MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Actinidin- the predominant protease in kiwifruit

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Studies serve for delight, for ornament, and for ability.

---------- Francis Bacon

ABSTRACT

Kiwifruit protein (actinidin) has been widely known as a protease. Kiwifruit protein has the potential of utilization in food industry as an enzyme that aids food digestion.

In this project, the soluble kiwifruit proteins were extracted from fresh Hayward and SunGold kiwifruit. Soluble kiwifruit proteins were analysed by the Hartree-Lowry method, SDS-PAGE, enzyme activity determination, ion-exchange chromatography and mass spectrometry. Anti-actinidin antibodies were raised by the injection of purified actinidin into rabbits. The main soluble kiwifruit protein was recognized by anti-actinidin antibodies using Western blot. Moreover, the effects of post-harvest storage on protein content, total enzyme activity and specific enzyme activity were investigated. Comparable studies on both Hayward and SunGold kiwifruit were also carried out in this project.

The results showed that Hayward and SunGold kiwifruit had a similar protein content. However, the total enzyme activity of Hayward kiwifruit was about 8 times higher than that of SunGold kiwifruit. The protein with enzyme activity (active actinidin) had a molecular weight of about 27 kDa according to SDS-PAGE and was one of main soluble proteins in Hayward and SunGold kiwifruit. This protease was purified from fresh kiwifruit by anion-exchange chromatography. A polyclonal antibody against actinidin was successfully generated in a rabbit using purified actinidin. Protein with a molecular weight of 27 kDa was recognized by the anti-actinidin antibodies. Post-harvest storage at 1 °C for up to 12 weeks significantly increased the total and specific enzyme activities of SunGold kiwifruit ($P<0.05$). By contrast, the total and specific enzyme activities of Hayward kiwifruit had a significant decrease after 16 weeks'

storage ($P<0.05$). Hayward kiwifruit had no significant changes in protein content after storage ($P<0.05$) while the protein content of SunGold kiwifruit fluctuated in a range from 5.04 to 5.84 mg/mL during post-harvest storage.

This study may help to understand the nature of kiwifruit proteins with enzyme activity, which contributes to a full understanding of the health benefits of kiwifruit.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

N-α-CBZ-lysine- pNP	N-α-carbobenzoxy-L-lysine-p-nitrophenyl ester
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
cDNA	Complementary DNA
mRNA	Messenger RNA
BSA	Bovine serum albumin
%	All percentages are weight/volume (w/v) unless otherwise stated
Tris	Tris (hydroxymethyl) amino methane

Amino acid abbreviations:

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine

Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

CHAPTER 1 INTRODUCTION

The first known kiwifruit plants in New Zealand were described in a personal diary dated 1910 (Ferguson, 2004). In the 1950s, kiwifruit had been used for fruit production in a few commercial orchards in New Zealand (Ferguson, 2004). As one of the most popular commercially produced fruits in the world, it has been suggested kiwifruit contributes health benefits to human beings. It has been found that there is high content of vitamin C in the fruit flesh and a large amount of vitamin E (α-tocopherol) in fruit seeds. Kiwifruit also provides folate, Vitamin K and minerals (Ferguson & Ferguson, 2002).

One of the major soluble proteins in kiwifruit, especially those with green flesh, is actinidin. Actinidin is a cysteine protease from kiwifruit, similar to bromelain in pineapple stem, ficain in fig or papain in papaya (Boland, 2013). This family of proteases shares similarities in the sequence and mode of action (Kamphuis, Drenth & Baker, 1985). So far, the physiological role of actinidin in kiwifruit is not clear. Actinidin was first reported by Arcus in 1959. The reason why he paid attention to this protein is because of a well-known household fact: fresh kiwifruit prevents table jelly setting (Arcus, 1959). Nowadays, the applications for actinidin range from its use as a meat tenderizer, to its use as an aid to human digestion. It has been proven that the proteolytic activity of actinidin enhances protein digestion both in vitro and in vivo (Kaur et al., 2010a, 2010b; Rutherfurd et al., 2011). Meanwhile, actinidin has been suggested to be the major potential allergen in kiwifruit. There is literature which has reported actinidin to be both immunogenic and allergenic at relatively low doses when compared to haemoglobin (Dearman et al., 2014).

Both active and inactive isoforms of actinidin are thought to be present in kiwifruit (Grozdanovic et al., 2012), as the protease is expressed as an inactive zymogen approximately 15 kDa larger than the native enzyme, with both N- and C-terminal extensions (Praekelt, McKee & Smith, 1988). Although mRNAs encoding actinidin isoforms have been identified (Praekelt, McKee & Smith, 1988), as far as we know, no studies have been done to understand the processes of post-translational modification of pre-pro actinidin and the mechanism(s) of activation in kiwifruit. Moreover, there is no explanation for different actinidin forms having different specific enzyme activities. The results of this study will be a guide to a further project which will involve the use of electrospray mass spectrometry of fruit extracts to identify the stages of post-translational processing of the actinidin proteins in ripening fruit.

The main objectives of my project were: a) determining protein content and enzyme activity of kiwifruit; b) effectively purifying actinidin from fresh kiwifruit; c) raising anti-actinidin antibodies using purified actinidin and identifying actinidin; d) investigating the relationship between actinidin activity and post-harvest storage. The purpose of this study was to understand the active forms of actinidin and the mechanism of actindin activiation during post-harvest storage at a preliminary level.

CHAPTER 2 LITERATURE REVIEW

2.1 Kiwifruit

Although now produced by a number of other countries, kiwifruit is an iconic agro-product of New Zealand. In the international trade today, two predominant kiwifruit cultivars are green-fleshed cultivar *Actinidia deliciosa* cv. 'Hayward' and yellow-fleshed cultivar *Actinidia chinensis* cv. 'Zesy002'. They are now commercialized under the names ZESPRI™ Green kiwifruit and ZESPRI™ SunGold kiwifruit respectively. It is necessary to note that SunGold kiwifruit (*Actinidia chinensis* cv. 'Zesy002') is a new and distinct kiwifruit variety which has been recently disclosed. Distinguished from the former commercially available yellow flesh kiwifruit (*Actinidia chinensis* cv. 'Hort16'), the fruit of this new kiwifruit variety is characterized by large fruit size, high yield potential, ovoid shape, yellow flesh colour, and early harvest maturity (Lowe, 2010). 2015 is the first year when it was commercially available.

Globally, the kiwifruit is very popular in the human diet because it has pleasant taste and it is a highly nutritious, and low calorie fruit. In green kiwifruit, actinidin is the predominant soluble protein. This cysteine protease has been suggested as meat-tenderizer and health-promoting diet supplement (Lewis & Luh, 1988a; Montoya et al., 2014).

2.2 Major soluble proteins in kiwifruit

Although kiwifruit is not a significant source of protein, kiwifruit protein is one of the most common causes of food allergic reactions (Bublin et al., 2010). Actinidin constitutes up to 30% - 60% of soluble protein in kiwifruit (Paul et al., 1995; Prestamo, 1995; Boyes, Strubi & Marsh, 1997b). It is a proteolytic enzyme that

belongs to the cysteine proteinase family (EC 3.4.22.14). Actinidin is concentrated in kiwifruit flesh (Prestamo, 1995). Results from immune-detection show that kiwifruit contains 3800 times more actinidin than leaf, and 11500 times more than apex tissue (Praekelt, McKee & Smith, 1988). So far, it is not clear where actinidin is located in the fruit cell. The large highly vacuolated nature of the *Actinidia* fruit cells, coupled with the absence of an efficient transformation system for this species, make it technically difficult to determine the precise location of actinidin and to determine the function of the actinidin pro-peptide sequences. Paul et al. (1995) characterized actinidin that had been cloned and expressed in transgenic tobacco. Their preliminary results showed that mature actinidin accumulates in the vacuoles of transgenic tobacco plants.

Kiwellin is a protein which accounts for 20% to 30% of total soluble protein in kiwifruit (Tamburrini et al., 2005). Kiwellin is a protein which has 189 amino acid residues. It appears as 28 kDa molecular mass in gel electrophoresis with the calculated mass of 19,968 from sequence data. As far as we know, there has been no literature reported on the biological function of kiwellin. Another soluble protein identified in kiwifruit is thaumatin-like protein. It has an apparent 24 kDa molecular mass on gel electrophoresis (Gavrovic-Jankulovic et al., 2002). Kirola is another kiwifruit protein which has been recently been purified and sequenced (D'Avino et al., 2011). Its true molecule mass is 17.460 Da, and is seen as a 17 kDa protein in gel electrophoresis.

2.2.1 Purification of Actinidin from Kiwifruit

When actinidin was first studied, it was prepared by centrifuging a homogenate of ripe fruit pulp following dialysis (Arcus, 1959). Later on, McDowall (1970) described crystalline anionic proteinase that had been prepared from thawed and

frozen ripe kiwifruit. Following centrifugation, clarified fruit juice was subjected to anion-exchange chromatography resin for further purification. Boland and Hardman (1972) added sodium tetrathionate and ethylenediaminetetraacetic acid (EDTA) into blended fresh kiwifruit during preparation, in order to prevent actinidin auto digestion. Actinidin was then concentrated by salting out and purified by dialysis and ion-exchange chromatography. Recently, the cysteine protease inhibitor E64 (N-[N-(L-3-trans-carboxyirane-2-carbonyl)-L-leucyl]-agmatine) and a cocktail of protease inhibitors (cOmplete Protease Inhibitor Cocktail Tablets, Roche, MI, USA) have been used in the extraction buffer to inhibit protease auto digestion (Grozdanovic et al., 2012; Maddumage et al., 2013).

2.3 The Biochemical Analysis of Actinidin

2.3.1 Molecular Analysis of Actinidin

It has been suggested that actinidin is encoded as a pre-pro-protein with an endoplasmic reticulum (ER)-targeting signal peptide and N- and C-terminal pro-peptides. Therefore, this enzyme is likely to be synthesized as a zymogen and processed into an active form after secretion or sequestration in the cell (Praekelt, McKee & Smith, 1988; Paul et al., 1995; Nieuwenhuizen et al., 2007).

The cDNA sequence for actinidin became available in the late 1980s (Praekelt, McKee & Smith, 1988; Podivinsky, Forster, & Gardner, 1989). Praekelt, McKee, and Smith (1988) reported amino acids differences between the proteins encoded by two cDNA clones, with 1145 and 809 bp lengths respectively. In the region containing most of the charged side chains affecting the isoelectric point (pI) of

the proteins, the protein encoded from the 1145 bp length cDNA clone differed from the other protein by losing two negative charges. Results from amino acid sequence analysis showed that there were two non-conservative replacements between these proteins, Asp-55 by Gly and Lys-181 by Glu. Since Lys-181 was highly conserved between all the cysteine proteinases and had been implicated in electrostatic interaction with positively charged side chains across the active site cleft, the authors thought its replacement by Glu may not be compatible with this role. Proteins encoded from both cDNA clones contained an additional 25 amino acids at the C-terminus, which contained a striking excess of Pro and Ser residues. And a 57 residue of N-terminal extension was detected from the protein encoded from the 1145 bp length cDNA clone. It was estimated that actinidin was synthesized as a precursor approximately 15 kDa larger than the mature protein.

Paul et al. (1995) found that actinidin protein in immature kiwifruit was a mixture of two proteins, one with an N-terminal sequence of Val-Leu-Pro-Ser and the other with sequence Leu-Pro-Ser-Tyr. The first form was more abundant than the second form. By having chimeric genes encoding pre-pro-actinidin or pre-pro-actinidin lacking a C-terminal pro-peptide expressed in tobacco, they found that the C-terminal pro-peptide was required for efficient processing of pre-pro-actinidin into a mature form. Using SDS-PAGE and immunoblotting methods, two bands with molecular weight of 29 kDa and 35 kDa were detected. The 29 kDa was suggested as mature actinidin while the 35 kDa band was N-pro-actinidin. The activity assay results also indicated that the N-pro-protein was less active than the mature protein (Paul et al., 1995).

Nieuwenhuizen et al. (2007) identified ten different actinidin mRNAs encoding mature actinidin from *Actinidia deliciosa* cv. ‘Hayward’, *Actinidia chinensis* cv.

'Hort16A' and *Actinidia chinensis* cv. 'EM4'. All mature protein sequences were of similar size, which was around 24 kDa. And all of them contained six conserved cysteine residues required for disulphide bridge formation and essential residues of the catalytic site triad (Figure 2-1). However, the predicted pIs of these proteins differed from 3.9 to 9.3. In addition, they reported that that both mRNAs for acidic and basic isoforms of actinidin were expressed at similar levels in the fruit of both Hayward and Hort16A varieties throughout ripening. But only acidic protein isoform encoded by act1a was detected at high levels in Hayward but was absent in Yellow A. This might explain why Hort16A shows less than 2% of the proteolytic activity of Hayward. Nieuwenhuizen et al. (2012) went on and partially restored cysteine activity in Hort16A fruit by expressing Act1a-1 cDNA (i.e. cDNA encoding high cysteine activity actinidin) in transgenic fruit.

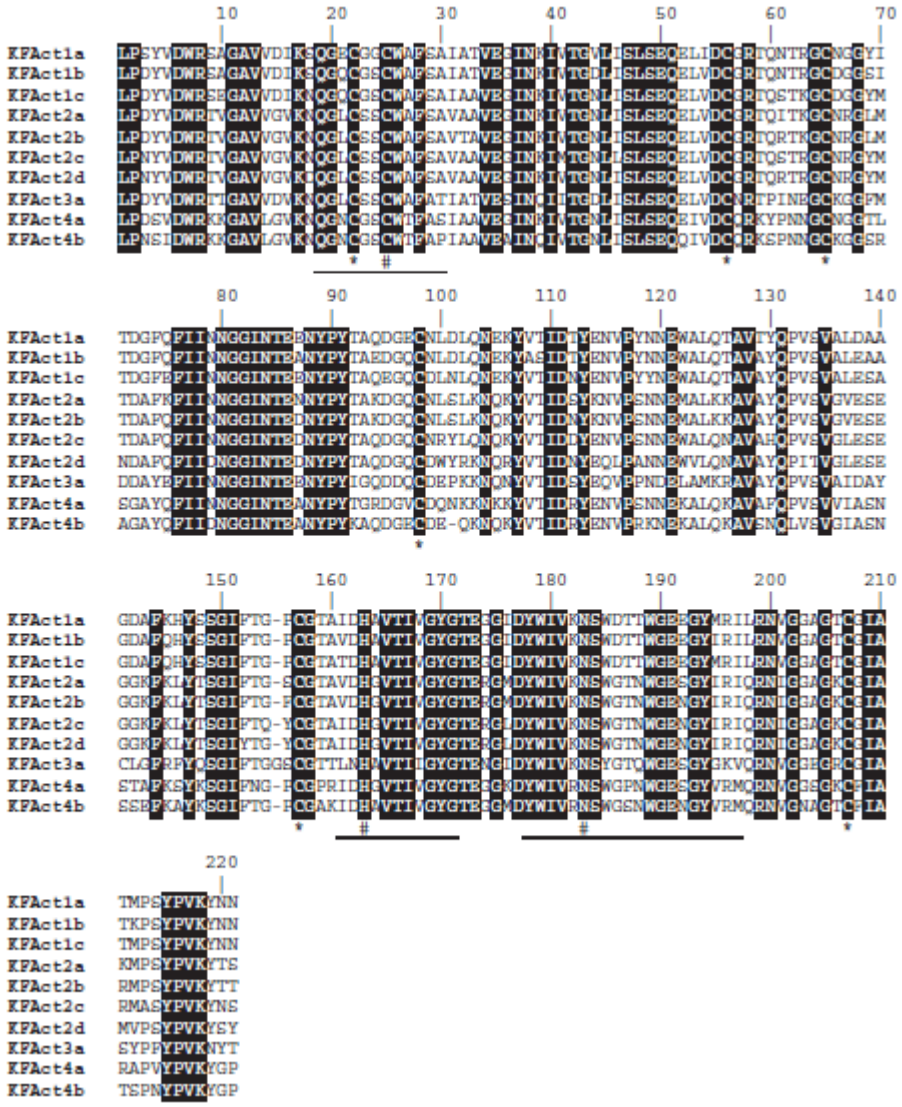

Figure 2-1 Comparison of amino acid sequences of actinidin isoforms from kiwifruit (Nieuwenhuizen et al., 2007). Identical amino acid residues are shaded. Conserved cysteine residues involved in disulphide bridge formation are marked by asterisks. Essential residues in the catalytic site triad are marked with "#".

