

Proteinase Activities of Kiwifruit, Pineapple and Papaya Using Ovalbumin, Soy Protein, Casein and Bovine Serum Albumin as Substrates

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Abstract Although there are several thousand publications on the proteinases of pineapple, papaya and kiwifruit dating back many decades, controversy remains over which fruit delivers the greatest proteinase activity against common dietary proteins. This is because of the variable experimental conditions used: pH, substrate, enzyme purity and enzyme quality. Therefore the proteinase activities of fresh green and gold kiwifruit, pineapple and papaya, sometimes marketed as dietary proteinase supplements, were directly compared for their proteinase activities against bovine β -casein, bovine serum albumin, chicken ovalbumin and soy protein across a pH range from pH 2 to pH 9. Proteinase activity was assessed using proteins labelled with the fluorescent dye bodipy-FL and by quantification of unlabelled protein digestion by SDS-PAGE. Bodipy-casein performed well as a substrate giving signal: noise ratios of 20:1. Bodipy-ovalbumin and bodipy-soy performed poorly as substrates. SDS-PAGE showed that bovine serum albumin and ovalbumin were readily digested by kiwifruit actinidin despite published reports to the contrary. Two per cent SDS promoted bromelain digestion of BSA at pH 7 but at pH 4 prevented BSA digestion at pH4. Gold kiwifruit was considerably better at digesting ovalbumin at pH 7 than green kiwifruit. Incubation of green kiwifruit homogenate at 37°C for 20 minutes led to a 45% loss of actinidin whereas no autocatalysis occurred at room temperature. Soy protein and casein were rapidly digested by kiwifruit and pineapple extracts. Papaya extract was very low in proteinase activity. Thus the evaluation of each fruit as an effective dietary proteinase supplement is greatly influenced by factors such as substrate choice, the presence of SDS and pH.

Keywords: *pineapple, papaya, kiwifruit, proteinase, ovalbumin, bovine serum albumin, soy, β -casein*

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1. Introduction

The study of the fruit proteinases papain, bromelain and actinidin (from papaya, pineapple and kiwifruit respectively) has been so extensive that thousands of publications are to be found in the scientific literature. The references date back to the 1900s for papain [1], the 1950s for bromelain [2] and the 1980s for actinidin [3]. The availability of this quantity of information would, at first sight, suggest that little further study of these enzymes' abilities to digest proteins, including food proteins, would be necessary. However, some of the published information contradicts our own in-house knowledge of actinidin. For example, it is reported that actinidin has limited activity against Bovine Serum Albumin (BSA) and that actinidin has a narrow substrate specificity [4]. Similarly, other reports suggest that some forms of actinidin can digest casein but not BSA or ovalbumin [5] yet our own experience is that BSA is indeed readily digested by actinidin. The reason for such discrepancies between reports will probably lie in the experimental procedures. A potential difficulty with

proteinase purification methods is that they may involve the inclusion of inhibitors [4] that might not be fully removed before assay of the enzyme. Alternatively, insufficient inhibitor concentration during a lengthy purification step might allow autocatalysis to take place. Choice of assay conditions may also greatly affect the observed activity, for example, one report shows weak actinidin activity used purified actinidin at pH 6 [5] whereas our assays show that its optimum pH is pH 4 – pH 4.5. The failure of synthetic substrates to predict actual catalytic activity against proteins was highlighted in a recent review [6]. In the same vein, a comparison of actinidin, bromelain and papain as meat tenderisers found substantial differences in the proteinases depending on whether connective tissue or muscle tissue protein was used as a substrate [7]. The same recent report examines proteolysis from the range pH 5 – pH 9 and is therefore of limited value in the acid conditions of the fruit homogenate and the stomach.

In this study we compare whole kiwifruit extract (actinidin), pineapple extract (bromelain) and papaya extract (papain) for their abilities to digest the common dietary proteins β -casein, egg-white albumin (ovalbumin)

and soy protein. Whole fresh fruit was chosen instead of purified proteinases because we wished to compare the actual proteinase content of foods as experienced by consumers of these products. The use of whole fruits has the added advantage that proteinase purification is likely to lead to some loss of proteinase function. We assign cysteine proteinase activity in the whole fruit extracts on the basis of sensitivity to the cysteine proteinase inhibitor E-64 [8]. The proteinase activities in the fruit extracts were compared over a pH range pH 2 – pH 9 because this encompassed the pH range of the human digestive system. Our first approach was to label the proteins with the fluorescent dye bodipy-FL, chosen because it is comparatively pH insensitive [9]. A protein bearing multiple fluor is minimally fluorescent due to the phenomenon of fluorescence resonance energy quenching. As the fluor labelled fragments are separated during digestion, the quenching phenomenon no longer occurs and a fluorescent signal is generated [10]. We also compare the unlabelled form of these protein substrates for digestibility on SDS-PAGE. Because our previous unpublished results disagree with the some literature reports, we have included BSA in the protein substrates studied by SDS-PAGE.

2. Materials and Methods

2.1. Materials

The following items were supplied by Sigma: 2-mercaptoethanol, Bis-Tris, bovine β -casein, bovine serum albumin, E-64 (a selective inhibitor of cysteine proteinases, [11]), glycine, ovalbumin, sodium acetate, Tris buffer, Igepal® detergent, PD10-desalting columns. Soy protein (Red8, Integria Healthcare) was supplied from Health & Vitality, Palmerston North. Fresh fruit (green ‘Hayward’ kiwifruit *Actinidia chinensis* var. *deliciosa*, gold ‘Zesy002’ kiwifruit SunGold™ *A. chinensis* var. *chinensis*, papaya and pineapple) were supplied by New World supermarket, Palmerston North. Papaya [also known as paw paw] and pineapple were produced in the Philippines. Four of each fruit were purchased. Bodipy-FL succinimidyl ester was supplied by Thermo Fisher Scientific Inc.

2.2. Methods

2.2.1. Bodipy Labelling Of Protein Substrates

Proteins were labelled with bodipy fluor as described in the E-64 actinidin assay method [12]. Briefly, 0.5mg protein was dissolved in 100 μ l of 0.1M carbonate buffer pH 9 and labelled at a fluor: protein molar ratio of 12.5:1; 5:1, and 2:1 (high, medium and low labelling). After 3h at 37°C the succinimidyl ester was blocked by addition of Tris pH 9 to a final molarity of 0.1M. The sample was diluted to 0.5ml and run on a PD-10 column equilibrated with phosphate buffered saline to separate labelled protein from excess, uncoupled fluor.

2.2.2. Fruit Homogenate Preparation

A scalpel and a glass Dounce homogeniser were used to produce kiwifruit, pineapple and papaya homogenate using

equal weights of kiwifruit to water containing 0.1% (14mM) 2-mercaptoethanol and 0.2% Igepal. The homogenised extract was microfuged at 14,000 rpm for 10 min and stored aliquoted at -80°C until used. No differences in caseinolytic activity could be detected among individual fruits of each type. Sample were therefore pooled.

