministry for Primary Industries Retrieved from <https://www.mpi.govt.nz/food-safety/food-act-2014/forms-and-templates/>.
- Muchenje, V., Dzama, K., Chimonyo, M., Strydom, P., & Raats, J. (2009). Relationship between pre-slaughter stress responsiveness and beef quality in three cattle breeds. *Meat Science*, *81*, 653-657.
- Naveena, B., & Mendiratta, S. (2004). The tenderization of buffalo meat using ginger extract. *Journal of Muscle Foods*, *15*, 235-244.
- Nieto, G., Díaz, P., Bañón, S., & Garrido, M. D. (2010). Effect on lamb meat quality of including thyme (*Thymus zygis* ssp. *gracilis*) leaves in ewes' diet. *Meat Science*, *85*, 82-88.
- Nieuwenhuizen, N. J., Maddumage, R., Tsang, G. K., Fraser, L. G., Cooney, J. M., De Silva,

- H. N., . . . Atkinson, R. G. (2012). Mapping, complementation, and targets of the cysteine protease actinidin in kiwifruit. *Plant Physiology*, *158*, 376-388.
- Nishimura, T., Hattori, A., & Takahashi, K. (1995). Structural weakening of intramuscular connective tissue during conditioning of beef. *Meat Science*, *39*, 127-133.
- Nishiyama, I. (2001). pH-Dependent Proteolytic Effects of Actinidin on Myofibrillar Proteins. *Journal of Home Economics of Japan*, *52*, 1083-1089.
- Nishiyama, I. (2007). Fruits of the Actinidia genus. *Advances In Food And Nutrition Research*, *52*, 293-324.
- Nute, G. (1996). Assessment by sensory and consumer panelling. In A. A. Taylor, A. Raimundo, M. Severini, & F. Smulders (Eds.), *Meat Quality and Meat Packaging* (pp. 243-255). Utrecht, Netherlands: ECCEAMST.
- Nute, G. R. (1999). Sensory assessment of poultry meat quality. In R. I. Richardson & G. C. Mead (Eds.), *Poultry Meat Science* (pp. 369-376). Wallingford, UK: CAB International.
- Ogino, K., & Fujihara, H. (1992). Biochemical reactions involving thioesters. In S. Oae & T. Okuyama (Eds.), *Organic sulfur chemistry: biochemical aspects* (pp. 71-136). Florida, USA: CRC Press.
- Ouali, A. (1990). Meat tenderization: Possible causes and mechanisms. A review. *Journal of Muscle Foods*, *1*, 129-165.
- Ouali, A., & Talmant, A. (1990). Calpains and calpastatin distribution in bovine, porcine and ovine skeletal muscles. *Meat Science*, *28*, 331-348.
- Paul, W., Amiss, J., Try, R., Praekelt, U., Scott, R., & Smith, H. (1995). Correct processing of the kiwifruit protease actinidin in transgenic tobacco requires the presence of the C-terminal propeptide. *Plant Physiology*, *108*, 261-268.
- Penny, I. F. (1980). The enzymology of conditioning. In R. A. Lawrie (Ed.), *Developments in Meat Science* (Vol. 1). London/New York: Elsevier Applied Science.
- Pietrasik, Z., Aalhus, J., Gibson, L., & Shand, P. (2010). Influence of blade tenderization, moisture enhancement and pancreatin enzyme treatment on the processing characteristics and tenderness of beef semitendinosus muscle. *Meat Science*, *84*, 512-517.