2.3.2 Actinidin amino acid sequences and molecular structures

The amino acid sequences of the tryptic peptides of actinidin were determined by the manual dansyl-Edman procedure (Carne & Moore, 1978). The actinidin molecule consists of a single chain of 220 amino acids (Figure 2-2). The polypeptide chain is folded into two domains, which are L-domain (domain I) and R-domain (domain II). Domain I consists of residues 19-115 and 214-218. It contains three helical regions. The main helix runs through the centre of the molecule at the interface of two domains. Domain II contains residues 1-18 and 116-213. This domain is made up of a twisted antiparallel β sheet barrel. The interface between the two domains is mostly hydrophilic, and contains the active site cysteine. The structure is further stabilized by the presence of three disulphide bonds: C22-C65, C56-C98 and C156-C206 (Baker, 1973, 1976, 1977, 1980; Boland, & Singh, 2013)

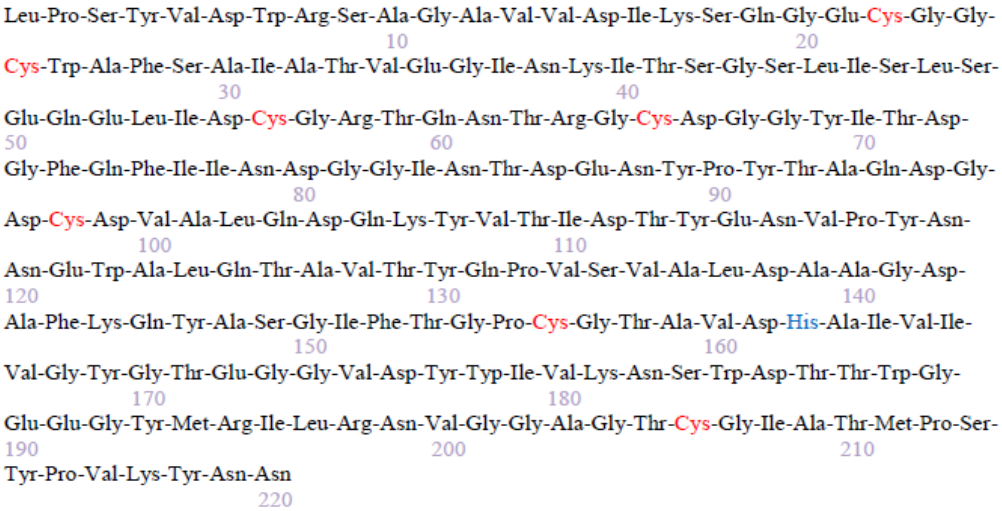

Figure 2-2 Amino acid sequence of actinidin from kiwifruit (Carne & Moore, 1978)

2.3.3 Actinidin Identification in Kiwifruit

In the literature, actinidin has mostly been recognized by molecular weight. Therefore, it should be first pointed out that different methods of molecular weight measurement could lead to different results. Due to the limitations of the methodology, SDS-PAGE and gel filtration provide only approximate results. In contrast, analytical ultracentrifugation (Boland & Hardman, 1972) and mass spectrometry (Grozdanovic et al., 2012) are able to provide relatively accurate measures of the protein molecular weight.

In previous studies, the molecular weight of actinidin was been reported as 26,000±500 Daltons by the high-speed equilibrium method (Boland & Hardman, 1972). Carne & Moore (1978) determined the amino acid sequence of actinidin using the Edman procedure. Based on the published sequence, actinidin should have a molecular weight of 23,500 Daltons.

A molecular weight of 27,000±3,000 Dalton was estimated for actinidin according to different studies. Some studies recognized 24 kDa bands on SDS-PAGE as actinidin (Nieuwenhuizen et al., 2007; Nieuwenhuizen et al., 2012; Maddumage et al., 2013), while other studies identified the band of about 30 kDa as actinidin (Praekelt, McKee & Smith, 1988; Paul et al., 1995). As far as we know, no explanation has been made of these differences. One reason could be that the protein molecules display an anomalous behaviour when subjected to electrophoresis in the presence of sodium dodecyl sulphate. The other possible reason is because of a lack of understanding of actinidin isoforms.

It has been reported that four actinidin isoforms were isolated by anion-exchange high-resolution liquid chromatography as homogeneous proteins with molecular weights of 27,000±1,000 Dalton. The circular dichroism spectrum analysis

showed that the folding of the polypeptide backbone of these four forms was very similar (Tello-Solís, Valle-Guadarrama & Hernández-Arana, 1995). Pastorello et al. (1998) collected four fractions from purified crude kiwifruit extract by analytic anion-exchange chromatography. Proteins with molecular weights of 17, 24, 28 and 30 kDa were detected in these four fractions. According to previous literature (Tamburrini et al., 2005; Gavrovic-Jankulovic et al., 2002; D'Avino et al., 2011), proteins identified in gel electrophoresis are kirola for 17 kDa, thaumatin-like protein of 24 kDa, kiwellin for 28 kDa and actinidin for 30 kDa. Pastorello et al. (1998) proved that proteins with lower molecular weight were not from actinidin self-digest, because there was no appearance of any new band shown on SDS-PAGE from the fraction containing 30 kDa protein after incubation at room temperature for different time periods. The authors described the 30 kDa component as actinidin based on the evidence provided by N-terminal amino acid sequences and isoelectric point determination. A very interesting observation reported in this study was that SDS-PAGE of the kiwifruit extract treated with the specific protease inhibitor E64 showed a large increase in the 30 kDa band but not in the 24 to 28 kDa band. Moreover, results of periodic acid-Schiff staining for glycosylation on the 30 kDa protein indicated that actinidin appeared to be an unglycosylated protein.

However, a different observation was reported by Lucas et al. (2007), who isolated native actinidin by affinity chromatography using thiopropyl Sepharose-6B covalent chromatography and ammonium sulphate precipitation. Meanwhile, recombinant acidic actinidin and basic actinidin were produced in *Escherichia coli*. The polyclonal antibody used in western blotting was raised against purified recombinant acidic actinidin protein in rabbit. It was observed

that purified native actinidin from Hayward kiwifruit ran at the same molecular weight of 25 kDa in both SDS-PAGE and Western blot analysis. The identification of this 25 kDa protein was confirmed by LC-MS showing identical fragments derived from recombinant acidic actinidin protein. However, for recombinant proteins, both 25 kDa and 30 kDa bands were observed on SDS-PAGE, but only the predominant protein produced at 30 kDa reacted with the polyclonal actinidin antibody. It was suggested that recombinant acid and basic forms of actinidin had a 6 x Histidine tag attached at the N-terminus that added approximately 5 kDa to the size of mature protein. Moreover, Western blot results showed antibodies also positively recognize a protein with a molecular weight around 38 kDa. But this protein was not detected on 2D gels by silver staining. The reason of this was unexplained. Another study reported that two-dimensional PAGE showed fragmentation of the 30 kDa protein, pI 3.6 into a 26 kDa protein with a pI value of 3.2, and both the protein and protein fragment showed actinidin proteolytic activity in a zymogram (Gavrovic-Jankulovic et al., 2005).

Sugiyama et al. (1996) reported that using proteomic tools and a combination of biochemical and immunochemical methods, actinidin isolated under native conditions from kiwifruit extract represented a mixture of active and inactive enzymes. Using ion-exchange chromatography actinidin can be separated into six different acidic actinidin isoforms, and all of them share similar molecular weight at 23,500 Dalton and the same N-terminal amino acid residues. A further study from the same authors showed that two of these isoforms were proved to have different kinetic parameters and pH optima against N-α-CBZ-lysine-pNP (Sugiyama et al., 1997).

Recently, it was reported that after ion-exchange chromatography purification, actinidin revealed two protein bands of about 22 kDa and 30 kDa on SDS-PAGE (Grozdanovic et al., 2012). These two bands revealed the same N-terminal sequence that responded to amino acids 127-132 of the complete sequence of actinidin. The molecular mass of isolated actinidin (22 kDa and 30 kDa) measured by mass spectrometry was 23,883.1 Daltons. In SDS-PAGE and 2D-PAGE under reducing conditions, an extraordinary difference in electrophoretic mobility (30 kDa /22 kDa) between inactive and active actinidin was noticed. By using SDS-PAGE analysis of the kiwifruit extract treated with E64 protease inhibitor, it was noticed that there was a large increase in the 30 kDa actinidin band (Grozdanović, Drakulić & Gavrović-Jankulović, 2013). After incubation with a cocktail of inhibitors, the 22 kDa protein showed the electrophoretic mobility of a 30 kDa protein. Active actinidin after thermal treatment also showed an apparent molecular weight of 30 kDa on SDS-PAGE (Grozdanovic et al., 2012). Mass analysis of tryptic digests of inactive and active actinidin revealed that both bands (22 kDa and 30 kDa) preserved the primary structure of mature actinidin. This phenomenon suggested that the difference in electrophoretic mobility between active and inactive actinidin may due to the conformational changes in the protein structure.

2.3.4 Actinidin Characterizations by Mass Spectrometry

Proteomics studies rely most of all on techniques that allow high-throughput analysis of the protein complement under investigation. Mass spectrometry is an indispensable analytical tool in proteomics field which aims to identify a compound from the molecular or atomic masses of its constituents. Under the conditions of certain mass spectrometric experiments, fragmentation of ions can

deliver information on ionic structure. Mass spectrometry can be coupled to separation methods such as high performance liquid chromatography (HPLC), and deliver high selectivity and low detection limits for the analysis of interesting compounds in the deconvolution of complex mixtures. Moreover, for structure elucidation, mass spectrometric experiments can be arranged consecutively to study mass-selected ions in tandem mass spectrometry. As one of the most frequently employed ionization methods in MS, electrospray ionization (ESI) is the most prominent technique among the group of atmospheric pressure ionization (API) methods and the leading method of choice for liquid chromatography- mass spectrometry coupling (LC-MS) (Gross, 2004). It is a soft ionization technique that accomplishes the transfer of ions from solution to the gas phase. The technique is extremely useful for the analysis of large, non-volatile, chargeable molecules such as proteins (Amad et al., 2000; Schalley, 2000; Cristoni & Bernardi, 2003). In addition, multiple charged ions are obtained, allowing extraordinary high-mass capability of ESI (Heck & van den Heuvel, 2004).

Grozdanovic et al. (2012) determined the molecular mass of actinidin by matrix assisted laser desorption/ionization mass spectrometry (MALDI TOF-MS) in the positive ion mode. They reported the molecular mass of isolated actinidin was 23,883.1 Da. To gain a deeper insight into the properties of kiwifruit proteases, more information was provided by Larocca, Rossano and Riccio (2010). Using MALDI TOF-MS, they identified several actinidin isoforms with the molecular mass of 31.2, 30.3, 29.3, and 23.9 kDa.

2.3.5 Actinidin activity

2.3.5.1 ***Enzyme activity assay***

Actinidin catalyses the hydrolysis of peptide bonds of proteins and also simple amides and esters (Boland & Singh, 2013). It has a broad pH optimum ranging from 5-7 when using N^2-p-Toluenesulphonyl-L-glutamine p-nitrophenyl ester (McDowall, 1973) and N-α-carbobenzoxy-L-lysine-p-nitrophenyl ester as substrates (Boland & Hardman, 1973). In addition, the proteolytic activity of actinidin has been determined using proteins, such as gelatine, haemoglobin and casein as substrates (Arcus, 1959; Grozdanovic et al., 2012; Nieuwenhuizen et al., 2007).

Generally, there are two common substrates used for measuring the activity of actinidin in literature. One is Casein, the other one is N-α-carbobenzoxy-L-lysine-p-nitrophenyl ester (N-α-CBZ-lysine- pNP).

Larocca et al. (2010) described how to assess total proteolytic activity by using a spectrophotometric method based on azocasein. However, casein is not stable at low pH, where the optimum pH for proteolysis by actinidin. The most convenient and repeatable substrate is using N-α-CBZ-lysine- pNP as substrate has been reported in several studies, as described by Boland et al. (1972).

2.3.5.2 Actinidin active site

Results from kinetic studies indicate both a sulfhydryl group and an imidazole group are required for actinidin catalysis (Boland & Hardman, 1973). Structural analysis of actinidin using X-ray diffusion showed Cys25 in domain I and His162 in domain II from the catalytic (Baker, 1977). Kaneda, Tomita and Tominaga (1986) reported that when they examined the effect of pH on the loss of activity

associated with the photo-oxidation of actinidin, the inflection point near pH 6.5 strongly suggested histidine residues were involved in enzyme inactivation.

