

Quantification of Functional Actinidin in Whole Kiwifruit Extract Using the Selective Cysteine Proteinase Inhibitor E-64

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Abstract Actinidin is a cysteine proteinase abundant in kiwifruit. It is of interest to the food industry as a meat tenderizer and an aid to digestion. The selective cysteine proteinase inhibitor E-64 allows the quantification of actinidin in whole extracts of commercial kiwifruit cultivars ‘Hayward’ (green) and ‘Zesy002’ (gold). The quantity of E-64 required to inhibit actinidin is a direct measure of the amount of actinidin in the sample. Actinidin can be quantified by measuring the inhibition of digestion of the fluorescent protein substrate bodipy-casein or the small-molecule fluorescent substrate Bz-Phe-Arg-aminomethylcoumarin. Use of the protein substrate appears to overestimate the quantity of enzyme by around 15%. The synthesis of the fluorescent protein β -casein labelled at a molar ratio of 5:1 (fluor: protein) greatly enhances the signal: noise ratio of substrate when it is digested with actinidin. The high quantity of actinidin in kiwifruit coupled with the selective nature of E-64 allows assay of extracts in a simple kinetic microplate assay. The method is particularly suitable for the comparison of Actinidia from different species since it reports the quantity of enzyme by mass and the relative activity of the enzyme against the chosen substrate. Gold kiwifruit are shown to have around half as much actinidin as green kiwifruit but the three-fold greater specific activity of green kiwifruit against these substrates results in an apparent six-fold greater actinidin activity of the green kiwifruit. This assay may simplify the reporting of actinidin activities which are variously reported as arbitrary fluorescence units, delta-optical density values and band intensities on polyacrylamide gels.

Keywords: actinidin, kiwifruit, E-64, bodipy-casein

Cite This Article: Harry Martin, “Quantification of Functional Actinidin in Whole Kiwifruit Extract Using the Selective Cysteine Proteinase Inhibitor E-64.” *Journal of Food and Nutrition Research*, vol. 4, no. 4 (2016): 243-250. doi: 10.12691/jfnr-4-4-8.

1. Introduction

The cysteine proteinase (CP) actinidin is abundantly expressed in kiwifruit and represents roughly 10% of total kiwifruit protein in the green-fleshed kiwifruit *Actinidia deliciosa* variety Hayward (referred to in this paper as green). Cysteine proteases (CPs) accumulate to high concentration in many fruit, where they are believed to play a role in fungal and insect defense. The fruit of *Actinidia* species (kiwifruit) exhibit a range of CP activities (e.g. the *Actinidia chinensis* variety Yellow A shows less than 2% of the activity of *Actinidia deliciosa* variety Hayward) [1] Actinidin has applications in meat tenderizing [2,3] and enhances the digestion of dietary protein [4]. Green kiwifruit has approximately ten-fold more protease activity than *Actinidia chinensis* var. *chinensis* ‘Hort16A’. Acidic and basic isoforms of actinidin were identified in *Actinidia deliciosa* ‘Hayward’ and *Actinidia arguta* ‘Hortgem Tahiti’, while only a basic isoform of actinidin was identified in *Actinidia chinensis* ‘Hort16A’. [5,6]. A major quantitative trait locus for CP activity was mapped to linkage group 16 in a segregating

population of *A. chinensis*. This quantitative trait locus co-located with the gene encoding actinidin, the major acidic CP in ripe Hayward fruit encoded by the ACT1A-1 allele [1]. Most genetic and biochemical characterization of actinidin has been carried out in the major commercial green-fleshed kiwifruit (*Actinidia deliciosa* var. Hayward), with multiple cDNA sequences.

Actinidin activity is frequently reported as the amount of cleavage of small-molecule fluorogenic [7] or colorimetric substrates [5,8]. Protein substrates such as azocasein [9] and bodipy-casein [2,10] are also used to measure actinidin activity. Actinidin is also quantified as a protein band on SDS-polyacrylamide gel electrophoresis or an antigenically detected band on Western blots [5,6]. The limitation of these approaches is that activity is quoted as arbitrary fluorescence units, delta-OD values or protein concentrations with unknown activity. None of these units is ideal because the results from one study cannot be compared with those of another. In addition, Azocasein [11] and bodipy-casein, although having the advantage of being proteins, have the drawback that they can vary substantially from batch to batch in labelling density and therefore digestibility and fluorescence intensity. Furthermore, the variable rate of colour or

fluorescence generation during digestion of a complex protein substrate creates problems for the accurate measurement of enzyme rate. Finally, the amount of actinidin protein does not correlate well with the specific activity of actinidin from different *Actinidia* species. Thus actinidin from two *Actinidia* species have substantially different proteinase activity while having the same concentration of actinidin protein [5].