2.2.3. Fluorescence Assays

Fluorescence readings were taken using a Tecan Safire2 microplate reader on white 384 well microplates (proxiplates, PerkinElmer) in 20 μ l volumes. Bodipy-casein fluorescence was measured at excitation and emission wavelengths of 502 and 515 nm (5 nm bandwidth). Coumarin fluorescence was measured at excitation and emission wavelengths of 351 and 430nm (bandwidth 20nm). Data were plotted in Excel or Origin® curve fitting software. Proteinase activity was measured in 100mM buffers; Glycine pH 2–3.5, Acetate pH 4–5.5, Bis-Tris pH 6–7, Tris pH 7.5–9.

2.2.4. SDS-Polyacrylamide Gel Electrophoresis

Protein samples were analysed reduced on step gradient polyacrylamide gels (10/15/20% acrylamide) using a Bio-Rad Mini-Protean® II electrophoresis system. Coomassie stained protein was detected by scanning on an Epson Perfection 4990 flatbed scanner and quantified using Multi Gauge Software, Fujifilm.

3. Results & Discussion

3.1. Labelling Protein Substrates with Bodipy-fluor

Crude soy protein, purified β -casein and purified ovalbumin substrates were labelled at three different densities with bodipy-succinimidyl ester as described. This soy food product has been heat treated during production and no longer contains any proteinase inhibitory activity. Only about 20% of the crude soy protein was soluble in the carbonate labelling buffer. Only carbonate-soluble soy protein was labelled and this altered the protein profile of the soy preparation somewhat (Figure 1).

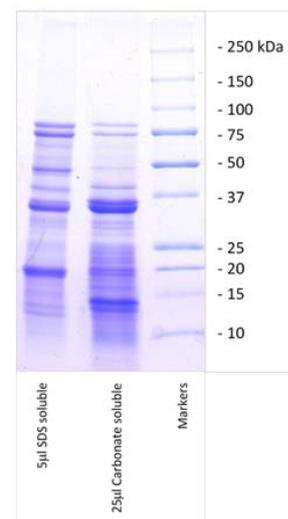


Figure 1. Comparison of SDS- soluble and carbonate-soluble protein in Soy Red8 by SDS-PAGE

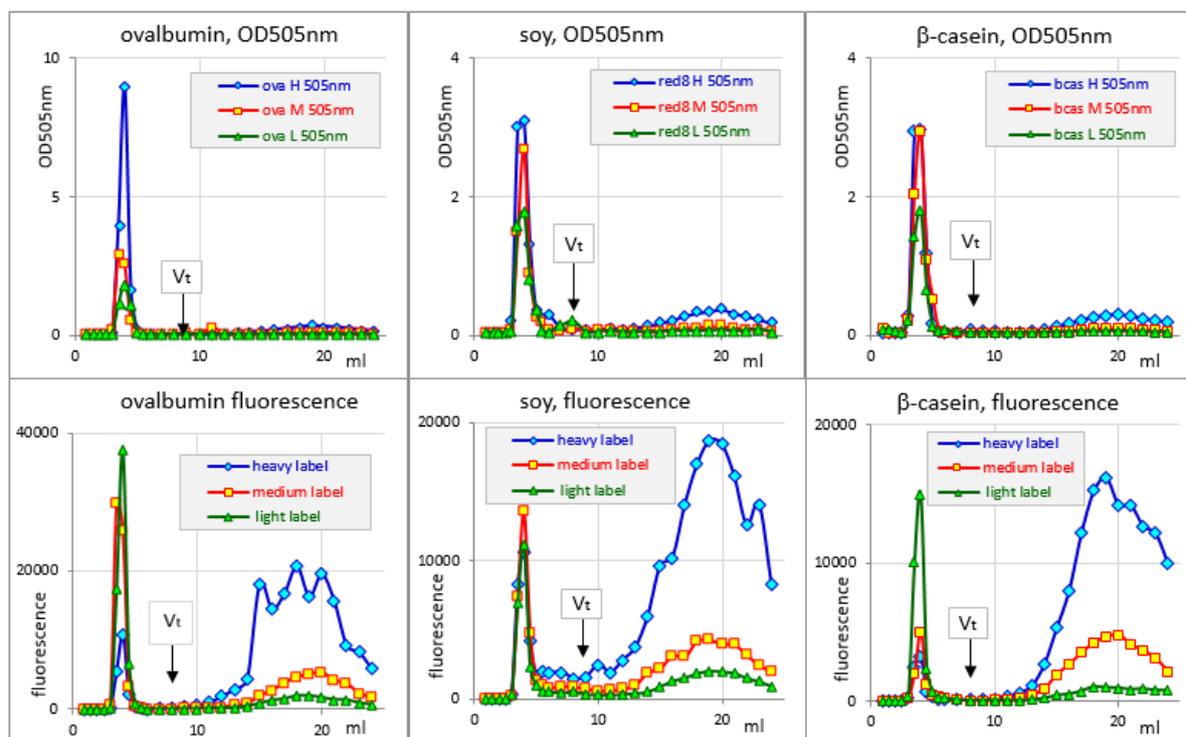


Figure 2. PD-10 fractionation of ovalbumin, soy protein and β casein labelled with three different concentrations of bodipy-FL

PD-10 fractions from each bodipy labelling were compared for absorbance at 505nm to determine the relative amount of bodipy-fluor in each fraction. Fluorescence readings were taken at 525nm to determine the amount of quenched fluor. The result (Figure 2) shows that the protein samples with the lowest fluorescence had the highest absorbance readings at 505nm, indicating that substantial fluorescence quenching was occurring. The very low levels of fluorescence and OD505nm readings of the free, uncoupled fluorescence fractions of the lightly labelled protein (around 20ml) indicate that almost all of the fluor in these samples has been taken up by the protein. Conversely the high fluorescence reading in the highly labelled samples in the same region show that fluor is now in excess of protein labelling sites. The free fluor eluting from the column well after V_t might be due to the hydrophobic bodipy dye binding to the Sepharose in the PD10 column.

3.2. Digestion of Bodipy-labelled Substrates with Pineapple and Papaya Extracts

The protein peaks (1.5ml) of the PD-10 samples were pooled and stored at -20°C . The bodipy-labelled substrates were then digested with pineapple, papaya and kiwifruit extracts (2 μl fruit extract + 50 $\mu\text{g}/\text{ml}$ substrate). Papaya and pineapple were assayed in 0.1M Bis-Tris buffer pH 6 and kiwifruit was assayed in 0.1M acetate pH 4.5. Comparing the dose-response curves in Figure 3, it is clear that the bodipy-labelled casein greatly outperforms the other two substrates in dynamic-range. It is also evident that the papaya extract is comparatively weak in proteinase activity (both pineapple and kiwifruit achieve a signal of six-fold fluorescence increase within a few minutes of casein digestion starting whereas papaya requires an hour to achieve this signal). For consistency in

all subsequent experiments, the high-bodipy-label was chosen because this gave the best overall signal:noise ratio.