2.4 Actinidin and kiwifruit post-harvest storage condition

The level of ethylene in storage ambient air is critical for kiwifruit storage. Retamales and Campos (1995) found that ethylene accelerated the softening of kiwifruits and ethylene-free ambient resulted in firmer fruits, but differences in responsiveness were evident along storage period. McDonald and Harman (1982) reported that atmospheres containing above 4% CO_2 with 15–20% O_2 caused a retardation in the softening of kiwifruit. Low O_2 (2–3%) with 3–5% CO_2 further delayed the rate of kiwifruit softening and increased storage life up to 3–4 months beyond normal air-storage life. Tavarini et al. (2008) evaluated the effect of harvest time and storage on the quality indices and nutritional content of kiwifruit. The firmness of kiwifruit stored under most of storage conditions was reduced at the end of cool storage and the soluble solids content significantly increased. Some nutritional characteristics such as vitamin C and carotenoids were also strongly influenced by storage. Boquete, Trinchero, Fraschina, Vilella, and Sozzi (2004) found that methylcyclopropene (1-MCP) suppressed the production of ethylene. 1-MCP treated fruit did not soften as much as control fruit. Core tissue of 1-MCP treated fruit softened more slowly than outer cortical tissue. 1-MCP severely retarded changes in lightness and chroma in the outer cortex. Soluble solid concentrations (SSC) remained low in 1-MCP treated fruit for about 14 days. Activity of three glucosidases (β-d-galactosidase (β-Gal), α-l-arabinofuranosidase (α-Af), and β-d-xylosidase (β-Xyl)) increased in control fruit during ripening but only to a limited extent, or not at all, in fruit treated with 1 μL l−1 1-MCP. Koukounaras and Sfakiotakis (2007) had the similar findings.

2.5 Application of Actinidin in Food Industry

The applications of the proteolytic activity of actinidin have been widely studied. It has been reported that actinidin is able to degrade all the cream milk proteins into fragments < 29 kDa suggesting its utilization as preparatory treatment in the production of milk derived products characterized by a reduced content of undesirable protein (Puglisi, Petrone & Piero, 2012). Purified actinidin was proved to completely degrade α-lactalbumin and 65.3% of β-lactoglobulin (Vázquez-Lara et al., 2003). However, Chalabi et al. (2014) reported that actinidin had no or limited proteolytic effect on globular proteins including whey proteins (α-lactalbumin and β-lactoglobulin). In the manufacturing process of cheese, actinidin had been characterized in view of its possible suitability as a coagulant enzyme (Piero, Puglisi & Petrone, 2011). Majdinasab et al. (2010) reported that actinidin significantly increased the nitrogen solubility index of soymilk in a dose-dependent manner, which suggested that actinidin could be used to improve the functionality of soymilk by increasing the solubility of soymilk proteins.

One of the most important applications of actinidin in food industry is to tenderize meat. Lewis and Luh (1987) first accessed the meat tenderizing ability of actinidin. Results showed actinidin significantly tenderize beef muscles. Later on, there are results indicating that actinidin was able to tenderize pork muscles by affecting both the myofibrils and connective tissue (Christensen et al., 2009). It has been reported that actinidin significantly increased protein solubility and water holding capacity of sausage product (Aminlari et al., 2009).

2.6 Actinidin and Health Benefits

Actinidin has been postulated as a digestive aid. In *in vitro* experimental trails, this protein has been proved to enhance the digestion of whey protein isolate,

zein, gluten and gliadin (Kaur et al., 2010a). Furthermore, it has been reported that dietary actinidin enhanced gastric digestion of some food protein in growing rat (Rutherfurd et al., 2011). Most recently, Montoya et al. (2014) showed that by feeding fresh green kiwifruit, which contain a large amount of active actinidin, to pigs, the digestion and rate of gastric emptying of meat proteins increased. These publications provide evidence that kiwifruit as part of meal may influence the digestion of food proteins in gastric conditions and also may help improve the over fullness feelings in the stomach (Kaur et al., 2010a, 2010b).

Overall, actinidin has different isoforms with varying levels of enzyme activity, which has been ascribed to small variations in amino acid sequence. During the maturation of kiwifruit, a certain amount of actinidin is activated. However, the activation mechanism (post-translational modification) has not been fully understood. A systemic investigation including isolation/purification of different isoforms/species of actinidin, protein sequencing/structuring and subsequent enzyme activity determination is required to understand the active forms of actinidin and the mechanism of actinidin activiation. In addition, the effect of the post-harvest storage on actinidin activation requires further study. Therefore, my research was to focus on the above research questions at a preliminary level.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

3.2 Chemicals

All reagents used were of analytical grade and were purchased from Sigma-Aldrich (Auckland, New Zealand), Merck Millipore (North Shore City, New Zealand), Bio-Rad Laboratories (Auckland, New Zealand), GE Healthcare (Auckland, New Zealand) or ThermoFisher Scientific (North Shore, New Zealand).

3.3 Kiwifruit

In this project, actinidin was extracted from two fresh kiwifruit cultivars. Green flesh kiwifruit Green kiwifruit (*Actinidia deliciosa* cv. 'Hayward') and yellow flesh kiwifruit (*Actinidia chinensis* cv. 'Zesy002') used in this study were provided by Zespri Co Limited. *Actinidia deliciosa* cv. 'Hayward' was referred as green kiwifruit and Hayward kiwifruit in this thesis. *Actinidia chinensis* cv. 'Zesy002' was referred as gold kiwifruit and SunGold kiwifruit in this thesis.

3.4 Methods

3.5 Actinidin Extraction Methods

Whole kiwifruit was processed using a Kenwood food processor (DeLonghi New Zealand Limited, Botany, New Zealand) with equal volume of 0.01M potassium phosphate buffer at pH 6 containing 10 mM EDTA and 150 ppm sodium metabisulfite. After stirring in a chiller room (4 °C) for 120 minutes, the mixture was centrifuged at 27,485 g at 0 °C (Sorvall Ultracentrifuge, Thermo Fisher

Scientific New Zealand Ltd, North Shore City, New Zealand) for 20 minutes. The supernatant was stored at 4 °C for further use.

Meanwhile, samples were prepared for future analysis of post-translational modification of actinidin by mass spectrometry. This preparation required the immediate and complete inactivation of the enzyme. Therefore, for this purpose, the extraction buffer was Milli-Q water containing DTT as reducing agent and a cocktail of protease inhibitor (cOmplete tablet, Roche, Auckland, NZ). Kiwifruit pieces were homogenized with equal weight of extract buffer using IKA Ultra Turrax Homogenizer (Thermo Fisher Scientific New Zealand Ltd, North Shore City, New Zealand) and were centrifuged at 21,000 g (Microcentrifuge, Thermo Fisher Scientific New Zealand Ltd, North Shore City, New Zealand) immediately. The supernatant was stored at -80°C for further analysis.

3.6 Protein Concentration Determination

In the beginning, protein content was determined by four different methods for comparison, which were described below.

3.6.1.1 Hartree-Lowry assay

The assay was conducted following protocol described by Sapan, Lundblad and Price (1999). Reagent A was prepared using 2 mg sodium potassium tartrate x 4 H_2O, 100 mg sodium carbonate, 500 mL 1N NaOH and H_2O to one litre. Reagent B was prepared using 2 mg sodium potassium tartrate x 4 H_2O, 1 mg copper sulphate ($CuSO_4$ x $5H_20$), 90 mL H_2O, 10 mL 1N NaOH (final concentrations 70 mM Na-K tartrate, 40 mM copper sulphate). Reagent C was prepared from 1 volume Folin-Ciocalteau reagent and 15 volumes water.

Because the protein content of the original kiwifruit extract was too high for this assay, the supernatant was diluted 20 times with water. A series of dilutions of 0.3 mg/mL bovine serum albumin was prepared with water, to give concentrations of 30 to 150 μg/mL. 1.0 ml of each dilution of standard sample was added to 0.90 mL reagent A in separated test tubes and mixed. The tubes were incubated in a 50 °C bath for 10 minutes, then were cooled to room temperature. 0.1 mL reagent B was added into each tube and mixed with further incubation at room temperature for 10 minutes. 3 mL reagent C was then rapidly added into each tube and mixed with further incubation at 50 °C for 10 minutes. After incubation, all tubes are cooled to room temperature. The final assay volume was 5 mL. The absorbance of the reagent was then measured at 650 nm in 1 cm cuvettes.

A standard curve of absorbance versus mg/mL protein was prepared (Figure 3-1).

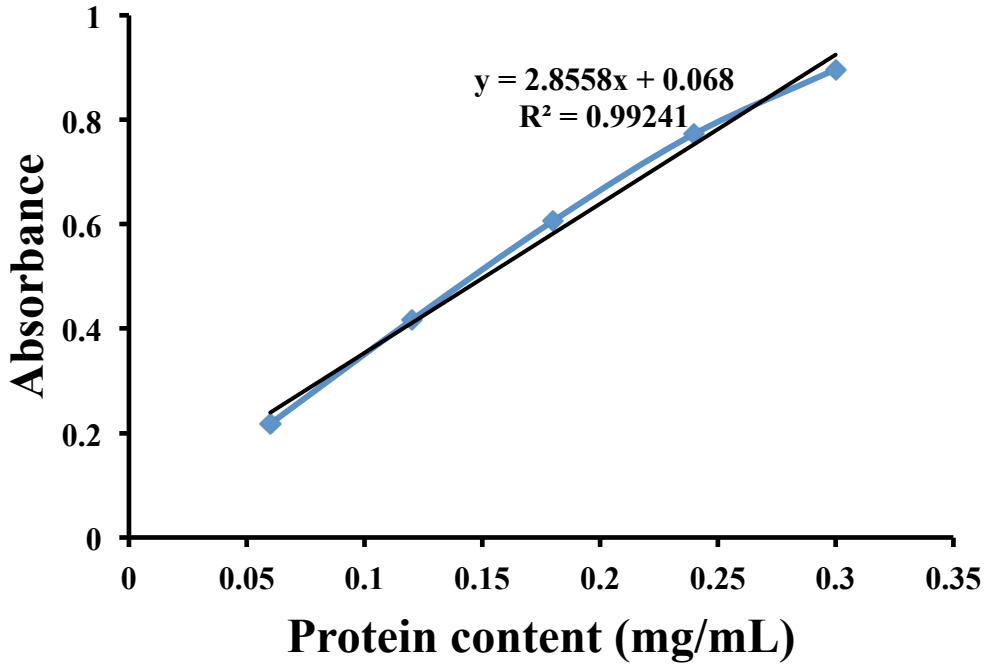

Figure 3-1 Standard curve for the Hartree-Lowry method.

3.6.1.2 Bradford method

100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL 95% ethanol and 100 mL 85% (w/v) phosphoric acid. Then this solution was diluted to 1 litre after the dye had been completely dissolved, and filtered through filter paper (Whatman #1, GE Healthcare, Auckland, NZ) just before use., The supernatant was diluted by 10 times with water because the protein content of the original kiwifruit extract was too high for this assay.

Standards were prepared containing a range of 5 to 100 micrograms protein (albumin globulin were used) in 100 µL volume. After adding 5 mL dye reagent and being incubated for 5 minutes, samples were measured the absorbance at 595 nm.

A standard curve of absorbance versus mg/mL protein was prepared (Figure 3-2).

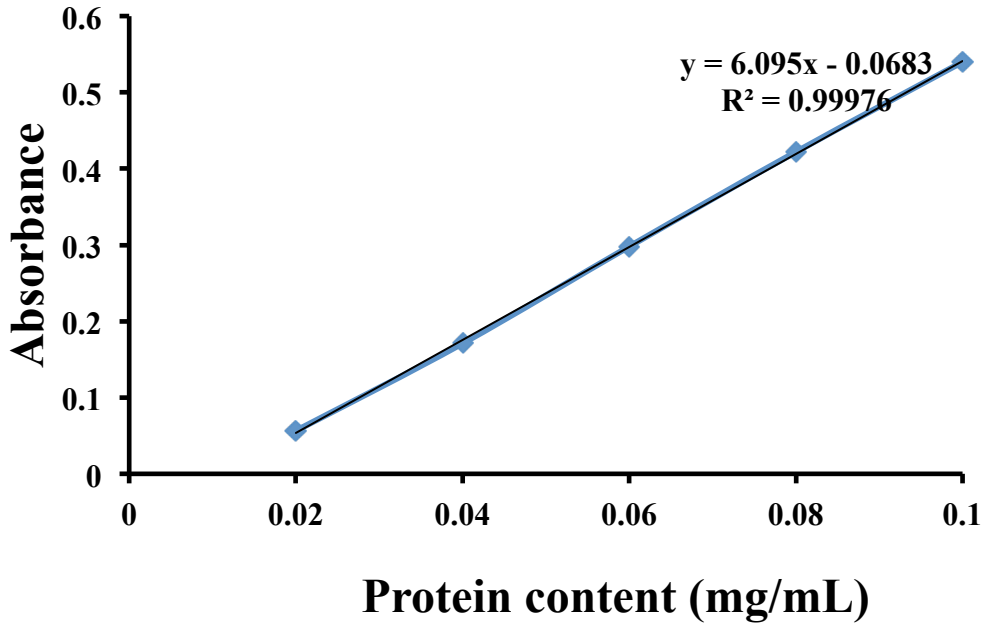

Figure 3-2 Standard curves for the Bradford method.

3.6.1.3 BCA method

The Pierce BCA protein assay kit from Thermo Fisher Scientific was used. The kit includes 1) BCA reagent A, which contained sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide; 2) BCA reagent B, which containing 4% cupric sulfate. Standards for this assay were bovine serum albumin solutions with concentrations at 0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL and 1 mg/mL. Kiwifruit juice supernatant was diluted 5 times with water. Mixed reagent A and reagent B with a ratio of A:B=1:20 for enough volume of using. 0.1 mL of each standard and unknown sample replicate was pipetted into an appropriately labeled test tube. 2.0 mL of the working solution was added to each tube and mixed well. Tubes were covered and incubated at 37°C for 30 minutes. With the spectrophotometer set to 562 nm, the absorbance of all the samples was measured within 10 minutes.

A standard curve of absorbance versus mg/mL protein was prepared (Figure 3-3).

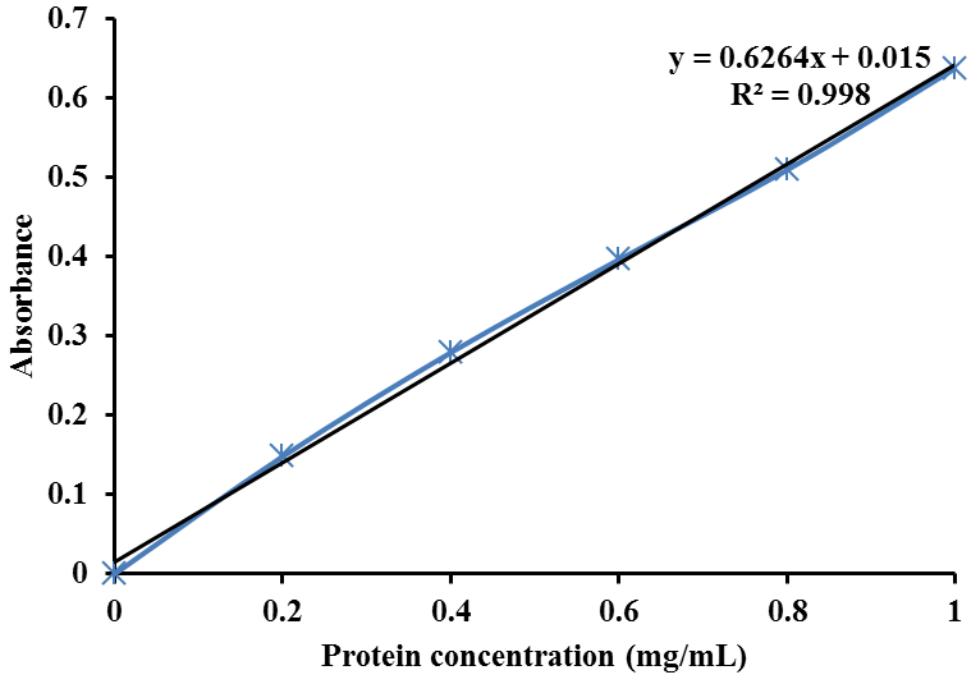

Figure 3-3 Standard curves for the BCA method.