E-64 is an irreversible inhibitor of cysteine proteinases isolated from the soil fungus *Aspergillus japonicus* [12] and has been used to quantify the concentrations of the cysteine proteinases papain and cathepsin [11,13]. E-64 is a dipeptide coupled to an epoxysuccinic acid group. The mechanism of inhibition is that the C2 carbon of the E-64 epoxy group becomes covalently attached to the sulfur atom of the enzyme active site cysteine [14]. E-64 is unreactive with free cysteine and also does not inhibit serine, aspartic or metallo-proteinases [13]. The selective action of E-64 coupled with the high actinidin content of kiwifruit suggest that E-64 could be used to measure the actinidin concentration in whole kiwifruit extract. The aim of this study was therefore to develop a simple assay for actinidin based on E-64 inhibition which simultaneously reports the concentration of actinidin in milligrams per milliliter but also reveals the different specific activity of actinidin from different *Actinidia* species.

2. Materials and Methods

2.1. Materials

Bodipy FL-labelled casein (product E-6638) and Bodipy FL succinimidyl ester (D-2184) were supplied by Thermo Fisher Scientific Inc. Bovine α , β , κ and crude caseins (products C8032, C6905 and C0406), Bz-Phe-Arg-aminomethylcoumarin (product C9521) (Bz-Phe-Arg-AMC), iodoacetamido-fluorescein (I9271), Igepal CA-630 (I3021) and E-64 (E3132) were supplied by Sigma-Aldrich Corporation. *Actinidia chinensis* var. *deliciosa* 'Hayward' (green) *A. chinensis* var. *chinensis* 'Hort16A' (gold) kiwifruit were purchased in local supermarkets in Palmerston North, New Zealand.

2.2. Methods

2.2.1. Kiwifruit Extract Preparation

Samples of kiwifruit flesh (2 g) were excised and pips removed. The tissue was diced with a scalpel and mixed with 2 ml of extraction buffer; 100 mM acetate pH 4.5 containing 0.2% Igepal detergent (w/v), 0.1% ascorbic acid (w/v), 0.1% 2-mercaptoethanol (v/v). The sample was homogenised using a loose and then tight fitting Dounce homogeniser. The homogenate was microfuged at 15,000 g for 5 minutes in 1.5 ml Eppendorf tubes. The actinidin containing supernatant (extract) was stored on ice until use on the same day. The same buffer was used as assay buffer.

2.2.2. Synthesis of Bodipy-casein

Casein protein was dissolved in 0.1 M carbonate buffer pH 9. Bodipy FL succinimidyl ester was dissolved DMSO

and mixed with 10 mg/ml casein at a concentration of 2 mM. Fluor was conjugated to protein for 3h at 37°C in the dark. Unconjugated succinimide was blocked by addition of 0.15 M Tris, pH 9. Sample volumes were adjusted to 0.5ml with phosphate buffered saline (PBS) and the labelled protein was separated from unbound Fluor by gel filtration on a G25 protein desalting column (PD 10) column equilibrated with PBS. Fluor was detected by absorbance at 505 nm and by fluorescence (excitation 502 nm, emission 515 nm). The fluorescence: optical density ratio of the unconjugated Fluor was 30-fold higher for the unconjugated Fluor than with the labelled protein. Fluorescent casein synthesized in-house at New Zealand Institute for Plant & Food Research Limited is described as PFR-bodipy-casein whereas bodipy-casein supplied by Thermo Fisher Scientific (MA, USA) is described as TF-bodipy-casein.

2.2.3. Fluorescence Assays

Fluorescence assays were performed using a Tecan Safire2 fluorescence microplate reader (Tecan, Grödig, Austria). 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). Initial enzyme rates were determined using the MonoMolecular Curve fit (Origin® v7.5, OriginLab, Northampton, MA, USA). Typically fluorescent casein substrate was used at a final concentration 5µg/ml, as recommended by the supplier, Thermo Fisher.

2.2.4. SDS-Polyacrylamide Gel Electrophoresis and Electro-blotting

Kiwifruit extract and casein samples were run on step gradient polyacrylamide gels (10/15/20% acrylamide) using a Bio-Rad Mini-Protean® II electrophoresis system [15]. Proteins were transferred onto nitrocellulose at 100 V for 60 min in 10 mM cyclohexyl-3-amino-propane-sulfonic acid (CAPS) buffer pH 11 containing 10% methanol. Fluorescent protein was detected by scanning on a Fujifilm FLA-5000 flatbed laser scanner. Protein concentrations on Coomassie stained SDS-gels were quantified using Multi Gauge Software, FujiFilm.