- Piggott, J. R., Simpson, S. J., & Williams, S. A. (1998). Sensory analysis. *International Journal Of Food Science & Technology*, *33*, 7-12.
- Polkinghorne, R., Philpott, J., Gee, A., Doljanin, A., & Innes, J. (2008). Development of a commercial system to apply the Meat Standards Australia grading model to optimise the return on eating quality in a beef supply chain. *Animal Production Science*, *48*, 1451-1458.
- Prækelt, U. M., McKee, R. A., & Smith, H. (1988). Molecular analysis of actinidin, the cysteine proteinase of *Actinidia chinensis*. *Plant Molecular Biology*, *10*, 193-202.
- Purslow, P. P. (2005). Intramuscular connective tissue and its role in meat quality. *Meat Science*, *70*, 435-447.
- Quaglia, G., Lombardi, M., Sinesio, F., Bertone, A., & Menesatti, P. (1992). Effect of enzymatic treatment on tenderness characteristics of freeze-dried meat. *Lebensmittel-Wissenschaft and Technologie*, *25*, 143-145.
- R Core Team. (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- Richardson, A., Marsh, K., Bolding, H., Pickering, A., Bulley, S., Frearson, N., . . . Macrae, E. (2004). High growing temperatures reduce fruit carbohydrate and vitamin C in kiwifruit. *Plant, Cell & Environment*, *27*, 423-435.
- Robbins, K., Jensen, J., Ryan, K. J., Homco-Ryan, C., McKeith, F. K., & Brewer, M. S. (2002). Enhancement effects on sensory and retail display characteristics of beef rounds. *Journal of Muscle Foods*, *13*, 279-288.
- Rutherford-Markwick, K. J., & Moughan, P. J. (2005). Bioactive peptides derived from food. *Journal of AOAC International*, *88*, 955-966.
- Rutherford, S. M., Montoya, C. A., Zou, M. L., Moughan, P. J., Drummond, L. N., & Boland, M. (2011). Effect of actinidin from kiwifruit (*Actinidia deliciosa* cv. Hayward) on the digestion of food proteins determined in the growing rat. *Food Chemistry*, *129*, 1681-1689.
- Sadana, A. (1988). Enzyme deactivation. *Biotechnology Advances*, *6*, 349-IN342.
- Salles, C., Tarrega, A., Mielle, P., Maratray, J., Gorria, P., Liaboeuf, J., & Liodenot, J. J. (2007). Development of a chewing simulator for food breakdown and the



- analysis of in vitro flavor compound release in a mouth environment. *Journal of Food Engineering*, 82, 189-198.
- Sarath, G., De La Motte, R. S., & Wagner, F. W. (1989). Protease assay methods. In R. J. Beynon & J. S. Bond (Eds.), *Proteolytic enzymes: a practical approach* (pp. 83-104). Oxford: IRL.
- Schwimmer, S. (1981). Applied enzymology of meat texture optimization. *Source book of food enzymology* (pp. 481-496). Westport, USA: AVI Publishing Company, Inc.
- Seggern, D. D. V., Calkins, C. R., Johnson, D. D., Brickler, J. E., & Gwartney, B. L. (2005). Muscle profiling: Characterizing the muscles of the beef chuck and round. *Meat Science*, 71, 39-51.
- Shackelford, S., Wheeler, T., & Koohmaraie, M. (1999). Evaluation of slice shear force as an objective method of assessing beef longissimus tenderness. *Journal of Animal Science*, 77, 2693-2699.
- Sigma-Aldrich. (2003). Ninhydrin reagent solution-product information. [https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product Information Sheet/2/n7285pis.pdf](https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product%20Information%20Sheet/2/n7285pis.pdf)
- Smith, J., & Hong-Shum, L. (2003). Enzymes. *Food additives data book* (pp. 389-462). Oxford, UK: Blackwell Science.
- Smith, S., & Judge, M. (1991). Relationship between pyridinoline concentration and thermal stability of bovine intramuscular collagen. *Journal of Animal Science*, 69, 1989-1993.
- Smyth, C., & Arbuthnott, J. (1974). Properties of Clostridium Perfringens (Welch II) Type-A  $\alpha$ -Toxin (Phospholipase c) Purified By Electrofocusing. *Journal of medical microbiology*, 7, 41-66.
- Storer, A., & Ménard, R. (1994). Catalytic mechanism in papain family of cysteine peptidases. *Methods in Enzymology*, 244, 486.
- Sugiyama, S., Ohtsuki, K., Sato, K., & Kawabata, M. (1996). Purification and characterization of six kiwifruit proteases isolated with two ion-exchange resins, Toyopearl-SuperQ and Bakerbond WP-PEI. *Bioscience, Biotechnology, and Biochemistry*, 60, 1994-2000.
- Sullivan, G. A., & Calkins, C. (2010). Application of exogenous enzymes to beef muscle