3.6.1.4 Dumas method

10 mL of kiwifruit supernatant was sent to the School of Food and Nutrition, Massey University for nitrogen determination using the Dumas method. The equipment used was a Leco Truspec elemental determinator (LECO, Castle Hill, NSW, Australia). The conversion factor from total nitrogen to total protein is 5.64 (Levey, Bissell & O'Keefe, 2000; Salo-väänänen & Koivistoinen, 1996; Sriperm, Pesti, & Tillman, 2011).

3.7 Enzyme activity determination

Total proteolytic activity was assessed by the method described by Boland and Hardman (1972) with some modifications. N-α-carbobenzoxy-L-lysine-p-nitrophenyl ester (N-α-CBZ-lysine-pNP) was used as substrate in the following experiment: 100 µL of substrate (1.2 g/L) was added to 2.85 mL of 0.05 M phosphate buffer, pH 6 directly in a cuvette. After measuring spontaneous hydrolysis of N-α-CBZ-lysine-pNP for 30 seconds, 50 µL of juice (enzyme solution) containing 25 µL of the 100 mM DTT was added and the change in absorbance was measured for 5 minutes (300s) at 348 nm using a spectrometer. Enzyme activity (µmol product formed per min per ml, or units per ml) of the extract was calculated using $\Delta\epsilon$=5400 $M^{-1}cm^{-1}$. Unit definition: one unit of actinidin activity will produce 1 µmol product per minute pH 6.0 at 24 °C measured as p-nitrophenyl products using CBZ-L-Lys-p-nitrophenol hydrochloride as substrate. Specific enzyme activity was calculated as enzyme activity (Units)/protein content (mg). Total enzyme activity was calculated as enzyme activity (Units)/kiwifruit (g).

3.8 Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were mixed with equal volume of sample buffer containing 12.5% 0.5M Tris-HCl buffer (pH 6.8), 25% glycerol, 0.001% bromophenol blue, 2% sodium dodecyl sulphate and 100 mM dithiothreitol (DTT). After the diluted samples being denatured by heating at 100 °C for 5 minutes, they were centrifuged at 10,000 rpm for 5 minutes. Samples were then loaded onto SDS-polyacrylamide gel and run in Tris-glycine running buffer (25 mM Tris, 192 mM glycine and 0.1% Sodium dodecyl sulphate) for 1 or 1.5 hours at 150 V using a Mini-PROTEAN 3 electrophoresis system (Bio-Rad, Auckland, NZ). Each gel was then stained with staining buffer (10% acetic acid, 20% isopropanol and 0.3% Coomassie brilliant blue R-250) for 1 hour at room temperature. Each gel was destained with destaining solution (10% acetic acid and 10% isopropanol) right afterwards overnight at room temperature.

3.9 Stability of Actinidin in Kiwifruit Extracts at different pHs

Actinidin was extracted from kiwifruit using 0.01M phosphate buffer containing 0.001M EDTA and 150 ppm metabisulfite as before. After centrifugation and filtration, the pH value of the supernatant was adjusted with 1 M HCl or 1 M NaOH to pH 2, pH 4 and pH 6. Meanwhile, the samples with no pH adjustment (pH=3.5) were used as control. Samples were incubated in a water bath at 25 °C. The enzyme activities in each sample were monitored over 22 hours.

3.10 Actinidin Purification by Ion-exchange Chromatography

Fresh kiwifruit was processed using Kenwood food processor with equal volume of 0.01M potassium phosphate buffer at pH 6 containing 10 mM EDTA and 150

ppm sodium metabisulfite. After stirring in the chiller room (4 °C) for two hours, the mixture was centrifuged at 27, 485 g at 0 °C for 20 minutes. The supernatant was then dialyzed for 2 hours at 4 °C against 0.01 M potassium phosphate buffer at pH 6 containing 10 mM EDTA and 150 ppm sodium metabisulfite followed by overnight dialysis against the same buffer. About 250 mL of this extract was then loaded on a DEAE-Sephadex A-25 column (25 x 3 cm) which had been equilibrated with 0.01 M potassium phosphate buffer (pH 6). All unbound proteins were eluted by applying 0.1 M potassium chloride in 0.01 M potassium phosphate buffer (pH 6) through the column. The actinidin was then eluted from the column with a linear gradient of 0.1 – 1.0 M potassium chloride (500 mL) in 0.01M potassium phosphate buffer (pH 6). The elution rate was 2.5 mL/min. The absorbance at 280 nm and the enzyme activity of all fractions were measured right after collection. The volume of each fraction is 6.5 mL. The fractions were stored at -80 °C for SDS-PAGE and mass spectrometry analysis.

3.11 Mass Spectrometry Analysis

Selected fractions from ion-exchange chromatography were desalted and concentrated. The samples were concentrated 10 times. They were analysed by mass spectrometry with direct injection using an ESI TOF mass spectrometer 6200 (Agilent Technologies, Auckland, New Zealand). 500 µL of the desalted and concentrated sample was centrifuged at 13, 000 g for 5 minutes and filtered (0.2 µm filter). 100 µL of the supernatant was then mixed with 900 µL methanol containing 0.1% formic acid. Then, 100 µL of the mixed fluid was directly injected onto the LC-MS system.

3.12 Raising Polyclonal Antibody against Actinidin

Anti-actinidin antibodies were generated using purified actinidin. The purposes were to recognize actinidin and actinidin analogues from kiwifruit and to make affinity columns for separation of actinidin isoforms. Proteins showing strong affinity for antibody will be separated and fully characterised in a future study.

3.12.1 Immunization

Ethics approval for the animal trials was obtained from the Animal Ethics Committee, Massey University, Palmerston North, New Zealand. The fractions from ion-exchange chromatography with the highest specific enzyme activity were desalted and concentrated using an ultrafiltration kit (Centriprep, Merck Millipore). 400 μg antigen emulsified with Freund's Incomplete Adjuvant (1:1) was injected into one rabbit. This was followed by booster injections of 150 μg antigen every two weeks thereafter. The blood was collected after 12 weeks.

3.12.2 Serum Preparation

Rabbit serum was sampled from the marginal ear vein 14 days after each immunization. Collected sample blood (about 3 mL) was allowed to clot for 60 minutes at room temperature, and then centrifuged at 10, 000 *g* for 15 minutes at 4 °C. The supernatant (i.e. rabbit anti-actinidin serum) was stored at -80°C. The presence of antibodies to actinidin was detected by western blot. After 6 boosters, final blood was collected from the euthanased rabbit, and the serum was prepared as described above, and then was kept in microtubes at -80°C until further use.

3.12.3 Immunoblotting

Western blotting was done according to the instructions provided with Immun-Blot® assay kit (Bio-Rad, Auckland, NZ) with some modifications.

Proteins which had been resolved by SDS-polyacrylamide gels under reducing conditions were electro-blotted onto a polyvinylidene fluoride (PVDF) membrane (Immun-Blot® PVDF membrane, pore size 0.20 μm, Bio-Rad, Auckland, NZ) in transfer buffer containing 0.025M Tris, 0.192M glycine and 10% methanol. The membrane was blocked with 12.5% skim milk powder in TBS (Tris buffered saline, 20mM Tris, 500mM NaCl, pH7.5) containing 0.05% Tween 20 at 4 °C overnight. After blocking, the membrane was incubated with rabbit anti-actinidin serum (1/500 dilution) in TBS buffer at 4 °C for 12 hours, and then incubated in secondary antibody (Goat anti-rabbit IgG (H+L) HRP, Bio-Rad, Auckland, NZ) at room temperature for 2 hours. After washing in TBS buffer, the Horseradish Peroxidase blots were then developed by immersing in the colour development solution in the kit.

3.13 Actinidin and Kiwifruit Post-Harvest Storage Conditions

3.13.1 SunGold Kiwifruit

SunGold kiwifruit (*Actinidia chinensis* cv. 'Zesy002') were collected at commercial harvest time from 5 different growers: Growers 1, 3, 4, 5, and 12. Fruit were then stored at 1°C. The enzyme activity of actinidin was measured on the day or on the day after the kiwifruit was received. Ten gold kiwifruit were mixed together for each test, and the supernatant was used for enzyme activity test and protein content determination. All tests were run triplicate.

3.13.2 Green Kiwifruit

Green kiwifruit (*Actinidia deliciosa* cv. 'Hayward') were collected at commercial harvest time from a single grower. Fruit were then stored at 1°C. The enzyme activity of actinidin was measured on the day when the kiwifruit was received and

2, 4, 8, 12, 16 weeks after storage. Ten green kiwifruit were mixed together for each test, and the supernatant was used for enzyme activity test and protein content determination. All tests were run triplicate.

3.14 Statistical analysis

Expermients including the measurements of enzyme activity and protein content and ion-exchange chromatography were run triplicate using different kiwifruit. Effect of grower and storage time on protein content and enzyme activity was analyzed by two-way ANOVA ($P<0.05$). The differences between different growers and storage time were compared by Duncan's new multiple range test ($P<0.05$). Error bars in the figures represent standard deviation. Standard deviation also is shown in the tables.

CHAPTER 4 ACTINIDIN EXTRACTION AND ENZYME ACTIVITY OF KIWIFRUIT

Abstract

In this chapter, actinidin extraction is described. Different methods for protein content determination are compared. The Hartree-Lowry method was shown to be the most suitable method for protein determination due to its accuracy, sensitivity, anti-interference ability and convenience in this project. Enzyme activity and protein composition of kiwifruit were also investigated. The SDS-PAGE results showed that the Hayward and SunGold kiwifruit had different protein compositions. Total enzyme activity of Hayward kiwifruit was about 8 times higher than that of SunGold kiwifruit. Enzyme activity stability was greatly affected by pH.

4.1 Introduction

Specific protein fractions and enzymes naturally present within kiwifruit are of much interest in relation to health. Of particular note in kiwifruit is actinidin. Actinidin is a cysteine protease of *Actinidia* spp. and is recognized for its ability to hydrolyze proteins. Actinidin catalyses the hydrolysis of peptide bonds of proteins. The standard method for determining food protein content for databases and nutritional labelling purposes is total nitrogen determination by the method of Kjeldahl or Dumas, However, the situation may not be quite as simple as that. The amount of "protein" measured in kiwifruit also depends very much on the type of measurement because other components in kiwifruit (e.g. sugar) may greatly influence the results. The amount of protein measured also varies to some extent on ripeness, on variety and on the conditions used by different growers.

Different methods of protein content determination were compared to the Dumas method. Protein composition was analysed by SDS-PAGE. Protein content, protein composition and specific enzyme activity of actinidin in kiwifruit extracts of Hayward and SunGold kiwifruit were compared. The effect of pH on actinidin activity of kiwifruit soluble protein was explored.

4.2 Results and discussion

4.2.1 Protein content of kiwifruit extract

The results of protein content of one batch of kiwifruit extract are shown in Table 4-1. In general, the protein content of green and gold kiwifruit was similar. Protein content measured by the Hartree Lowry and Bradford methods was similar to that measured by the Dumas method, which was consistent with previous studies on protein content of kiwifruit juice (~2-3 mg/mL) by the Coomassie assay or Folin-Lowry method (Boyes, Strübi, & Dawes, 1997; Wilson & Burns, 1983). The value of protein content measured by the BCA method was much higher than that measured by the other three methods. This was ascribed to the interference of reducing sugars during the BCA assay. However, the reducing sugars had limited effect on protein determination using the Dumas, Hatree-Lowery and Bradford methods (Walker, 2009). The Hartree Lowry was used for protein content determination in this study.

Table 4-1 Protein content measured by different methods

Methods	Protein content of green kiwifruit extract (mg/mL)	Protein content of gold kiwifruit extract (mg/mL)
Bradford assay	2.36 ± 0.2	1.94±0.12
Hartree-Lowry method	4.04 ± 0.1	5.54±0.6
BCA	7.52 ± 0.4	10.36±0.69
Dumas	4.11 ± 0.18	3.77±1.17

4.2.2 SDS-PAGE protein patterns of kiwifruit extract

Figure 4-1 shows SDS-PAGE protein patterns of kiwifruit extract. The green and gold kiwifruit showed the similar SDS-PAGE protein patterns. Almost all proteins in kiwifruit were smaller than 30 kDa. There were four main bands in SDS-PAGE for gold kiwifruit: 26.6, 23.2, 17.9 and 14.6 kDa. Their band intensity was 21.8, 25.3, 24.5 and 12.5%, respectively. There were four main bands in SDS-PAGE for green kiwifruit: 27.1, 24.6, 22.7 and 14.9 kDa. Their band intensity was 33.2, 22.1, 18.2 and 11.3%, respectively. The green and gold kiwifruit had the same three bands at around 27, 24 and 14.7 kDa. The intensity of proteins at 27 kDa in green kiwifruit was much higher than that in gold kiwifruit. For both green and gold kiwifruit, the proteins at 24 and 27 kDa were main proteins in kiwifruit.