3.3. Digestion of Bodipy-casein with all Three Extracts across a pH Range of pH 2–pH 9

Looking across the pH range pH 2 to pH 9 in 0.5 pH unit steps with bodipy- β -casein as a substrate, we see that pineapple extract proteinase activity has a very broad pH range and a very strong activity. By comparison green kiwifruit achieves the same caseinolytic activity as pineapple at pH 4 but has a comparatively narrower pH range. Gold kiwifruit has a noticeably weaker activity against β -casein and a slightly higher pH optimum. Papaya has a very weak activity which peaks around pH 6.0 (Figure 4A). In this experiment 1 μl of fruit extract was mixed with 1 μl of β -casein (high label) (containing 0.33 μg of protein). The reaction rates of pineapple and green kiwifruit fall off very rapidly away from the pH optimum of the enzymes. The collapsing rate may indicate an instability of the enzyme at non-ideal pH values. The unevenness of the line of pH optimum of pineapple over this wide range may be due to the buffer changes across the pH series. The buffers were used at the high concentration of 100mM to minimise the effects of the extracts on the pH. Although 100mM buffers were used across the pH series, the conductivity will change from buffer to buffer. In addition, the nature of the buffer ions themselves can affect enzyme rates irrespective of pH and conductivity [13]. The diffuse violet vertical lines in Figure 4B indicate a change in buffer. Thus it is possible that acetate depresses pineapple proteinase activity somewhat. The data in Figure 4A are simplified by comparing the fluorescence intensity at 30 minutes (Figure 4B [inset]).

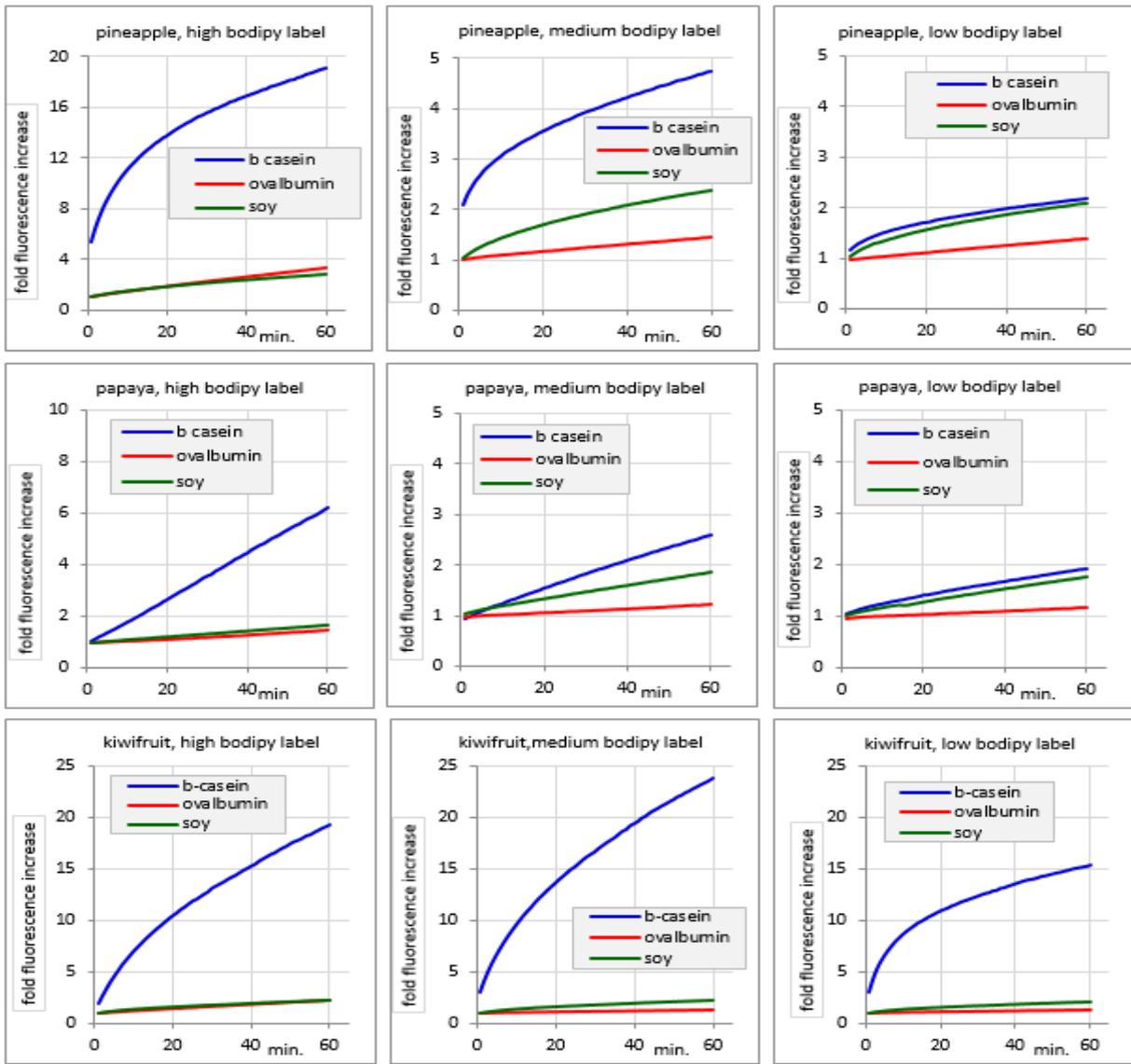


Figure 3. Digestion of bodipy-labelled substrates with pineapple, papaya & kiwifruit extracts

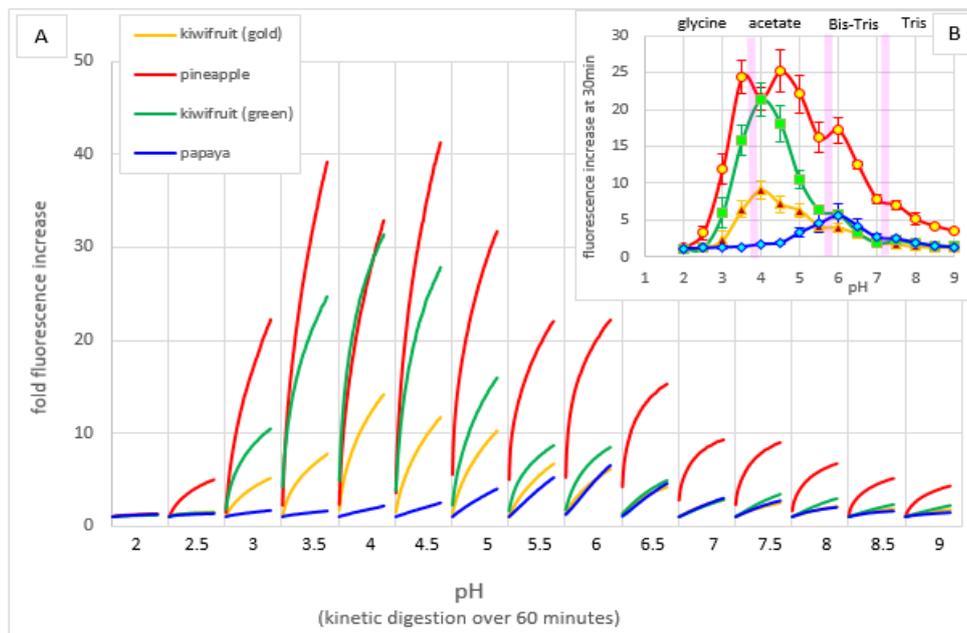


Figure 4. (A) Digestion of bodipy-β-casein with pineapple green kiwifruit, gold kiwifruit and papaya extracts across a pH range pH2 – pH9 (B) [inset] (Fluorescence at 30minutes)