3. Results & Discussion

3.1. Actinidin Digestion of Bodipy-casein; Time Course of Inhibition by E-64 Using TF-Bodipy-casein

Either 9 µM or 3 µM of E-64 was mixed with 25µl of kiwifruit extract in a volume of 133µl for various durations to determine how long E-64 requires to inactivate the actinidin in an extract. Then 67 µl of Life Technologies bodipy-casein was mixed with the sample in a black 96-well microplate to a final amount of 5 µg/well. The results (Figure 1) show that an incubation period of 25 min is sufficient time under these conditions to allow E-64 to inhibit actinidin. In subsequent assays E-64 was allowed to bind to actinidin for 30 min before assay.

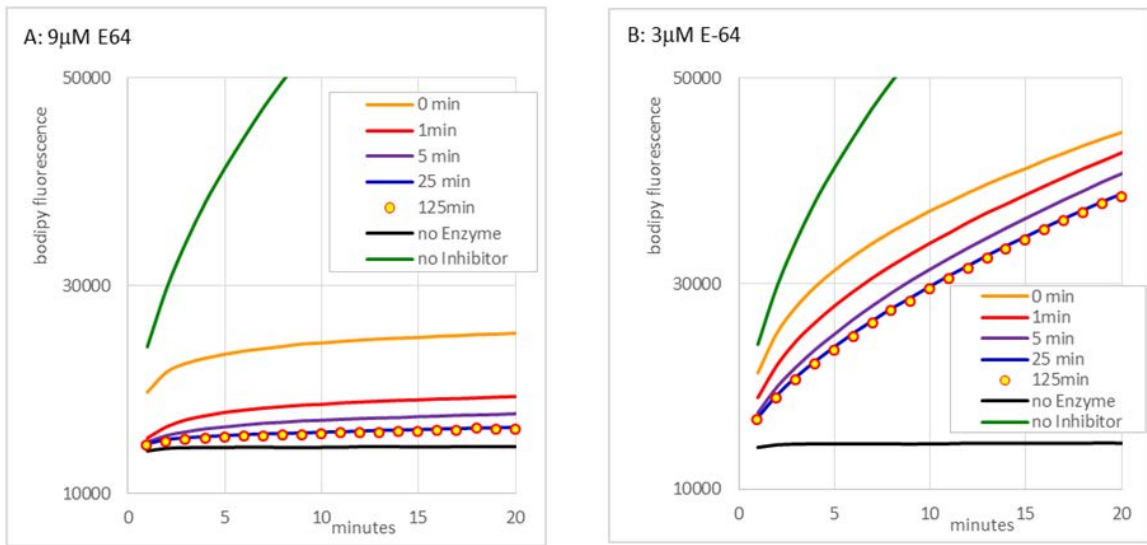


Figure 1. Time course of E-64 inhibition of actinidin in kiwifruit extract at 9µM (A) or 3µM (B) E-64

3.2. Comparison of Protein (TF-casein) and Peptide (Bz-Phe-Arg-AMC) Substrates

TF bodipy-casein and BZ-Phe-Arg-AMC were compared in E-64 actinidin inhibition assay (Figure 2A & B). The results show very similar behavior of the two substrates. However, with TF bodipy-casein there is a noticeable time-dependent increase in baseline fluorescence even after maximal inhibition with E-64 has been achieved.