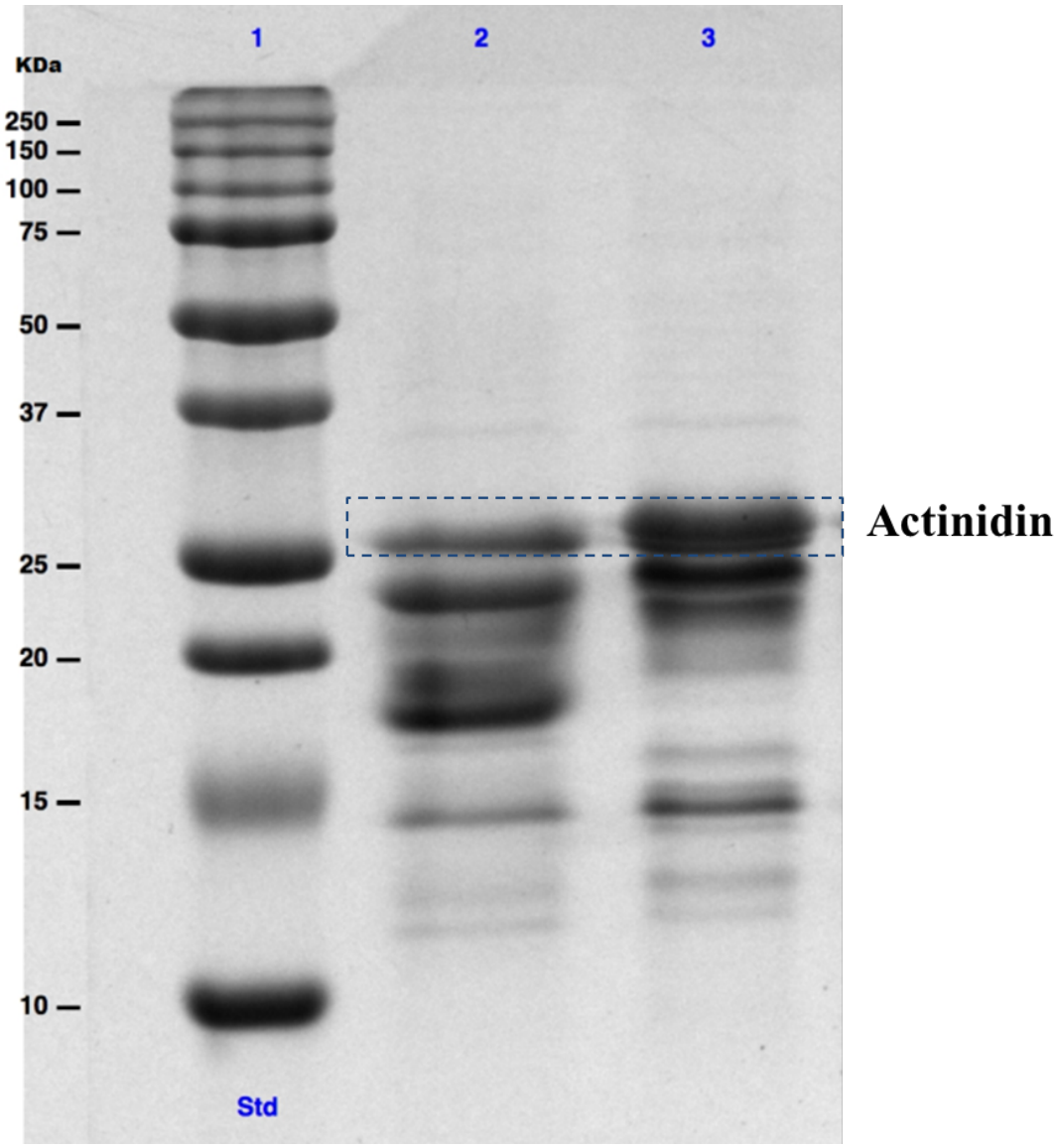

Figure 4-1 Analysis of soluble protein content in fresh kiwifruit by SDS-PAGE under reducing conditions. Lane 1 is protein marker. Lane 2 is the SDS-PAGE protein pattern of kiwifruit extract of gold kiwifruit. Lane 3 is the SDS-PAGE protein pattern of kiwifruit extract of green kiwifruit.

4.2.3 Enzyme activity of different cultivars of kiwifruit

Figure 4-2 shows total enzyme activity of green and gold kiwifruit. Total enzyme activity of green and gold kiwifruit was 55.6 and 8.8 U/g kiwifruit, respectively. Results showed that total enzyme activity of green kiwifruit is about 8 times higher than that of gold kiwifruit.

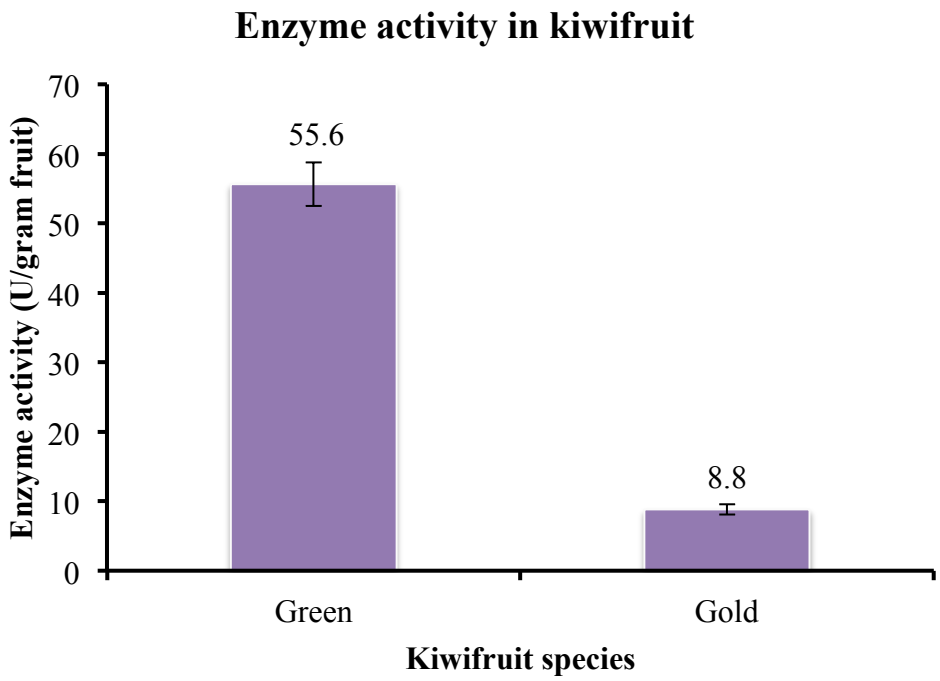

Figure 4-2 Total enzyme activity of green and gold kiwifruit. Five kiwifruit of each species were sampled in this test.

4.2.4 Effect of pH and incubation on enzyme activity

Figure 4-3 shows the specific enzyme activity changes of kiwifruit extracts at different pH values over time. The specific enzyme activity was monitored over 22 hours. During incubation, the kiwifruit extract as control and the kiwifruit extract at pH 4 showed a similar trend. There was a slight increase of specific enzyme activity after 4 or 6 hours' incubation. This may be because more active sites of actinidin were activated. For kiwifruit extract at pH 2 and 6, the actinidin was not stable and its specific enzyme activity decreased with incubation time. At pH 6, after 6 hours' incubation, kiwifruit extract lost all enzyme activity. At pH 2, initial specific enzyme activity of actinidin at 0 min was much lower than that of control kiwifruit extract and kiwifruit extract at pH 4 and 6. After 2 hours' incubation, the kiwifruit extract lost all enzyme activity. This study implies that decreasing pH to 2 rapidly denature actinidin and increasing pH up to 6 can slowly denature actinidin.

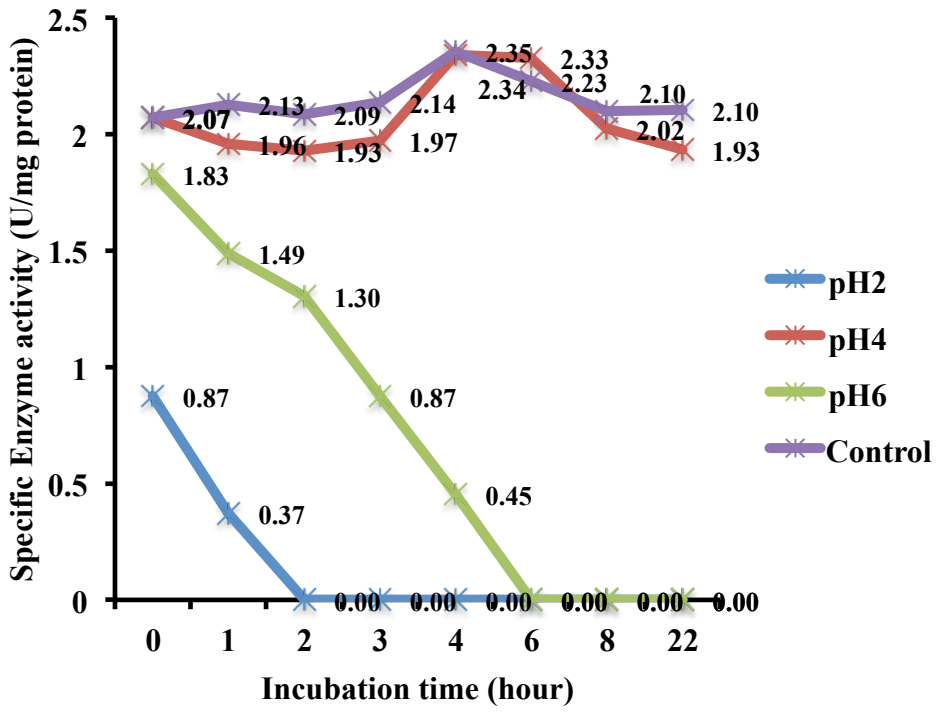

Figure 4-3 Actinidin activity over time in different pH environments. The pH value for the control was 3.5.

4.3 Conclusions

This chapter has provided general information about soluble proteins in kiwifruit. Protein content of green and gold kiwifruit was similar. The green and gold kiwifruit had different protein composition. Proteins of 27 and 24 kDa for both green and gold kiwifruit were the main proteins in kiwifruit. Total enzyme activity of green kiwifruit was much higher than that of gold kiwifruit. At pH 3-4, which is the pH of kiwifruit, the enzyme (actinidin) in kiwifruit extract was stable for up to 22 hours' incubation. Decreasing or increasing the pH led to loss of stability of active actinidin.

CHAPTER 5 ACTINIDIN PURIFICATION AND IDENTIFICATION

Abstract

Soluble proteins were prepared by grinding kiwifruit, centrifuging kiwifruit extract and dialyzing supernatant of kiwifruit extract. The soluble proteins were separated and purified by ion-exchanged chromatography. Protein fractions eluted by different volumes were analysed by SDS-PAGE. Specific enzyme activity of different protein fractions was determined. Purified actinidin was analysed by mass spectrometry. The results showed that molecular weight of actinidin with enzyme activity was at around 27 kDa determined by SDS-PAGE. Actinidin was composed of proteins with and without enzyme activity that had similar molecular weights. The molecular weight of the main peak of mass spectrometry was 23.5 kDa and is believed to be actinidin. It was interesting to note that proteins at around 27-28 kDa were recognized by anti-actinidin antibodies.

5.1 Introduction

Chapter 4 shows that there are many types of proteins in kiwifruit proteins. Protein separation and purification are one of the most important steps to understand which protein has enzyme activity and what actinidin are. Purification is very important for investigating the structure, physical properties and biological properties of kiwifruit proteins. Purification should yield a sample of protein containing only one type of molecule. Proteins can be purified according to solubility, size, charge, and binding affinity. There are several common methods for protein purification described below:

1. Salting Out

Most proteins are less soluble at high salt concentrations (salting out). The salt concentration at which a protein precipitates differs from one protein to another. Therefore, salting out can be used to separate proteins. Salting out is also useful for concentrating dilute solutions of proteins. Usually, dialysis is used to remove the salt if necessary.

2. Dialysis

Proteins can be separated from small molecules by dialysis through a semipermeable membrane with pores. Molecules with greater radius than the pore size of membrane are retained inside the dialysis bag, whereas smaller molecules traverse the pores of the membrane and emerge in the dialysate outside the bag.

3. Gel-Filtration Chromatography

Gel filtration chromatography separates proteins, and peptides on the basis of molecule size. The sample is applied to the top of a column consisting of porous beads made of an insoluble but highly hydrated polymer. Molecules move through a bed of porous beads, diffusing into the beads to greater or lesser degrees.

Smaller molecules can enter into the holes of the beads and therefore move through the bed more slowly, while larger molecules cannot and thus move through the bed more quickly. Both molecular weight and three-dimensional shape contribute to the degree of retention. Gel filtration chromatography may be used for analysis of molecular size, for separations of components in a mixture, or for salt removal.

4. Ion-Exchange Chromatography

Ion-exchange chromatography retains molecules on the column based on ionic interactions. The stationary phase surface displays ionic functional groups (R-X) that interact with analyte ions of opposite charge. For example, proteins that have a low density of net positive charge will tend to emerge first in the column with negative charges, followed by those having a higher charge density.

For green kiwifruit, actinidin is the predominant soluble protein comprising up to 40-50% of soluble proteins. Lewis and Luh (1988) found that the specific activity of given actinidin as about 40% of protein was about 4.2 U/mL, whereas the purified protein is described as having a specific activity of 57 U/mL, implying "pure" actinidin as determined by enzyme activity is less than 10% of the "actinidin". This discrepancy is unexplained.

In this chapter, ion-exchange technique was used to separate and purify kiwifruit soluble proteins. Enzyme activity of purified protein fractions was determined. Purified protein fractions were further characterized by SDS-PAGE and HPLC-MS/MS. Anti-actinidin antibodies were generated using purified actinidin. The purposes were to recognize actinidin and actinidin analogues from kiwifruit and to make affinity columns for separation of actinidin isoforms.

5.2 Results and discussion

5.2.1 Ion-exchange chromatography of kiwifruit proteins

Ion-exchange chromatography of kiwifruit soluble proteins on DEAE-Sephadex A-25 is shown in Figure 5-1. During the first 220 mL elution, a small portion of proteins without enzyme activity was eluted, indicating these fractions did not have active actinidin. In elution chromatography, the peak appeared between 250 and 350 mL of elution volume (fractions 33 to 47). Each fraction contains 7.5 mL solution. Proteins with enzyme activity began to elute at 220 mL (fraction 29). Between elution volumes of 250 to 350 mL (fractions 33 to 47), most active actinidin was eluted. Specific activity increased with elution volume and reached a plateau after 350 mL of elution volume. Ion-exchange chromatography indicated that actinidin may have different species or isoforms. Fractions from 33 to 45 (indicated as highlighted area in Figure 5-1) were further analysed by gel electrophoresis. Fractions 37, 41 and 46 (indicated as purple diamond in Figure 5-1) were selected for mass spectrometry analysis. This was consistent with the study of Lewis and Luh (1988a). They found that the specific enzyme activity of purified protein by ion-exchange chromatography was greatly increased.