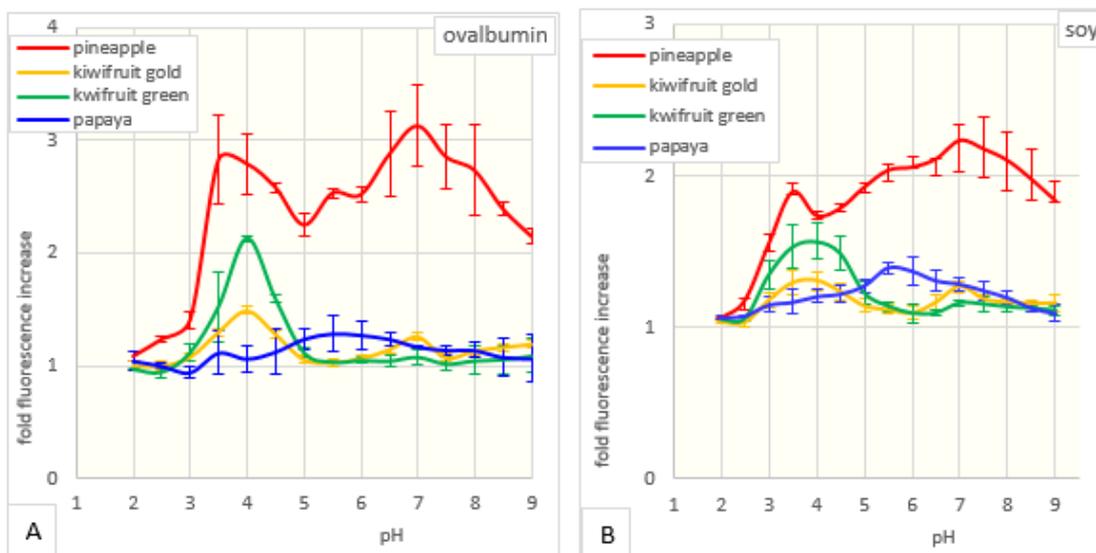


Figure 5. Effect of pH on proteinase activity of pineapple, green kiwifruit and papaya extracts using bodipy ovalbumin (A) & bodipy soy protein (B) as substrates

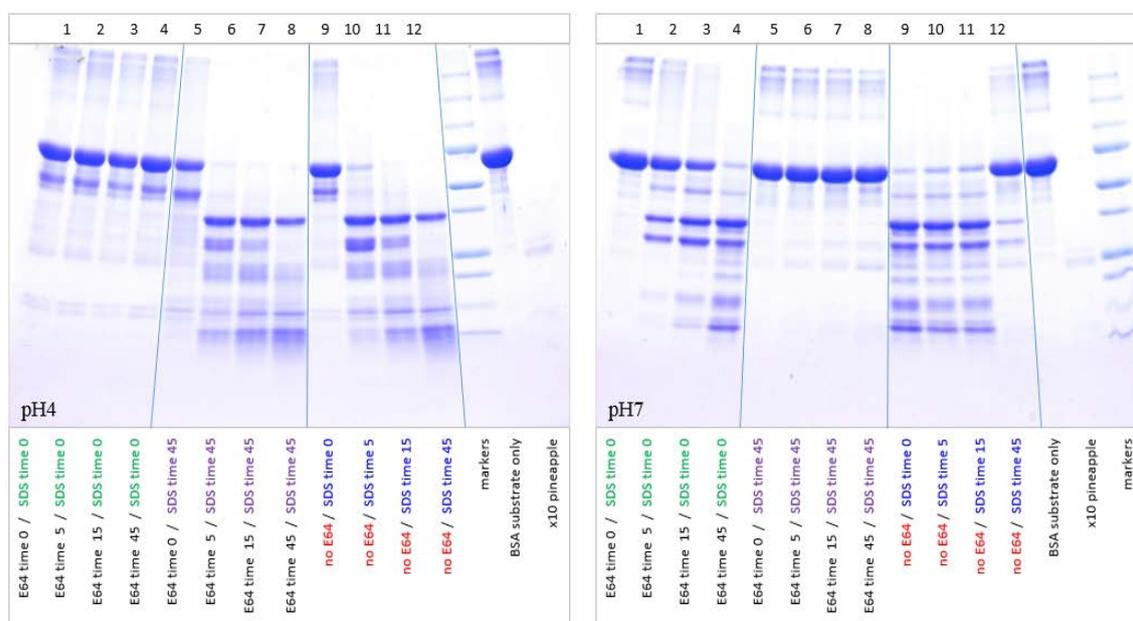


Figure 6. Effect of 2% SDS on digestion of BSA by pineapple extract at pH4 and pH 7

The earlier observation of the weak fluorescence increase during digestion of the bodipy-ovalbumin and bodipy-soy substrates when assayed at a single pH (Figure 3), suggested that these are poor substrates for assessing proteinase activity. Figure 5A and 5B shows the relative change in bodipy-ovalbumin and bodipy-soy after 30 minutes' digestion from pH 2 – pH 9. The data confirm that unlike β -casein, bodipy-labelled ovalbumin and soy protein are not useful substrates for these enzymes due to the low signal generated during digestion which is only around two to three times the fluorescence of the undigested substrate.

The poor performance of bodipy-labelled ovalbumin and soy protein necessitated the use of SDS-PAGE to assess the proteinase activities of the fruit extracts against these proteins. Addition of 2% SDS was sufficient to immediately inhibit proteinase activity in kiwifruit and papaya extracts (data not shown) but when 2% SDS was used to block pineapple proteinase activity, contrasting effects were observed depending on the pH of the

digestion buffer (Figure 6). At pH 4 SDS inhibited protein digestion by pineapple extract which, in the absence of SDS would proceed. Conversely, at pH 7 BSA resisted digestion by pineapple extract in the absence of SDS but when SDS was present then digestion occurred. 100 μ M E-64 was used to stop all proteinase reactions directly prior to the addition of SDS. This phenomenon would be explained if bromelain could retain tertiary structure (and function) in the presence of SDS at pH 7 but not pH 4, but BSA structure is opened up at pH 7 for bromelain access. The profile of BSA breakdown in Figure 6A and Figure 6B are not the same, implying that different regions of BSA are exposed to proteolysis under the different pH and SDS conditions. BSA, ovalbumin, β -casein and soy protein were all digested at 1mg/ml by fruit extracts at a final dilution of 1/10 (therefore the fruit content was 5%) at 37°C for either 5 minutes or 60 minutes before inactivation with E-64 and analysis by SDS-PAGE (Figure 7 - Figure 11).