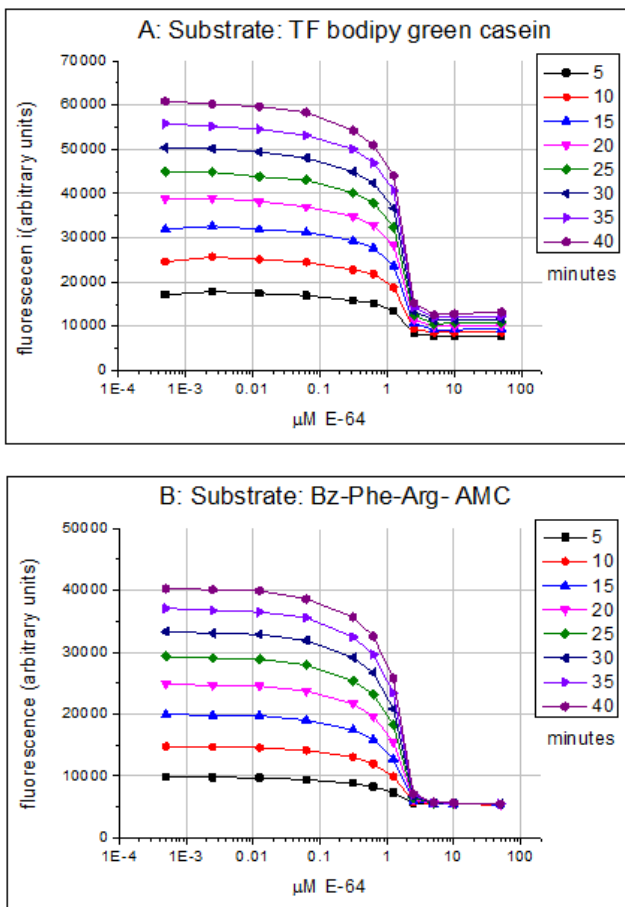


Figure 2. Comparison of (A) TF bodipy-casein and (B) Bz-Phe-Arg-AMC

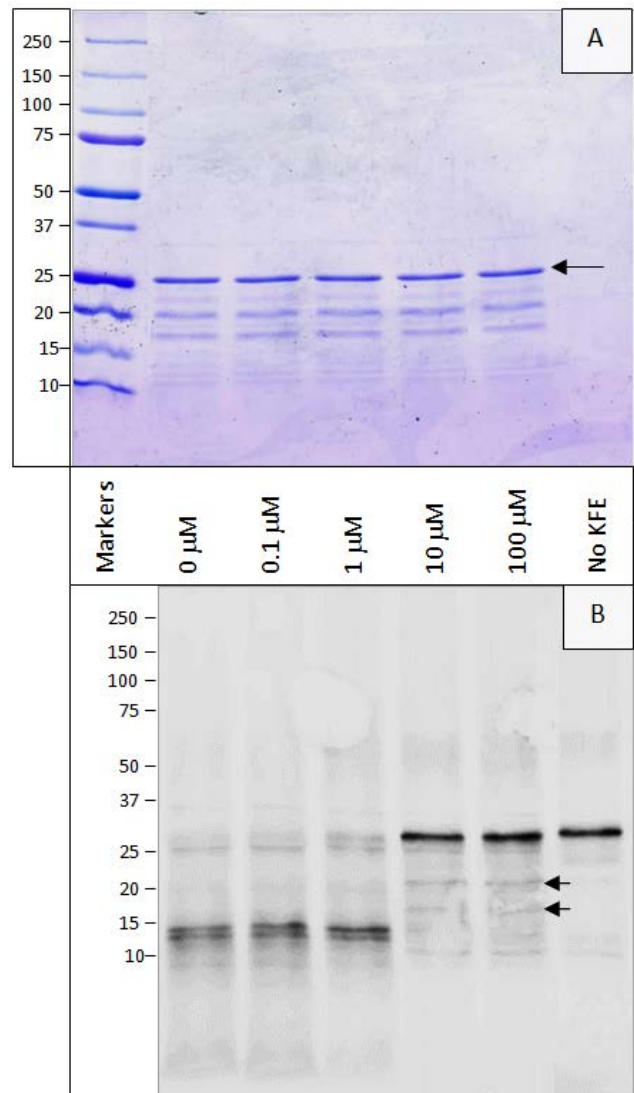


Figure 3. Digestion of TF-Bodipy casein with Kiwifruit extract (A) Coomassie stain (Kiwifruit protein) (B) fluorescence scan (bodipy-casein)

This increase in fluorescence might be due to a non-enzymatic change in casein structure during incubation in kiwifruit extract. However since casein is almost aperiodic and without much tertiary structure this explanation is

unlikely. More probably the increase in fluorescence observed in Figure 2B will be due to a non-cysteine proteinase in the kiwifruit extracts (KFE). Incubation of the casein substrate in assay buffer alone does not lead to any fluorescence change. Analysis of the bodipy-casein sample on SDS-PAGE shows that there is evidence for very slight proteolysis of the casein (Figure 3). The major band at 27 kDa in the Coomassie stained gel is actinidin (arrowed in Figure 3A). The molecular weights of bovine alpha-S1 and beta caseins which make up the majority of casein protein are around 23–24 kDa. The concentrations of these protein in the sample are too low to be detected by Coomassie staining. The fluorograph (Figure 3B) shows the effectiveness of 10 μ M and 100 μ M E-64 compared with lower concentrations in preventing casein hydrolysis.