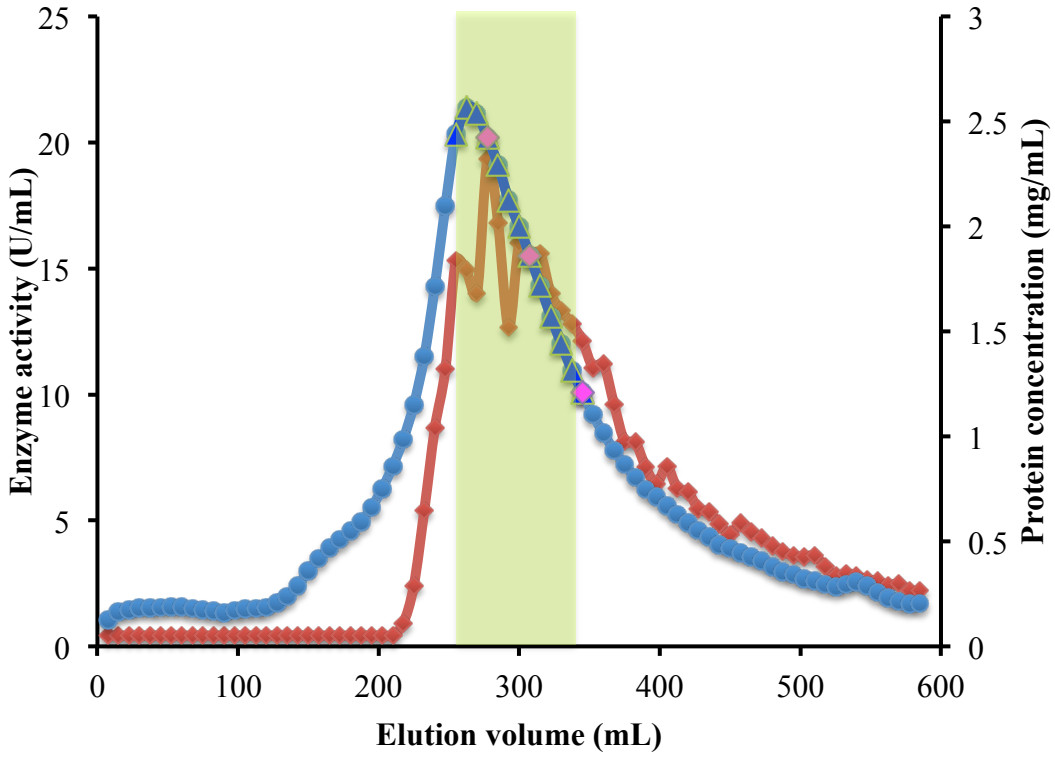

Figure 5-1 Ion-exchange chromatography of Hayward kiwifruit soluble proteins on DEAE-Sephadex A-25. Protein concentration was indicated by blue line. Enzyme activity per mL of fraction against CBZ-L-Lys-p-nitrophenol hydrochloride is indicated by red line. Fractions marked with triangle in green highlight area were further analysed by SDS-PAGE. Fractions marked with pink diamond in green highlight area were further analysed by mass spectrometry.

5.2.2 SDS-PAGE of Hayward kiwifruit soluble proteins

Fractions with different levels of enzyme activity were analysed by SDS-PAGE right after separation. As shown in Figure 5-2, selected fractions presented one single main band with similar molecular weight (around 27.5 kDa). The intensity of each band accounted for greater than 95% in each lane, indicating that the actinidin had been purified.

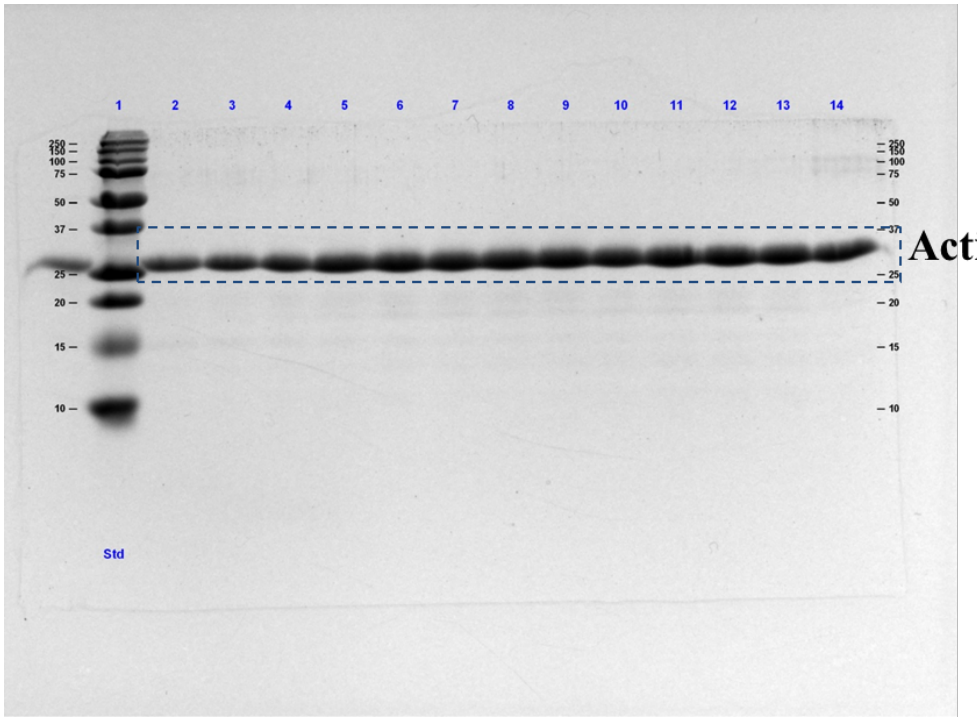

Figure 5-2 SDS-PAGE analysis of actinidin fractions purified by ion-exchange chromatography. Lane 1 was loaded with protein standards. Lane 2-14 were loaded with fractions collected from elution volume of 250 mL to 350mL (Fraction 33 to 45). No visible difference was observed between fractions by electrophoresis.

5.2.3 Actinidin Analysis by Mass Spectrometry (Hayward kiwifruit)

Figures 5-3, 5-4 and 5-5 show the mass spectra of purified protein fractions with different enzyme activities. All three fractions showed the same main peak with the molecular weight of 23,884 Da. This is consistent with the previous study of Grozdanovic et al (2012). They purified kiwifruit soluble proteins using HPLC, 1-D and 2-D gel electrophoresis. The main peak of purified proteins by ion-exchange chromatography had a different molecular weight compared to the main band of SDS-PAGE. This can be attributed to the difference of two methods. SDS-PAGE has two main limitations in estimating molecular weight of proteins. The first limitation is amount of SDS bound to proteins is not constant. The second one is protein mobility in the gel is more a function of molecular size (which is a function of both weight and length) than of molecular mass.

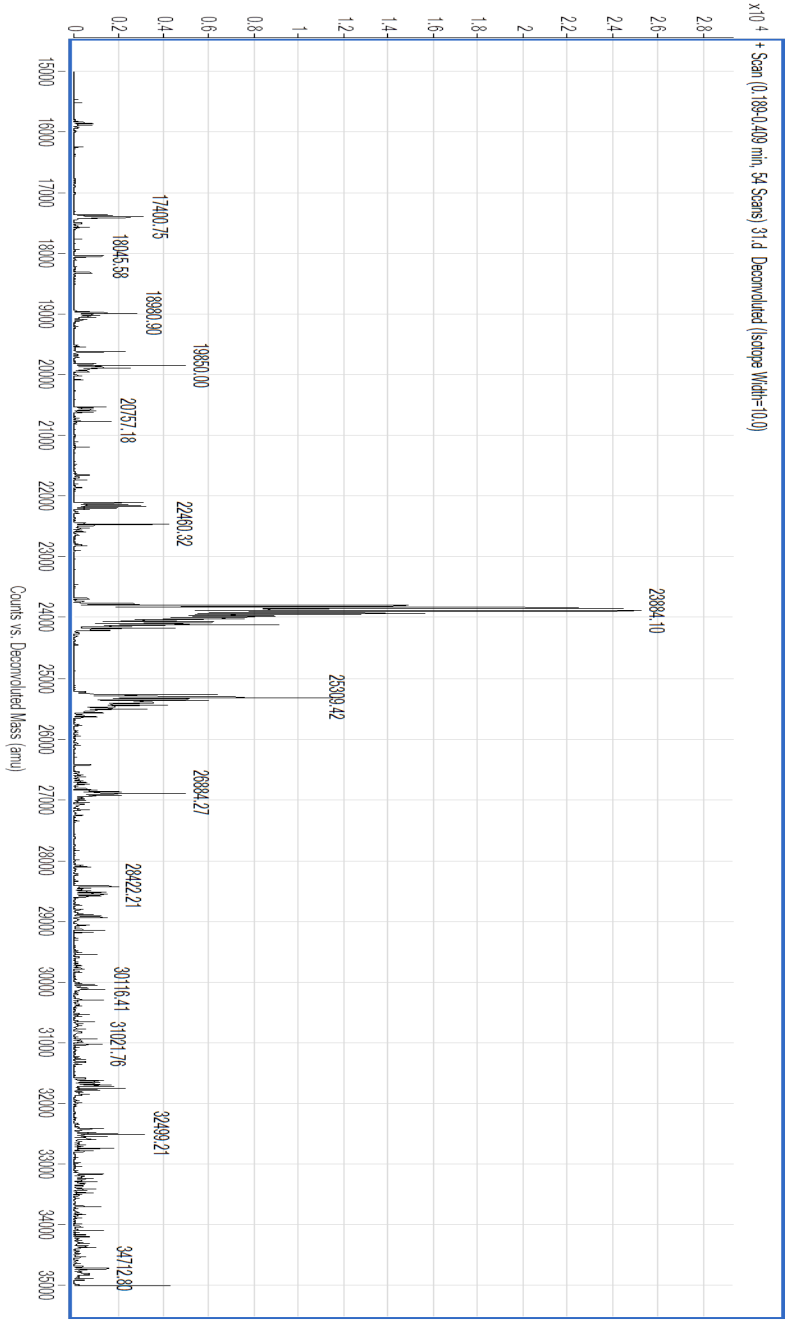

Figure 5-3 Mass spectrum of fraction (#46) with specific enzyme activity of 9.6 U/mg

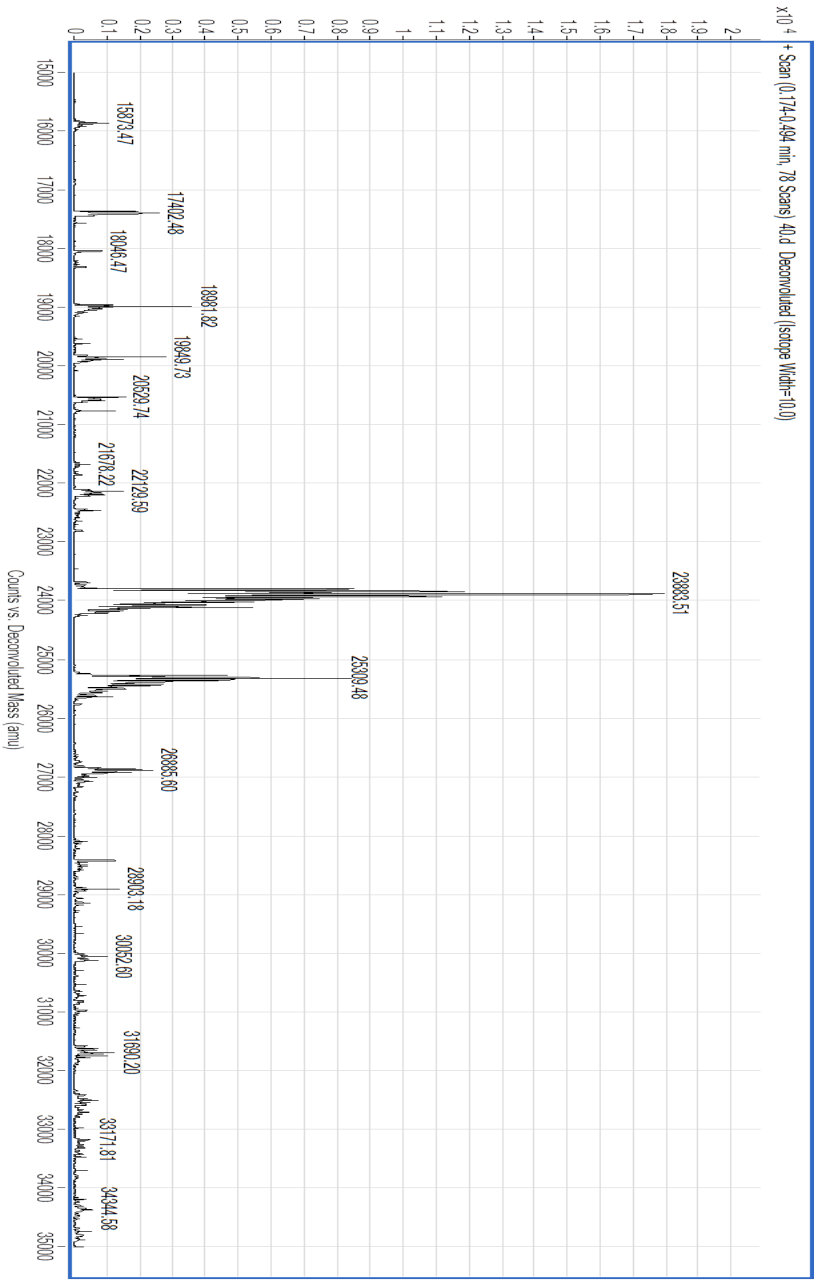

Figure 5-4 Mass spectrum of fraction (#41) with specific enzyme activity of 8.1 U/mg.

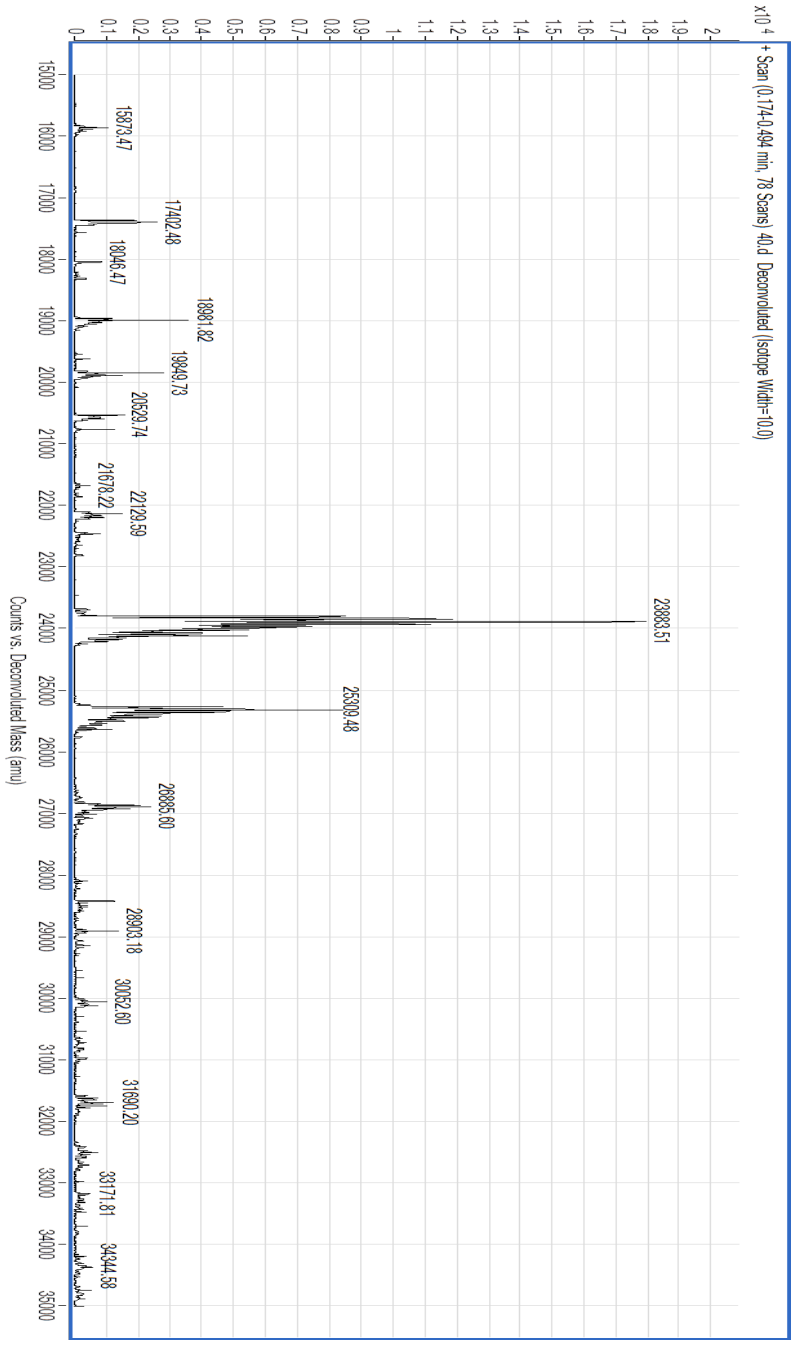

Figure 5-5 Mass spectrum of fraction (#37) with specific enzyme activity of 6.6 U/mg.