- of high and low-connective tissue. *Meat Science*, *85*, 730-734.
- Sun, Q., Zhang, B., Yan, Q., & Jiang, Z. (2016). Comparative analysis on the distribution of protease activities among fruits and vegetable resources. *Food Chemistry*, *213*, 708-713.
- Takagi, H., Kondou, M., Hisatsuka, T., Nakamori, S., YING-CHIEH, T., & Yamasaki, M. (1992). Effects of an alkaline elastase from an alkalophilic *Bacillus* strain on the tenderization of beef meat. *Journal of Agricultural and Food Chemistry*, *40*, 2364-2368.
- Thompson, E., Wolf, I., & Allen, C. (1973). Ginger rhizome: A new source of proteolytic enzyme. *Journal of Food Science*, *38*, 652-655.
- Toohy, E., Kerr, M., van de Ven, R., & Hopkins, D. (2011). The effect of a kiwi fruit based solution on meat traits in beef m. semimembranosus (topside). *Meat Science*, *88*, 468-471.
- Torrescano, G., Sánchez-Escalante, A., Giménez, B., Roncalés, P., & Beltrán, J. A. (2003). Shear values of raw samples of 14 bovine muscles and their relation to muscle collagen characteristics. *Meat Science*, *64*, 85-91.
- Varnam, A., & Sutherland, J. (1996) *Meat and meat products: technology, chemistry and microbiology* (pp. 46-120). London: Chapman and Hall.
- Veiseth, E., Shackelford, S., Wheeler, T., & Koochmarai, M. (2004). Factors regulating lamb longissimus tenderness are affected by age at slaughter. *Meat Science*, *68*, 635-640.
- Venable, J. H., & Coggeshall, R. (1965). A simplified lead citrate stain for use in electron microscopy. *The Journal of Cell Biology*, *25*, 407.
- Wada, M., Suzuki, T., Yaguti, Y., & Hasegawa, T. (2002). The effects of pressure treatments with kiwi fruit protease on adult cattle semitendinosus muscle. *Food Chemistry*, *78*, 167-171.
- Ward, C., & Courtney, D. (2013). Kiwifruit: taking its place in the global fruit bowl. *Advances in Food and Nutrition Research*, *68*, 1-14.
- Warriss, P. D. (2001). *Meat science* (2nd ed.). Oxfordshire, UK: CABI.
- Wattenbarger, C. J. (1965). U.S. Patent No. US3216826 A. Washington, DC: U. S. Patent.
- Wretling, B., & Wadström, T. (1977). Purification and properties of a protease with elastase activity from *Pseudomonas aeruginosa*. *Microbiology*, *103*, 319-327.

- Xiong, Y. (1997). Structure-function relationships of muscle proteins. In S. Damodaran & A. Paraf (Eds.), *Food Proteins and Their Applications* (pp. 341-392). New York: Marcel Dekker Ltd.
- Yeh, C., Yang, M., & Tsai, Y. (2002). Application potency of engineered G159 mutants on P1 substrate pocket of subtilisin YaB as improved meat tenderizers. *Journal of Agricultural and Food Chemistry*, *50*, 6199-6204.
- Zhang, B., Sun, Q., Liu, H., Li, S., & Jiang, Z. (2017). Characterization of actinidin from Chinese kiwifruit cultivars and its applications in meat tenderization and production of angiotensin I-converting enzyme (ACE) inhibitory peptides. *LWT-Food Science and Technology*, *78*, 1-7.

# Appendices

## Appendix A. Letter of low risk notification



Date: 05 May 2017

Dear Xiaojie Zhu

Re: Ethics Notification - **4000017710** - **Sensory study on kiwifruit enzyme-treated Sous vide brisket steak**

Thank you for your notification which you have assessed as Low Risk.

Your project has been recorded in our system which is reported in the Annual Report of the Massey University Human Ethics Committee.

The low risk notification for this project is valid for a maximum of three years.

If situations subsequently occur which cause you to reconsider your ethical analysis, please go to <http://irms.massey.ac.nz> and register the changes in order that they be assessed as safe to proceed.

Please note that travel undertaken by students must be approved by the supervisor and the relevant Pro Vice-Chancellor and be in accordance with the Policy and Procedures for Course-Related Student Travel Overseas. In addition, the supervisor must advise the University's Insurance Officer.

**A reminder to include the following statement on all public documents:**

*"This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researcher(s) named in this document are responsible for the ethical conduct of this research."*

*If you have any concerns about the conduct of this research that you want to raise with someone other than the researcher(s), please contact Dr Brian Finch, Director - Ethics, telephone 06 3569099 ext 86015, email [humanethics@massey.ac.nz](mailto:humanethics@massey.ac.nz).*

Please note, if a sponsoring organisation, funding authority or a journal in which you wish to publish requires evidence of committee approval (with an approval number), you will have to complete the application form again, answering "yes" to the publication question to provide more information for one of the University's Human Ethics Committees. You should also note that such an approval can only be provided prior to the commencement of the research.

Yours sincerely

Dr Brian Finch  
Chair, Human Ethics Chairs' Committee and Director (Research Ethics)

Appendix B. Permission from Elsevier for reprinting **Table1 & Figure 1.**

7/27/2017

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Jul 26, 2017

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