There are two faint but discernible bands of casein breakdown (arrowed in Figure 3B) which are present in the 10 and 100 μ M E-64 tracks. This is evidence of a small amount of enzymatic cleavage of casein occurring despite the presence of E-64. This would account for the slight increase in bodipy fluorescence observed at high E-64 concentrations in Figure 2A and would indicate the presence of a small amount of proteinase activity in addition to actinidin in the kiwifruit extract.

3.3. Synthesis of α , β , κ and Mixed Caseins Labelled with Bodipy-green

α , β , κ and crude mixed caseins were labelled lightly with bodipy green casein at a ratio 5 moles Fluor to 1 mole casein. The light labelling was chosen because lysine residues derivitized by the bodipy succinimidyl ester are also potential cleavage sites for actinidin [16,17]. Thus heavy labelling of casein could suppress digestion of the substrate. This phenomenon was clearly observed with casein labelled using 50 moles fluor per mole of substrate (data not shown). Each of the four casein substrates was digested in a kinetic assay with kiwifruit extract. The data show that light labelling of individual caseins results in very high signal: noise ratios (Figure 4). The maximal signal: noise ratio of the crude mixed caseins reached about six to one, whereas the highest signal: noise ratio of fifteen to one was achieved with β -casein by 45 minutes.

In subsequent experiments PFR β casein was used as a substrate in preference to the other PFR caseins. The reasons for the much smaller signal: noise ratio of the mixed caseins is unknown. Conceivably the mixed caseins are able to adopt tertiary and quaternary structures that result in sub-optimal labelling with the fluor, resist actinidin digestion or suppress fluorescence due to proximity of proteolytic fragments.

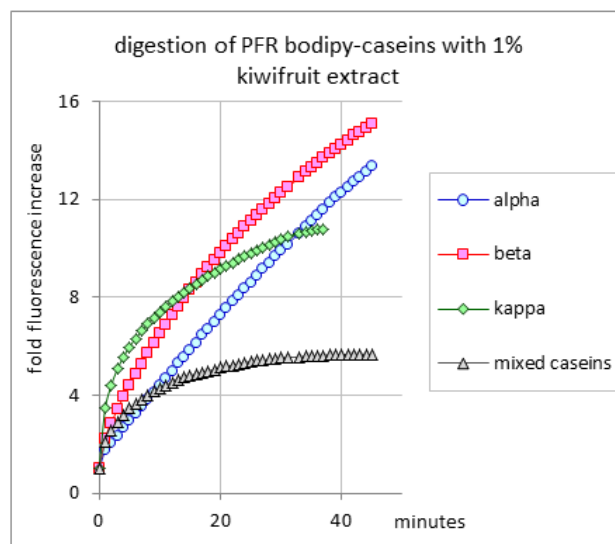


Figure 4. Digestion of PFR α , β , κ and crude (mixed) bodipy caseins with kiwifruit extract

3.4. Comparison of Bodipy-labelled PFR- β Casein Labelled with Commercial Bodipy Casein

The PFR β -casein was compared in an assay with the same concentration of TF-casein. The data (Figure 5) show that the TF-casein is similar to the PFR-mixed-casein in giving a dynamic range of about one third that of the PFR- β -casein. Although PFR- β -casein is clearly preferable for use in an actinidin assay, in general we find no advantage of PFR- β -casein over the commercial LT-casein in assays with trypsin and chymotrypsin (data not shown).