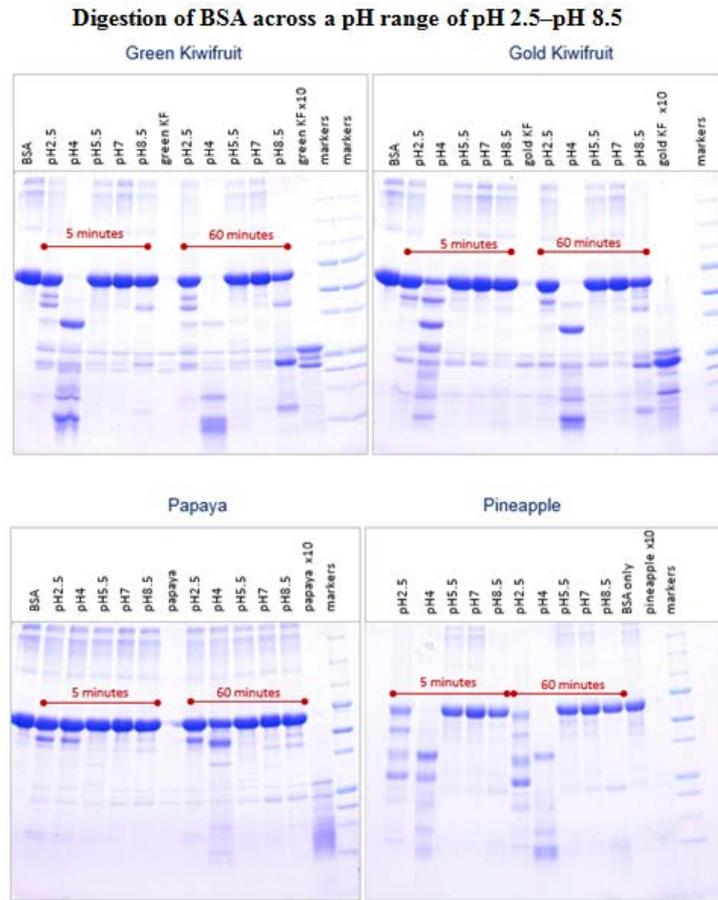


Figure 7. SDS-PAGE of BSA digested with green kiwifruit, gold kiwifruit, papaya and pineapple extracts for 5 and 60 minutes

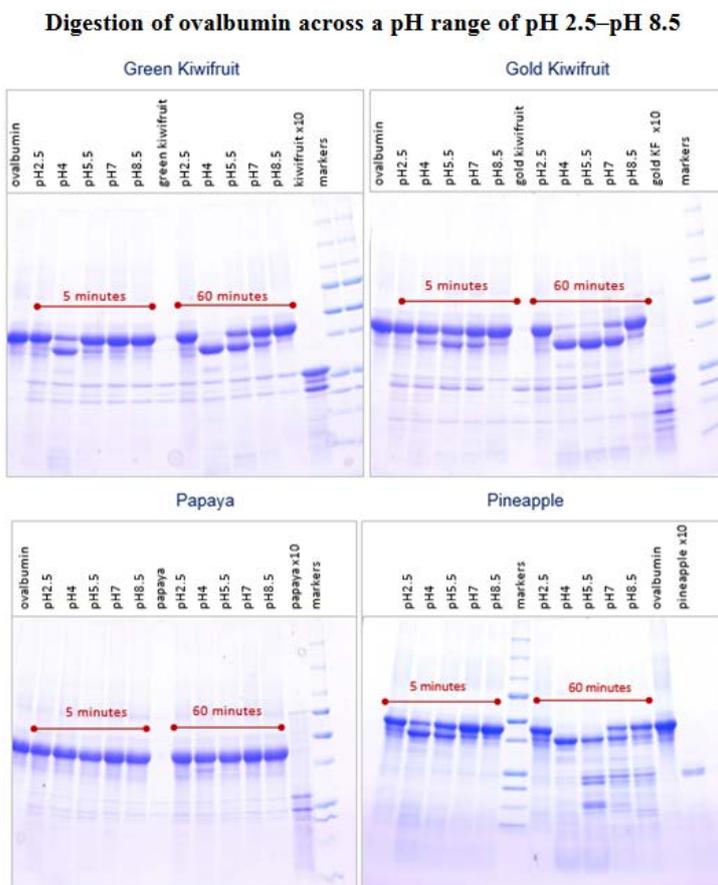


Figure 8. SDS-PAGE of ovalbumin digested with green kiwifruit, gold kiwifruit, papaya and pineapple extracts for 5 and 60 minutes

Digestion of β -casein across a pH range of pH 2.5–pH 8.5

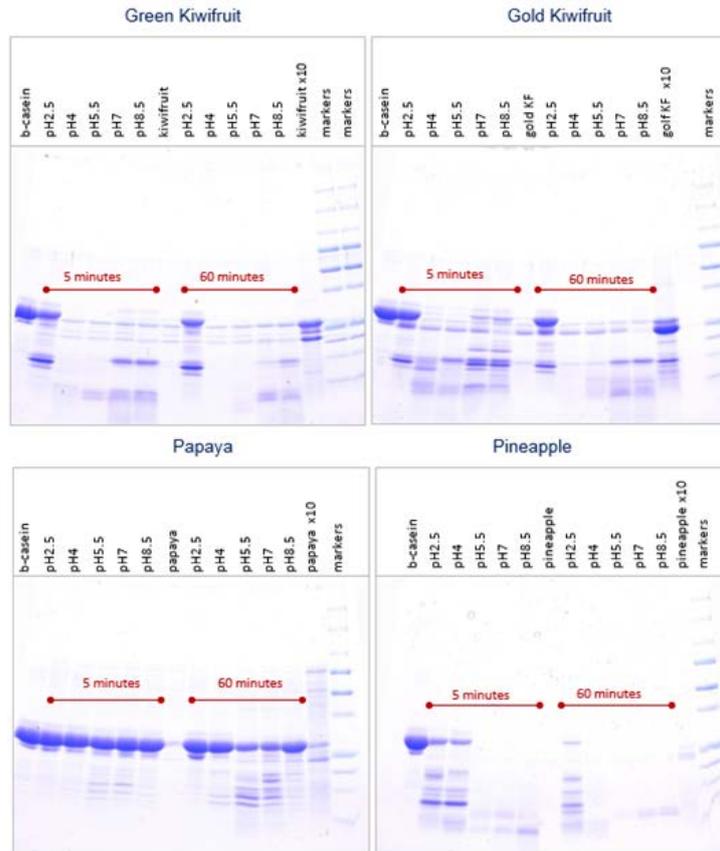


Figure 9. SDS-PAGE of β -casein digested with green kiwifruit, gold kiwifruit, papaya and pineapple extracts for 5 and 60 minutes