5.2.4 Anti-rabbit actinidin antibody generation

No non-specific binding was observed from background testing (Figure not shown). Figure 5-6 is the result of the Western blot analysis of protein extracted from Hayward kiwifruit and SunGold kiwifruit. The results showed antibody was successfully generated. It was interesting to note that anti-actinidin antibodies from Green kiwifruit extract recognized proteins at around 27-28 kDa, while 25 kDa bands were recognized from SunGold kiwifruit extract. Moreover, for both kiwifruit cultivars, several other bands were able to be observed on the membrane. It is unclear if these proteins were actinidin degradations which contain a specific epitope that can be recognized by the antibodies or prepro actinidin.

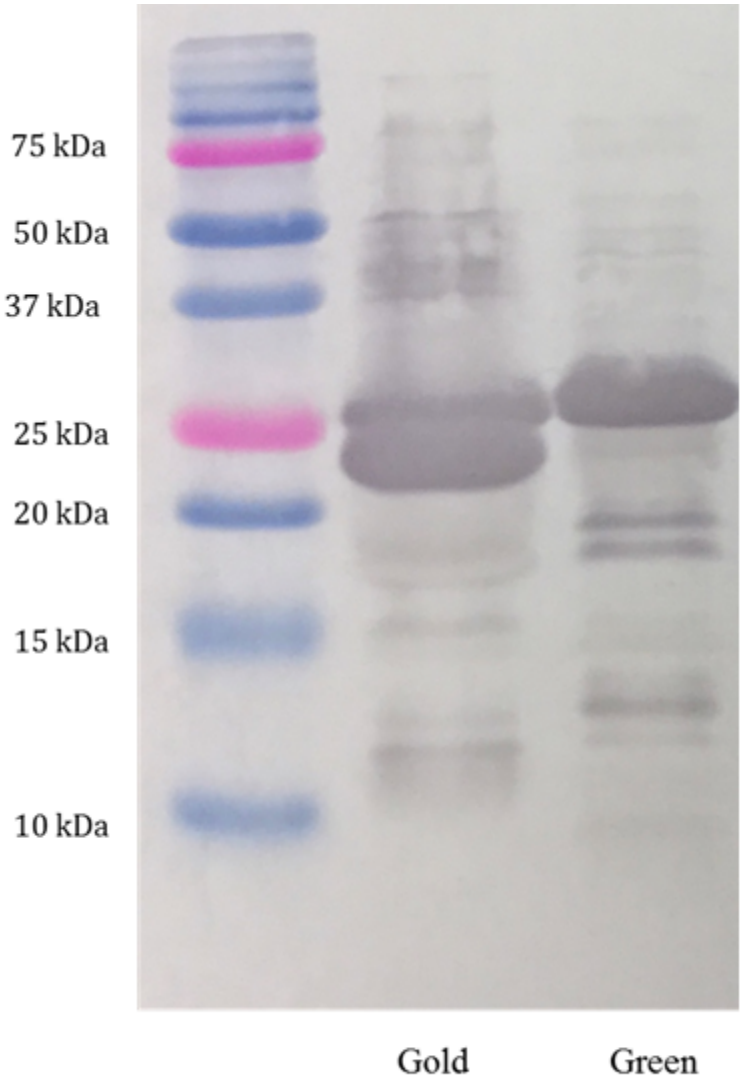

Figure 5-6 Western blot analysis of proteins extracted from Hayward kiwifruit and SunGold kiwifruit. Kiwifruit varieties are as indicated in lanes 2 and 3. Lane 1 is the standard.

5.3 Conclusions

The molecular weight of proteins with enzyme activity was about 27 kDa measured by SDS-PAGE and about 24 kDa detected by mass spectrometry, respectively. In Western blotting, a protein band of 27 kDa from green kiwifruit was detected. Meanwhile, protein bands at 27 kDa and 24 kDa from SunGold kiwifruit were detected. bovine serum albumin These results indicated these proteins contained the same epitope. Further investigation is needed for a more precise conclusion.

The different protein fractions separated by ion-exchange chromatography had different specific enzyme activity, while mass spectrometry results showed that they had similar molecular weights. This observation is consistent with several previous studies (Tello-Solís, Valle-Guadarrama & Hernández-Arana., 1995; Sugiyama et al., 1996), which also successfully separated numbers of actinidin isoforms from kiwifruit extract. Results of mass spectrometry analysis of fraction with different specific enzyme activity showed the molecular mass of main protein in these fractions was similar, which was around 23,883 Da. It is consistent with a previous study (Grozdanović et al., 2013), which reported the molecular mass of isolated actinidin was 23,883 Da measured by mass spectrometry. This may suggest that actinidin was composed of different species or isoforms; but only some had enzyme activity.

CHAPTER 6 EFFECT OF POST-HARVEST STORAGE ON THE ACTINIDIN ACTIVITY AND PROTEIN CONTENT

Abstract

In this chapter, the effect of post-harvest storage on the enzyme activity of kiwifruit was investigated. SunGold and Hayward kiwifruit were selected for the study. SunGold kiwifruit from 5 different growers was stored at 1 °C for 0, 1, 2, 4, 8 and 12 weeks. Hayward kiwifruit from one grower was stored for 0, 1, 2, 4, 8, 12 and 16 weeks. Protein content, specific enzyme activity of soluble kiwifruit proteins and total enzyme activity of kiwifruit with the storage time were determined. In general, the post-harvest storage had a significant effect on protein content and kiwifruit enzyme activity.

6.1 Introduction

Post-harvest storage greatly influences the quality of kiwifruit. The effect of various storage conditions on the maturity and storability of kiwifruit has been investigated. There has been literature indicating that during 3 weeks postharvest ripening at 5 degrees Celsius, actinidin activity of green kiwifruit increased 3 – fold while specific enzyme activity decreased by 15% (Lewis & Luh, 1988b). Boyes, Strübi and Marsh (1997a) reported that there were small changes in actinidin activity during up to 2 months' storage. Results from another study indicated that postharvest handling might influence protein concentration in green kiwifruit (Ciardiello et al., 2009). However, to date, there has been limited research on effect of storage conditions on enzyme activity changes of golden kiwifruit during post-harvest storage. In this chapter, effect of post-harvest storage time on specific enzyme activity of kiwifruit soluble proteins and total enzyme activity of kiwifruit was investigated. Two cultivars of kiwifruit (i.e. Hayward and SunGold) were used for the comparison.

6.2 Results and discussion

6.2.1 Variability of enzyme activity among fruits

Specific enzyme activity of different individual fruit is shown in Figure 6-1. Specific enzyme activity of actinidin of randomly selected kiwifruit was significantly different at $P<0.05$.

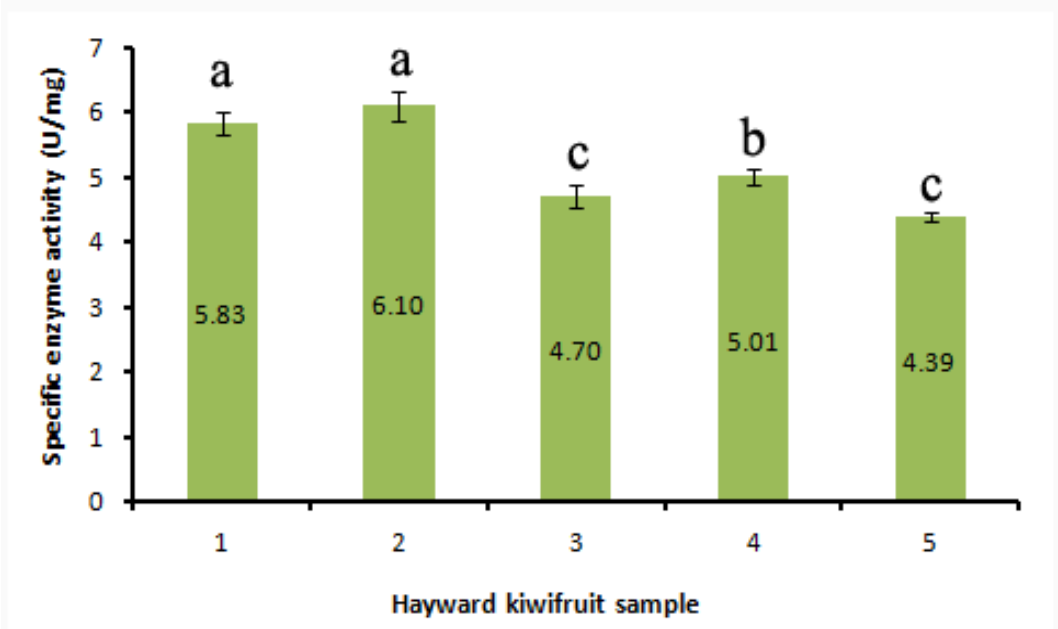

Figure 6-1 Specific enzyme activity of different individual kiwifruit. Different alphabetical letters represent significant difference at $P<0.05$.

6.2.2 Effect of post-harvest storage on actinidin enzyme activity of SunGold kiwifruit from different growers

Specific enzyme activity of soluble protein from SunGold kiwifruit at commercial harvest time (Day 0) and after 1, 2, 4, 8 and 12 weeks' storage is shown in Table 6-1. SunGold kiwifruit (*Actinidia chinensis* cv. 'Zesy002') was collected at commercial harvest time from 5 different growers. Kiwifruit were stored at 1 °C for 0, 1, 2, 4, 8 and 12 weeks, respectively. Ten kiwifruit were sampled for each

test. In general, both grower and storage time had significant influence on the specific enzyme activity of actinidin ($P<0.0002$). There were no significant differences among 0, 1 and 2 weeks' storage ($P<0.05$). Specific activity had a significant increase at 4 weeks, reached the highest value at 8 weeks and then decreased significantly at 12 weeks ($P<0.05$). Boyes et al. (1997a) found that up to 2 months' storage after the commercial harvest period, the kiwifruit remained hard, and the change in actinidin levels was small. My results were different from those of Lewis and Luh (1988b). In their study, kiwifruit were obtained directly postharvest (Winters, California) in the firm state and ripened at 5 °C in air for three weeks. They found a 3-fold increase in actinidin activity but 14.3 % decrease in specific activity over 3 weeks ripening. They ascribed the decrease of specific activity to the release of cell wall and membrane proteins.

Table 6-1 Specific enzyme activity of SunGold kiwifruit

Week/Grower	Specific enzyme activity (U/mg)				
	1	3	4	5	12
0	0.69±0.04	0.50±0.03	0.74±0.03	0.78±0.05	0.70±0.03
1	0.66±0.03	0.71±0.04	0.67±0.02	0.66±0.04	0.63±0.01
2	0.80±0.02	0.70±0.05	0.68±0.04	0.66±0.02	0.61±0.03
4	0.79±0.02	0.90±0.03	0.89±0.01	0.87±0.03	0.74±0.06
8	0.86±0.02	1.13±0.01	0.82±0.07	1.01±0.11	0.84±0.04
12	0.80±0.01	0.85±0.03	0.79±0.01	0.66±0.03	0.87±0.02

Total enzyme activity of kiwifruit is shown in Table 6-2. In general, both storage time and grower influenced total enzyme activity significantly ($P<0.001$). Total enzyme activity increased significantly from 0 to 8 weeks ($P<0.05$) which was consistent with the study of Lewis and Luh (1988b). There was no significant difference between 8 and 12 weeks ($P<0.05$).

Table 6-2 Total enzyme activity of SunGold kiwifruit

Week/Grower	Enzyme activity (U/g)				
	1	3	4	5	12
0	6.21±0.40	5.50±0.34	7.23±0.25	8.21±0.55	6.93±0.28
1	6.30±0.32	7.27±0.45	7.53±0.27	8.24±0.52	7.39±0.16
2	7.69±0.24	7.60±0.54	8.09±0.47	8.39±0.24	8.12±0.39
4	9.07±0.25	8.47±0.24	8.95±0.14	9.42±0.34	9.29±0.71
8	8.58±0.25	9.72±0.05	9.32±0.84	10.30±1.13	9.76±0.45
12	7.87±0.12	9.79±0.31	9.79±0.11	8.52±0.38	10.21±0.24

6.2.3 Effect of post-harvest storage on actinidin enzyme activity of Hayward kiwifruit

Figure 6-2 shows the results of trend of actinidin enzyme activity in green kiwifruit. The green kiwifruit showed a different trend of enzyme activity compared with gold kiwifruit. Specific enzyme activity significantly decreased from 5.8 to 5.0 U/mg for 16 weeks' storage ($P<0.05$). Total enzyme activity of kiwifruit significantly decreased from 59 to 51 U/g for 16 weeks' storage ($P<0.05$).

Importantly, the specific enzyme activity of soluble proteins in Hayward kiwifruit (green) and enzyme activity of kiwifruit was about 8 times higher than that in SunGold kiwifruit. This indicated that green kiwifruit had a much higher content of active actinidin than SunGold kiwifruit.