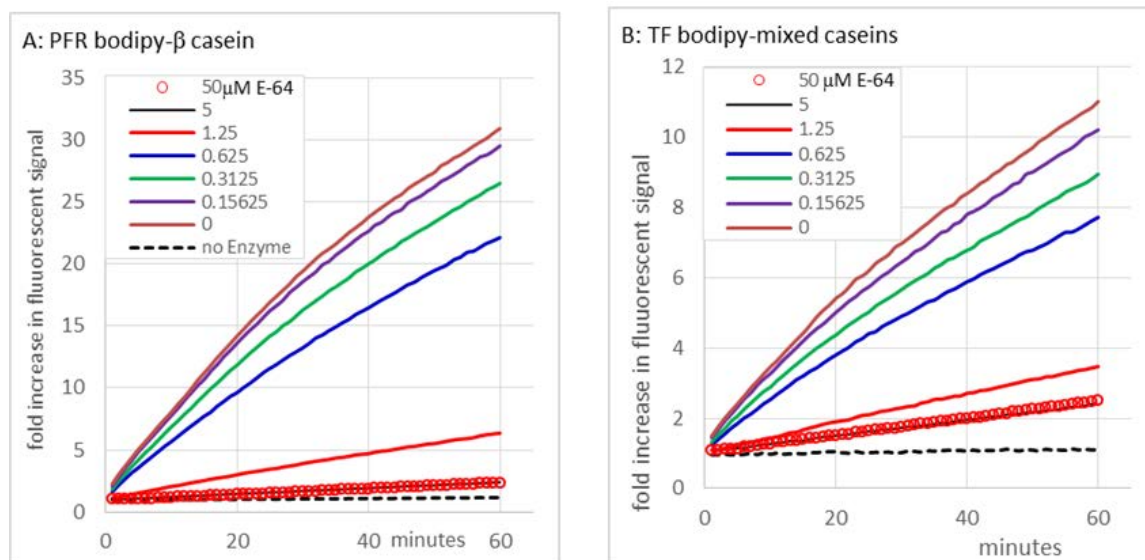


Figure 5. Comparison of PFR- β -casein (A) and TF-bodipy-casein (B) for actinidin inhibition by E-64

In the assay shown in Figure 5, 8% kiwifruit extract was used instead of 1% as used in Figure 4. It is also evident from Figure 5 that the contribution to the background fluorescent signal (at higher E-64 concentrations) is higher in the TF-bodipy-casein than the PFR-bodipy-casein. This would imply that although PFR-β-casein is a better substrate for actinidin than TF-bodipy-casein, the E-64 resistant proteinase in kiwifruit extract generates fluorescent signals from each substrate at similar rates.

3.5. Actinidin Quantification by E-64: Comparing Bz-Phe Arg-AMC and PFR-bodipy β-casein Substrates

Kiwifruit extract was incubated with a dilution series of E-64 and assayed for inhibition of cleavage of Bz-Phe-Arg-AMC and PFR-β-casein substrate. An estimate of the maximum fluorescence value when all of the Bz-Phe-Arg-AMC substrate was cleaved, was determined by digestion of the substrate with trypsin followed by dilution into acetate buffer. The initial rates were then plotted against

the substrate concentration as described in the method of Barret [13]. The raw data from two separate dilution series (A & B) are shown in Figure 6A (Bz-Phe-Arg-AMC substrate) and Figure 6B (PFR-β-casein). The initial rates are plotted in Figure 7.

The results of the analysis show that the two substrates give similar but not identical results; the bodipy-casein substrate yields a final actinidin concentration of 0.88 mg/g in the original kiwifruit +/- 0.04 mg/g while the Bz-Phe-Arg-AMC substrate data suggest the actinidin concentration is 0.74 mg/g +/- 0.03 mg/g. The calculation used to estimate the original concentration of actinidin in the kiwifruit are shown in Table 1. Origin® curve fitting software was used to determine the Standard Error values of the Y-axis intersect and the slope. The X-axis standard errors were calculated using this formula in excel:

$$\text{Intercept SE} = -\text{slope} * \text{SQRT} \left(\frac{((Y - \text{interceptSE} / Y - \text{intercept})^2)}{+((\text{slopeSE} / \text{slope})^2)} \right)$$