Digestion of soy protein across a pH range of pH 2.5–pH 8.5

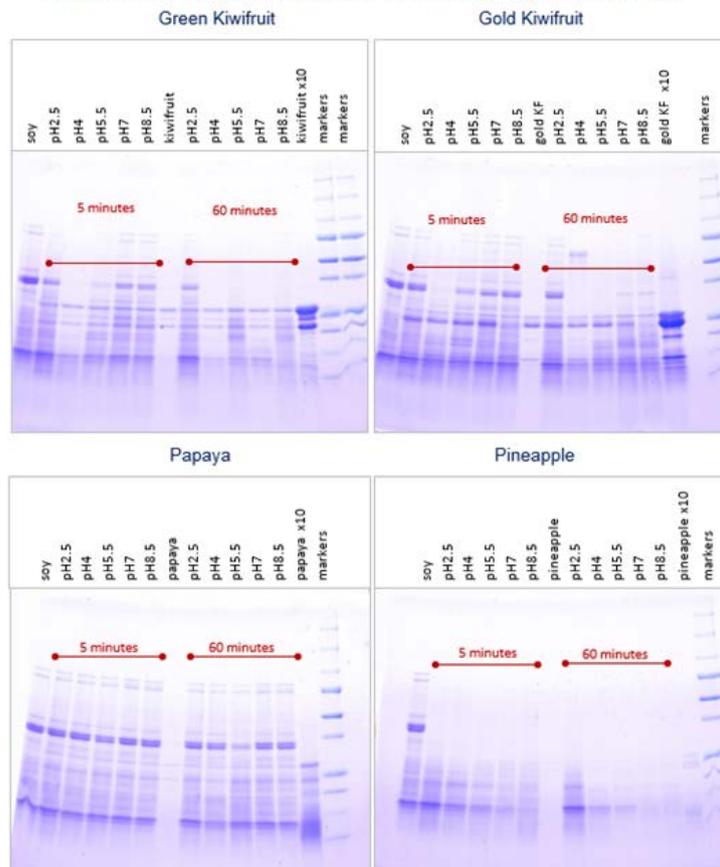


Figure 10. SDS-PAGE of soy protein digested with green kiwifruit, gold kiwifruit, papaya and pineapple extracts for 5 and 60 minutes

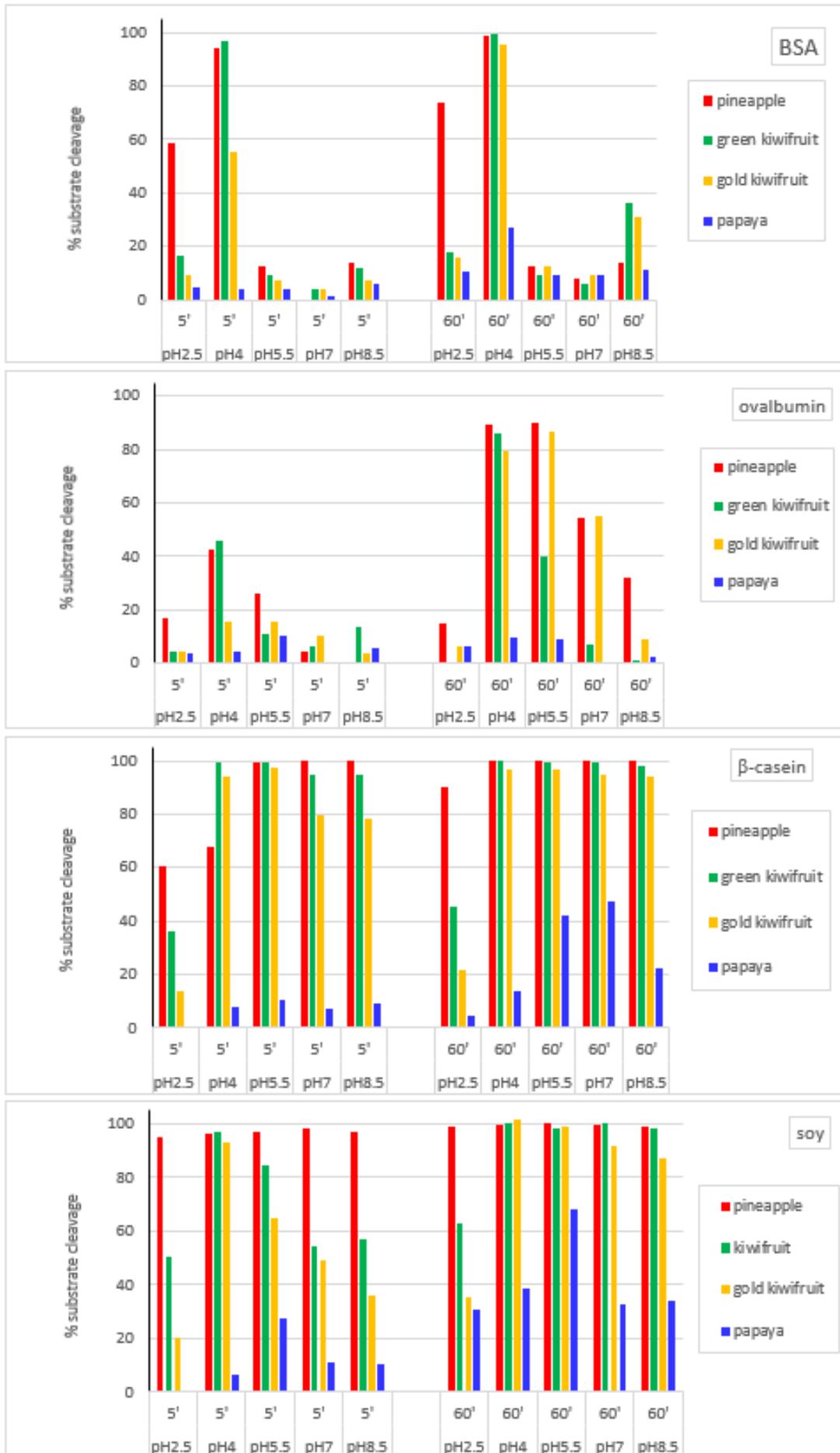


Figure 11. Densitometry summary of SDS-PAGE data

From Figure 11 the following can be concluded:

- The fruits vary greatly in their proteinase activity; while BSA, β -casein and soy protein are entirely digested within 5 minutes (at optimal pH) by green kiwifruit and pineapple these protein substrates are barely affected by papaya in this time period.
- The substrates vary greatly in their susceptibility to digestion; kiwifruit and pineapple will digest 100% of the casein at pH 5.5 within 5 minutes but at the same pH BSA will be almost wholly undigested in 1 hour.
- The pH optimum of papaya proteinase activity is around 5.5 – 7, about 1.5 – 3 pH units higher than that of the other fruits.
- The apparent pH optimum of proteinase varies with the choice of substrate. For example, using ovalbumin as substrate, the maximal digestion by gold kiwifruit is achieved at pH 5.5 whereas very little BSA digestion occurs at this pH. In addition, the spread of the pH optimum across the pH range is also dependent on the choice of substrate. Digestion of BSA by gold kiwifruit occurs to a substantial degree only at pH 4.5 but the same gold kiwifruit digests ovalbumin over a much wider range at pH 4, pH 5.5 and pH 7. Similarly, pineapple extract achieves almost full digestion of soy and casein from pH 2.5 – pH 8.5 but leaves BSA almost intact at pH 5.5 and pH 7.5
- With BSA as substrate, gold and green kiwifruit have a biphasic distribution of proteinase activity. The majority of proteinase activity occurs at pH 4 but there is a minor peak of activity at pH 8.5. In the SDS gels there is a different fragment profile in the pH 4 and pH 8.5 tracks.
- Although green and gold kiwifruit are related actinidia species, when ovalbumin is used as substrate quite different proteinase activities emerge. At pH 7 green kiwifruit is very poor at digesting ovalbumin (7% digestion) but gold kiwifruit digests more than 50% in 1 hour. So with ovalbumin but

not BSA, gold kiwifruit has a higher and broader pH optimum than green kiwifruit proteinase activity.