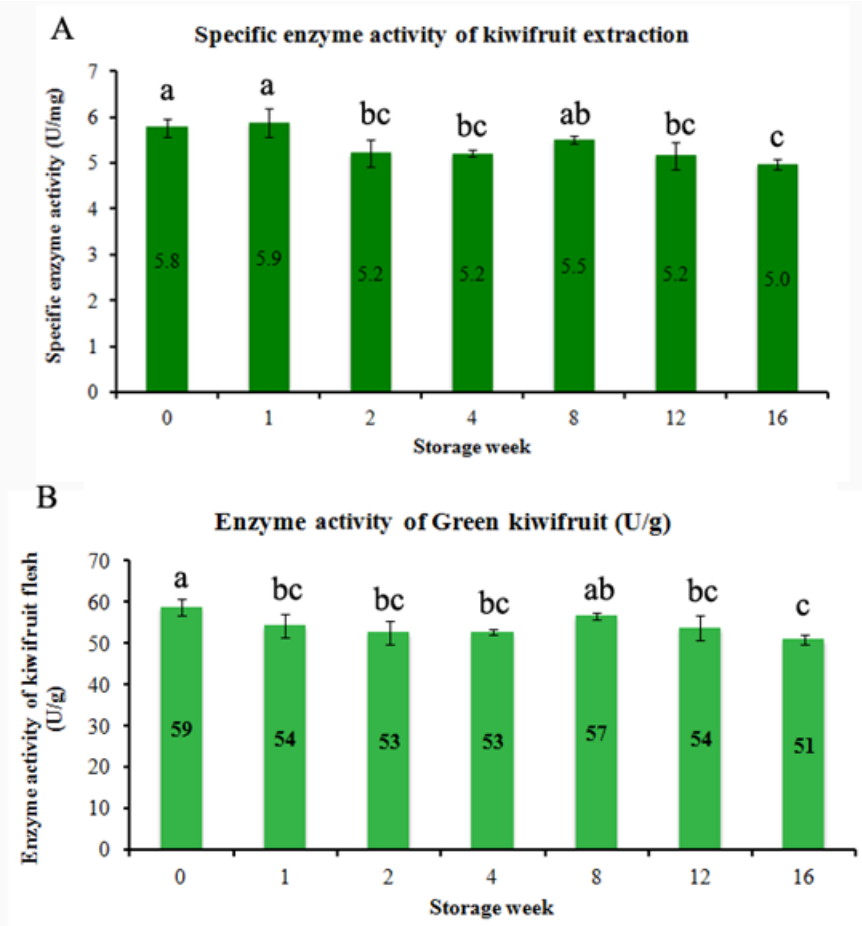

Figure 6-2 Specific enzyme activity of soluble protein from Hayward kiwifruit (A) and total enzyme activity of Hayward kiwifruit (B) at commercial harvest time (Day 0) and after 1, 2, 4, 8, 12 and 16 weeks' storage. Ten kiwifruit were sampled for each test. Different alphabetical letters represent significant difference at $P<0.05$.

6.2.4 Changes of Soluble Protein Content with Storage Time

Changes of protein content in SunGold kiwifruit extract during post-harvest storage are shown in Table 6-3. In general, both storage time and grower had a significant influence on protein content of SunGold kiwifruit ($P<0.001$). For SunGold kiwifruit from 5 growers, protein content fluctuated between 5.04 and 5.84 mg/mL. Protein changes in Hayward kiwifruit extract during post-harvest storage are shown in Figure 6-3. There were no significant changes in protein content (around 5.0 mg/mL) during 16 weeks of storage at $P<0.05$ (except 1 week storage). In general, SunGold kiwifruit had significantly lower protein content than Hayward kiwifruit before storage ($P<0.05$). After storage, SunGold kiwifruit had significantly higher protein content than Hayward kiwifruit ($P<0.05$). This strongly supported that Hayward kiwifruit had much higher amount of active actinidin.

Ciardiello et al. (2009) found that gold kiwifruit protein content was a little higher than that of green kiwifruit. They also reported that the post-harvest storage could increase the protein content of kiwifruit. After 2 months' storage at 4 °C, the protein content for gold and green kiwifruit measured by the Bradford method was about 3.2 and 3.5 mg/g tissue, respectively.

Table 6-3 Protein changes in SunGold kiwifruit extract during post-harvest storage

Week/Grower	Protein content (mg/mL)				
	1	3	4	5	12
0	4.52±0.17	5.53±0.12	4.88±0.04	5.29±0.11	4.98±0.10
1	4.80±0.01	5.11±0.14	5.65±0.04	6.27±0.19	5.90±0.10
2	4.79±0.03	5.42±0.02	5.99±0.11	6.34±0.16	6.62±0.19
4	4.95±0.03	4.41±0.13	5.62±0.08	5.99±0.02	6.38±0.02
8	4.99±0.49	4.31±0.17	5.65±0.04	5.12±0.29	5.79±0.16
12	4.92±0.14	5.76±0.16	6.16±0.18	6.47±0.03	5.90±0.20

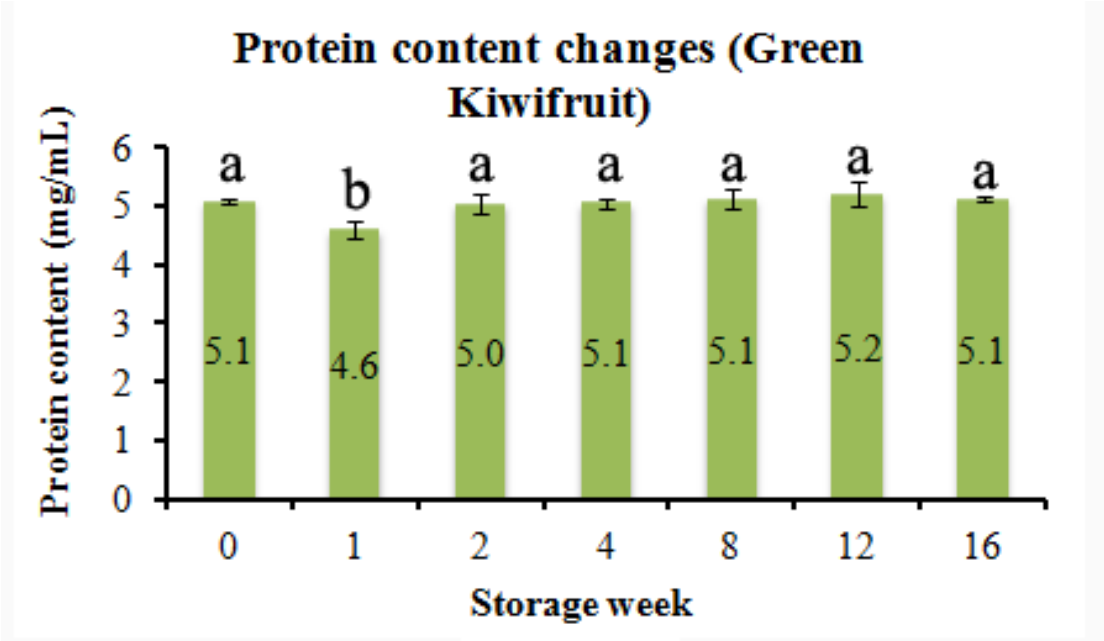

Figure 6-3 Protein changes in Hayward kiwifruit extract during post-harvest storage at 1°C. Ten kiwifruit were sampled for each test. Different alphabetical letters represent significant difference at $P<0.05$.

6.3 Conclusions

After harvest, the total enzyme activity of SunGold kiwifruit significantly increased with storage time. However, total enzyme activity of Hayward kiwifruit decreased significantly with storage time. In general, the specific enzyme activity of soluble proteins in SunGold kiwifruit had a significant increase after storage time (4, 87 and 12 weeks) while that in Hayward kiwifruit had a significant decrease. Protein content of SunGold kiwifruit fluctuated over a small range while that of Hayward kiwifruit had no significant changes. These findings indicate that more active actinidin was generated during storage in SunGold kiwifruit compared with Hayward kiwifruit. In general, the postharvest storage had a significant influence on the enzyme activity of both SunGold and Hayward kiwifruit. In addition, different growers also significantly influenced enzyme activity of kiwifruit.

CHAPTER 7 OVERALL CONCLUSION AND DISCUSSION

7.1 Overall Conclusions and Discussion

7.1.1 Protein Determination of Kiwifruit Extract

This project provides an overall picture of the soluble proteins in both Hayward and SunGold kiwifruit, which will contribute to the understanding of kiwifruit proteins. Four different protein determination methods were compared in this project. The Dumas method is a method for the quantitative determination of nitrogen in chemical substances. It can accurately measure total nitrogen of samples that is converted into protein content. One major disadvantage of the Dumas method is its high cost. The results of the Hartree-Lowry and Bradford methods were similar to those of the Dumas method. The Hartree-Lowry and Bradford methods were believed to be the most accurate method for protein determination of kiwifruit proteins because reducing sugars in kiwifruit had little interference in these two assays. Protein content determined by the BCA method was higher than that measured by the other three methods, which was ascribed to the high sensitivity of the BCA method to reducing sugars and subsequent interference of protein quantification (Sapan et al., 1999). Protein content of SunGold kiwifruit was found to be similar to that of Hayward kiwifruit. Total enzyme activity of SunGold kiwifruit was 8 times lower than that of Hayward kiwifruit. This implies that SunGold kiwifruit has much less active actinidin than Hayward kiwifruit.

7.1.2 Enzyme Activity

The pH had a great impact on enzyme activity of kiwifruit proteins. Lowering the pH of kiwifruit extract (pH at around 3.5) to 2 led to a fast loss of enzyme activity. After 2 hours' incubation, the kiwifruit proteins lost all enzyme activity. Increasing the pH of kiwifruit extract to 6 also decreased enzyme activity of kiwifruit proteins. After 6 hours' incubation, the kiwifruit proteins lost all enzyme activity. This implies that a severe acidic or neutral environment would cause the loss of enzyme activity of kiwifruit proteins. As far as we know, there is no reference data in the literature about the actinidin stability as a function of pH. There is a limitation for the method of enzyme activity determination, i.e., the substrate for enzyme activity determination was not stable (spontaneous hydrolysis) during the assay. However, this method is accurate in general because the spontaneous hydrolysis of the substrate was very slow at acidic or neutral pH.

7.1.3 Kiwifruit Soluble Protein Composition and Actinidin Purification

Soluble kiwifruit proteins of Hayward and SunGold kiwifruit were analyzed by SDS-PAGE. Hayward and SunGold kiwifruit had different SDS-PAGE protein patterns. The main band of both Hayward and SunGold kiwifruit was at about 27 kDa.

Soluble proteins of kiwifruit were separated and purified by ion-exchange chromatography. The fractions collected at different elution volume had different specific enzyme activity. These fractions had a similar molecular weight that was at around 27 kDa determined by SDS-PAGE. This implied that the proteins at 27 kDa had enzyme activity and they contained inactive proteins without enzyme activity. The proteins at 27 kDa without enzyme activity may be isoforms of proteins with enzyme activity. Other researchers have reported the actinidin has a

molecular weight of 30 kDa (Paul et al., 1995) or 24 kDa (Nieuwenhuizen et al., 2007) determined by SDS-PAGE. This difference may be because gel electrophoresis cannot provide an exact molecular weight. During gel electrophoresis, there are many factors (e.g. protein purity, buffer pH and SDS concentration) possibly influencing mobility of proteins, thereby leading to different observed molecular weights. In addition, different protein standards may affect the calculation of molecular weight of proteins. Of note, the molecular weight of actinidin determined using mass spectrometry in this and previous studies is around 23 kDa. This indicates that actinidin of different molecular weights determined by SDS-PAGE (24, 27 or 30 kDa) are same. The molecular weights of Kiwellin, thaumatin-like protein and Kirola determined by SDS-PAGE (24.7, 22.6 and 14.9 kDa) are also different from that reported by previous work (28, 24 and 17 kDa) (Paul et al., 1995). In addition, 1D SDS-PAGE cannot separate proteins with similar molecular weights. To better characterize the kiwifruit proteins, 2D SDS-PAGE is recommended for the future study.

7.1.4 Effect of Post-harvest Storage on Enzyme Activity and Protein Content

The post-harvest storage of SunGold kiwifruit at 1 °C up to 12 weeks significantly increased total and specific enzyme activities of actinidin. In contrast, the post-harvest storage up to 16 weeks significantly decreased total and specific enzyme activities of actinidin in Hayward kiwifruit. With up to 16 weeks' storage, the protein content of Hayward kiwifruit had no significant changes while that of SunGold kiwifruit fluctuated over a small range during the storage. This implies that maturation of kiwifruit during post-harvest storage can activate actinidin and generate more soluble proteins in SunGold kiwifruit; however, post-harvest

storage led to the loss of enzyme activity in Hayward kiwifruit. Ciardiello et al. (2009) observed that kiwifruit protein concentration increased slowly after the stage of commercial harvest time, and the actinidin concentration (measured by relative abundance in SDS-PAGE) did not change much during this stage. In contrast, results from other literature reported that both protein concentration and actinidin activity increased more than 3-fold after 3 weeks post-harvest storage at 5 °C (Lewis & Luh, 1988b).

In addition, kiwifruit production is greatly affected by climate (e.g. altitude, daily temperature, rainfall, humidity) and soil (e.g. soil type and pH). Moreover, kiwifruit product is influenced by how it is cultured such as planting, spacing, pollination, irrigation and fertilizer. These factors may lead to the variation of protein content and enzyme activity of kiwifruit from different growers (Ferguson, & Seal, 2008; Ferguson, 2013).

Enzyme activity and protein content were monitored at the different time points during storage and the kiwifruit were selected randomly at each time point. That is to say the selected kiwifruit for each time point may have had different ripeness. Futher study may be carried out by selecting kiwifruit with the same ripeness (i.e. same firmness) to better undestand the effect of post-harvest storage on the enzyme activity and protein content.

7.2 Recommendation for future work

7.2.1 Separation and identification of actinidin isoforms

This project is the preliminary study of a Ph.D. project. Actinidin separation and purification may be improved in a future study. Since the resolution of ion exchange chromatography is not satisfactory. The ion-exchange resin

DEAE-Sephadex can be substituted by other resin: “Source Q” (GE health), which might have better resolution. HPLC is another alternative for higher resolution of the separation.

7.2.2 Effect of ripening and postharvest on enzyme activity of actinidin

To investigate the relationship between actinidin isoforms/activity and the maturity of kiwifruit, further test samples could be collected before commercial harvest time. In addition to storage/harvest time, some tests need to be carried out to evaluate the maturity of kiwifruit, such as Brix, dry Matter, seed colour, weight, flesh colour and firmness. To identify the pure actinidin with enzyme activity, HPLC-MS/MS is recommended to be used in the future study.

7.3 Implications of the research

Enzymes from plant sources are becoming increasingly popular in food industry as e.g. meat tenderising and digestive enzymes. My research has shown that: 1) the actinidin has different species/isoforms with different levels of enzyme activity; 2) the optimal pH for stability of actinidin is around 3.5; 3) green kiwifuit have a much larger amount of active actinidin than gold kiwifruit. This could contribute to the isolation/seperation of actinidin and its wide use in food industry. Post-harvest storage is very important for the maturity and quality of kiwifruit. The change of protein content and enzyme activity of kiwifruit during post-harvest storage is one of the factors affecting post-harvest quality of kiwifruit. Interestingly, the post-harvest storage significantly increases the protein and enzyme activity of gold kiwifruit with a slight decrease in green kiwifruit. This would contribute to maintaining the post-harvest quality of kiwifruit.

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