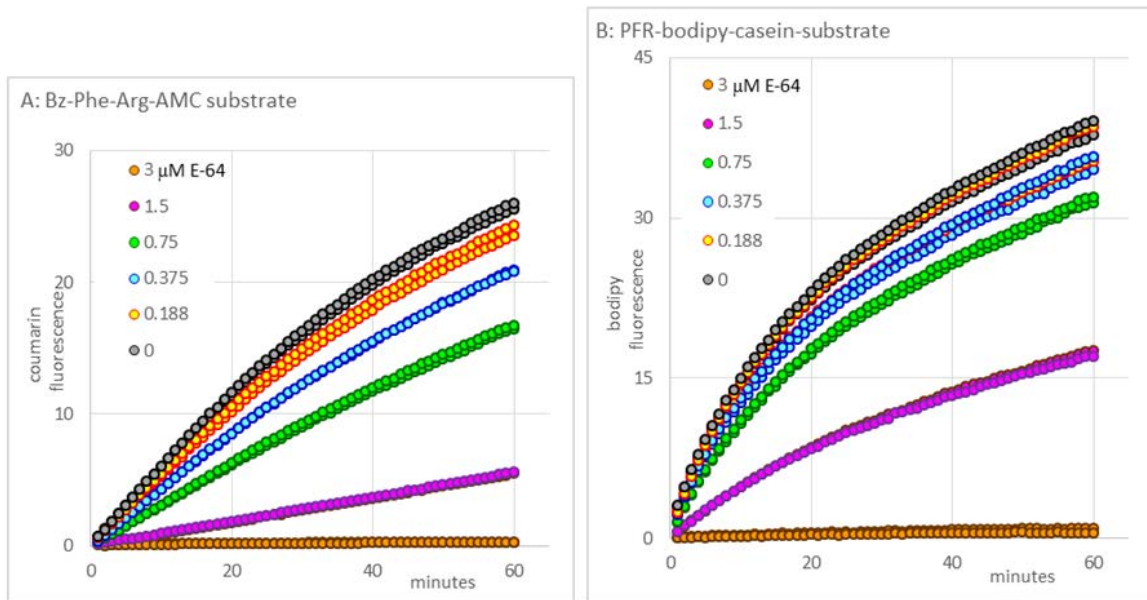


Figure 6. Actinidin quantification with E-64 inhibitor using Bz-Phe-Arg-AMC substrate (A) and PFR-bodipy β-casein substrate (B)

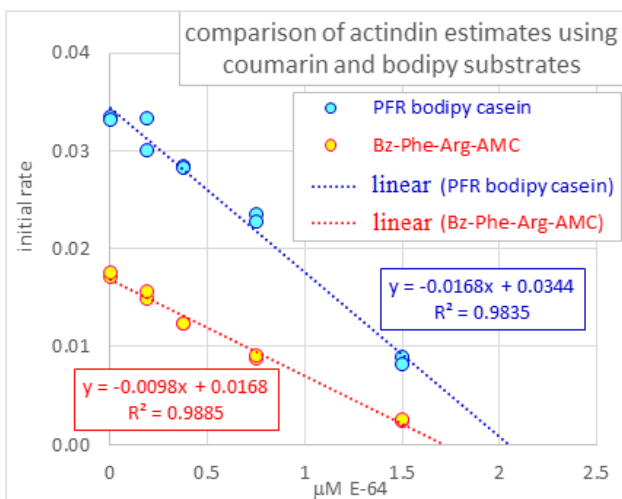


Figure 7. Initial rates from Figure 6

Table 1. estimate of mg/g concentration of actinidin

| substrate | Bz-Phe-Arg-AMC | PFR-bodipy β-casein |
|------------------------------------|----------------|---------------------|
| Y-axis intersect | 0.017 | 0.034 |
| Y intersect S.E. | 0.00029 | 0.00060 |
| slope | -0.00981 | -0.01675 |
| Slope S.E. | 0.00037 | 0.00077 |
| (A) X-axis intersect | 1.71 | 2.05 |
| X intersect S.E. | 0.072 | 0.101 |
| C of V (%) (X intersect) | 4.9 | 4.1 |
| (B) 50% extract correction | x2 | x2 |
| (C) Microplate well dilution | x8 | x8 |
| (D) Actinidin μmolar correction | x0.027 | x0.027 |
| mg actinidin/g kiwifruit (AxBxCxD) | 0.74 +/- 0.03 | 0.88 +/- 0.04 |

The reason for the estimated ~15% difference in actinidin concentrations between the two substrates may

lie in a combination of factors. For example, the bodipy-casein assay detects a slight contribution for additional proteinases in the kiwifruit sample which the Bz-Phe-Arg-AMC substrate does not. As a protein substrate the bodipy-casein digestion does not yield a constant output of fluorescence during digestion in the same way that the cleavage of Bz-Phe-Arg-AMC does; bodipy-casein is a heterogeneous substrate while Bz-Phe-Arg-AMC is a single chemical entity. The Bz-Phe-Arg-AMC value is the more reliable estimate of actinidin concentration although some food scientists and regulatory authorities prefer that a dietary proteinase activity is measured against a dietary protein substrate [2,18].

3.6. Estimate of Actinidin Concentration in Kiwifruit Extract by SDS-PAGE

To examine the predictions of the actinidin concentrations in green kiwifruit a sample of the same extract used in Figure 6 and Figure 7 was analysed by SDS-PAGE alongside two marker proteins: bovine serum albumin and mixed bovine casein. The data show that in comparison with BSA as a standard the total actinidin protein is estimated to be 1.68 mg/g and using casein as a protein standard the actinidin estimate is 0.87 mg/g (Figure 8). In the figure, the 27kDa actinidin is arrowed in the kiwifruit extract (KFE) tracks. The difference in Coomassie binding between the two standard proteins will be due in part to the different amino acid composition of the proteins [19]. Thus the figure indicates that the E-64 actinidin concentration estimate of is 0.74 mg is approximately correct and fortuitously agrees closely with the casein standard.