3.4. Recovery from Exposure to low pH.

Since green kiwifruit is reported to aid digestion in the stomach and also the small intestine [14,15], the performance of actinidin after exposure to acidic pH is of interest. Green and gold kiwifruit show little activity against ovalbumin and BSA at pH 2.5. The activity against casein and soy protein is substantial but short-lived at pH 2.5 since almost all of the proteolysis occurs in the first five minutes and very little additional proteolysis occurs in the following 55 minutes. To test the ability of pineapple, green and gold kiwifruit to tolerate low pH expected in the stomach, these extracts were placed for 10 minutes in glycine buffer at pH 2 and pH 2.5. After this time the sample was diluted in acetate buffer, the pH was adjusted 4.5 and the remaining proteinase activity assessed by digestion of bodipy-casein (Figure 12). The results show that kiwifruit proteinase activity is irreversibly lost after 10 minutes at pH 2 and even at pH 2.5 only a trace of proteinase activity remains. Pineapple tolerates low-pH conditions better with only a minor loss of activity at pH 2.5 and a trace of activity remaining after exposure to pH 2. The loss of proteinase activity at pH 2.5 is surprising since the pH of a kiwifruit homogenate is pH 3.7. This loss in actinidin activity is all the more striking because there is no pepsin present in these assay which is likely to reduce actinidin activity even further. Conceivably the actinidin is protected in a higher pH compartment in the fruit or is stabilised by protecting factors in the kiwifruit which are lost during homogenisation. This finding would suggest that kiwifruit are unlikely to make much of a contribution to duodenal protein digestion since the human gastric pH is lower than pH 2 [16]. However there is ample opportunity for kiwifruit and pineapple to contribute to gastric digestion since the post-prandial time required to reach pH 2.5 is around 1 hour [16].

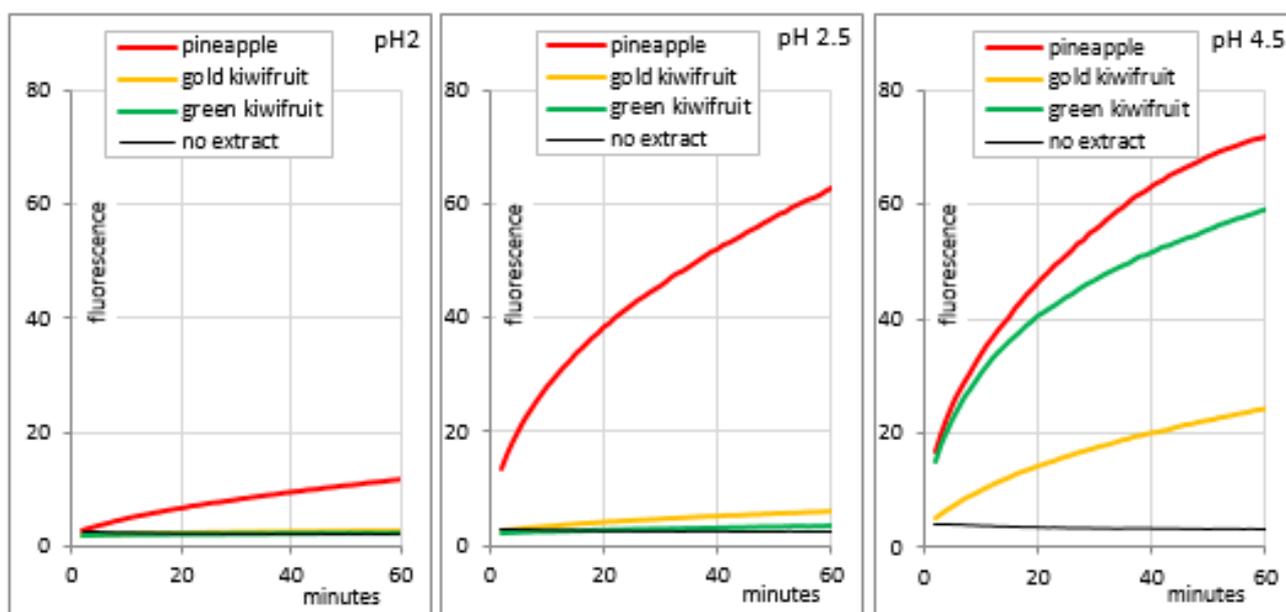


Figure 12. Resilience of fruit extract proteinases to acid conditions. Extracts were placed in pH 2, pH 2.5 or pH 4.5 as shown for 10 minutes at room temperature, then the pH was adjusted to pH 4.5 before the addition of bodipy-casein substrate

3.5. Non-cysteine Proteinase Activity in Extracts

Recently the actinidin concentration in kiwifruit was quantified by measuring the amount of E-64 necessary to inhibit proteinase activity [12]. In that publication it was shown that E-64 fully inhibited the proteinase activity of the green and gold kiwifruit extracts. To determine if the same approach might be used in measuring the enzyme concentration of pineapple and papaya extracts, these extracts were exposed to increasing concentrations of E-64 and then assayed for proteinase function. Acetate buffer, pH 4.5 was used for kiwifruit and pineapple and Bis-Tris, pH 6 was chosen as a buffer for papaya because these pH conditions gave strong activity in (Figure 4). The data (Figure 13) shows that, unlike in kiwifruit, a substantial proportion of proteinase activity in pineapple and papaya is not inhibited by E-64. Therefore although E-64 can quantify actinidin in whole kiwifruit and purified papain [17], it cannot so easily be used to measure total proteinase concentration in pineapple or papaya, probably because there is significant

amount of non-cysteine proteinase activity in these fruits. Whether the kiwifruit proteinase activity observed at pH 8.5 against BSA in Figure 7 is E-64 sensitive was not tested.

Green kiwifruit is almost entirely inhibited by E-64 whereas a substantial minority of pineapple and papaya proteinase activity is not inhibited by E-64 treatment (shown by the red arrow). The rather weak proteinase activity of papaya is not due to a failure of papain to digest the bodipy substrate but to a low concentration of active proteinase in this particular commercial source. When purified papain is used at the same enzyme concentration as actinidin found in green kiwifruit (around 1mg/ml in the fruit), it generates strong dose response curves very similar to those of actinidin when used at similar enzyme concentrations (data not shown). In addition, when fruit of mountain papaya (*Vasconcellea pubescens*) is used, very strong proteinase activity is evident (data not shown). Mountain papaya is a related species in the same family (Caricaceae) as commercially distributed papaya (*Carica papaya*).

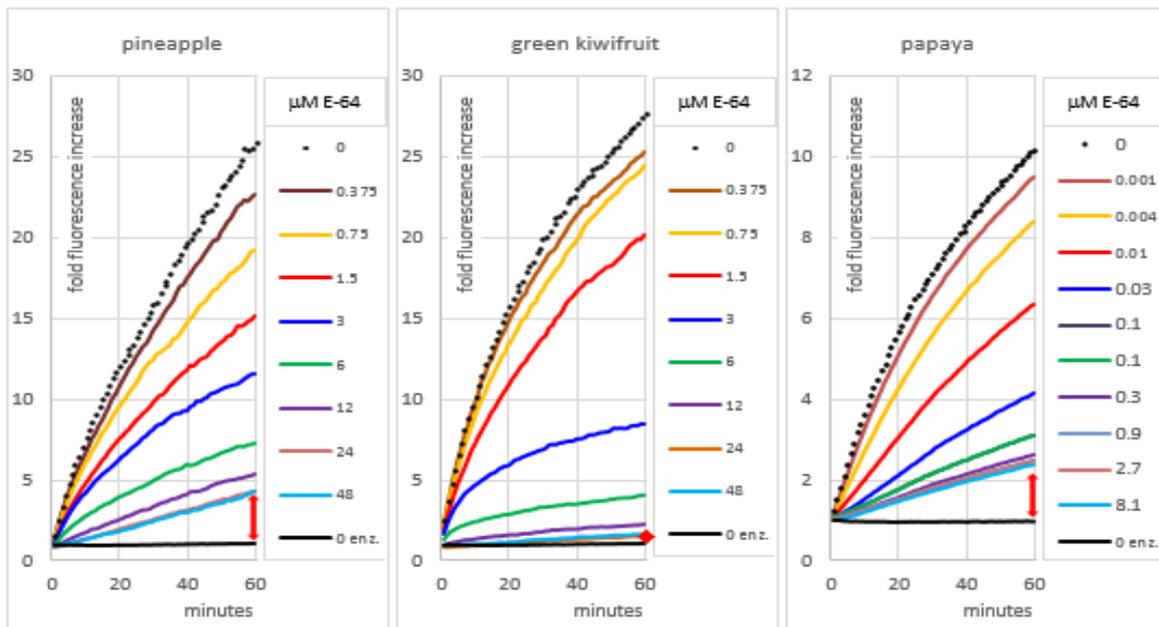


Figure 13. E-64 treatment of pineapple, green kiwifruit and papaya to inhibit cysteine proteinase activity

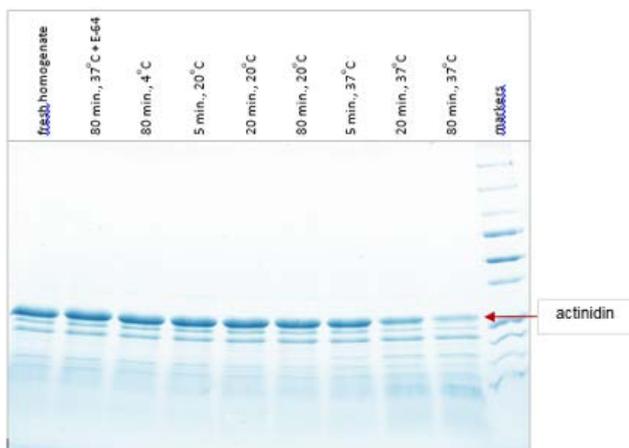


Figure 14A. SDS-PAGE of kiwifruit homogenate after incubation at different times and temperatures

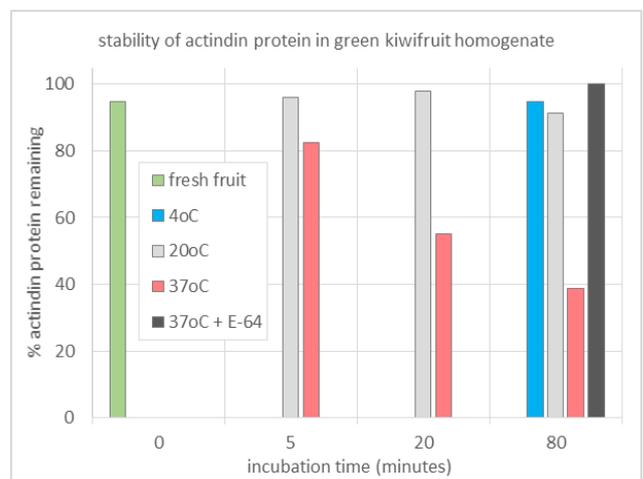


Figure 14B. Densitometry of actinidin band in Figure 14 A

3.6. Auto Digestion of Actinidin in Green Kiwifruit

The spontaneous loss of actinidin protein in green kiwifruit homogenate was investigated. Proteinase activity in undiluted homogenate was measured after incubation at 4°C, 20°C and 37°C for up to 5, 20 and 80 minutes. The actinidin protein remaining in the samples was measured by SDS-PAGE Figure 14A and quantified by densitometry (Figure 14B). The results show that about 45% the actinidin in homogenised green kiwifruit is lost within 20 minutes at 37°C due to autocatalysis. E-64 treatment of the homogenate prevents autodigestion. From the practical point of view of handling and storing kiwifruit homogenate, it is clear that actinidin is stable at 4°C and 20°C for more than 1 hour. However in concentrated form, kiwifruit would be expected to lose much of its activity through autocatalysis and then acid denaturation before reaching the duodenum. Some additional loss of actinidin in the stomach due to the action of pepsin would be expected. However in the presence of non-kiwifruit protein then autocatalysis will be minimal because the additional protein provides an alternative substrate to actinidin itself and also dilutes the kiwifruit. No evidence of autocatalysis is seen in Figure 7 – Figure 10 when kiwifruit is diluted in excess protein substrate.

4. Conclusions

1. Bodipy-labelled casein performs admirably as a substrate against all fruit proteinases but bodipy-ovalbumin and bodipy-soy protein are of little use as indicators of proteinase activity. This may be because of a combination of a) resistance to proteolysis, b) internal disulfides which hold cleaved polypeptides together, c) an unlucky distribution of labelled lysines relative to cleavage sites on these proteins which generate quenched proteolytic fragments and d) destruction of lysine cleavage sites due to the labelling process which derivatises lysine residues.
2. Kiwifruit proteinase activity is destroyed in minutes after exposure to pH at or below pH 2.5 whereas pineapple proteinase activity can recover from pH 2.5 but not pH 2.
3. The papaya imported from the Philippines to New Zealand supermarkets has very little proteinase activity due to the low functional papain concentration in the fruit.
4. Green kiwifruit and pineapple have similarly potent proteinase activities against all substrates tested except that pineapple extract tolerates very acid conditions (pH 2.5) somewhat better.
5. Gold kiwifruit has a markedly better ability to digest ovalbumin at the higher pH values of 5.5 and 7 than green kiwifruit. This enhanced activity of gold kiwifruit over green kiwifruit at these higher pH values is not manifested with the three other substrates.

6. The actinidin in kiwifruit homogenate will rapidly autocatalyse if warmed to 37°C (almost 50% loss in 20 minutes) but the actinidin in homogenate is stable at room temperature.
7. The complex interactions of these well-studied proteinases and substrates may explain the lack of consensus as to the susceptibility of the substrates to the enzymes. SDS can either prevent BSA digestion by pineapple extract or promote it, at different pH values. A 1.5 unit pH change can make the difference between 100% or 0% digestion of BSA by green kiwifruit extract.
8. As sources of supplementary dietary proteinase, kiwifruit and pineapple are much more effective than papaya imported to New Zealand from the Philippines. This is probably an indication that the papaya cultivated for export from the Philippines has a low papain content.

Acknowledgments

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