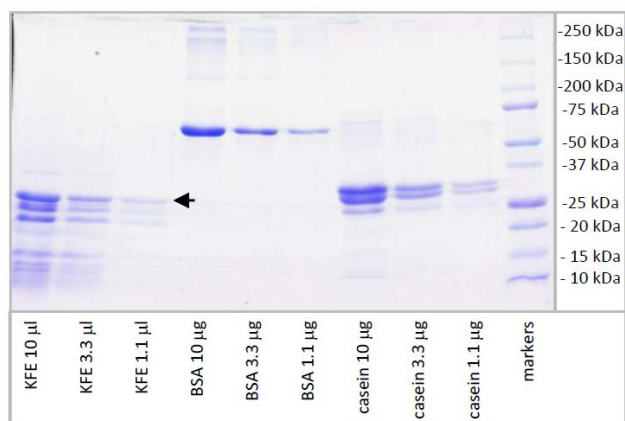


Figure 8. Coomassie stained SDS-PAGE; kiwifruit extract, bovine serum albumin and bovine casein

3.7. Blocking of Actinidin Active Site Cysteine with E-64: SDS-PAGE and Electroblothing

To confirm that the dominant band at 27 kDa is actinidin, iodoacetamidofluorescein (IAF) was used to label this band selectively. Actinidin has a free sulfhydryl group in its active site that should be alkylated with iodoacetamide [20]. Because prolonged incubations at high temperature will lead to labelling of other residues, the kiwifruit extract was exposed to 0.5 mg/ml IAF for only 10 min at room temperature in 0.1 M Bis-Tris buffer pH 7. In the production of this kiwifruit extract, mercaptoethanol was omitted from the extraction buffer so

that it would not interfere with the labelling of the actinidin active site cysteine [21]. Before dilution of the kiwifruit extract into Bis-Tris buffer containing iodoacetamidofluorescein, the concentrated kiwifruit extract was exposed to a dilution series of E-64 for 30 min. After a 10-min incubation, IAF was blocked by the addition of excess 2-mercaptoethanol. The sample was then analysed by SDS-PAGE (Figure 9).

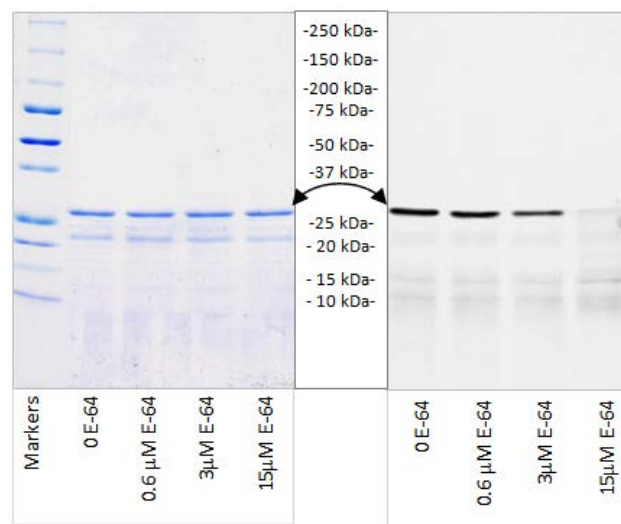


Figure 9. Blocking IAF labelling of actinidin with E-64 Left hand side: Coomassie stained samples Right hand side: Fluorograph of electroblotted samples

The data confirm that the major 27 kDa protein band (arrowed) is actinidin since this band contains a free sulfhydryl blocked by E-64. The higher concentrations of E-64 required to block the actinidin in this assay are due to the need to maintain a high protein concentration in this sample allow detection by Coomassie staining (i.e. the E-64 was used in a more concentrated sample than in the functional assays shown in earlier figures).

3.8. Comparison of Gold and Green Kiwifruit for Actinidin Activity and Concentration

Gold and green kiwifruit extracts were compared using Bz-Phe-Arg-AMC for actinidin concentration and specific activity. The data (Figure 10) show that this sample of green kiwifruit has 0.85 mg actinidin per gram of fruit flesh compared to 0.39 mg actinidin per gram of gold kiwifruit flesh. The data show that the gold kiwifruit have about half as much actinidin (by mass) as green kiwifruit whereas the green kiwifruit actinidin is three times as efficient as the gold kiwifruit actinidin in cleaving the Bz-Phe-Arg-AMC substrate at pH4.5. Thus although the gold kiwifruit sample has almost half as much actinidin as green kiwifruit on a mg/g basis, the weaker specific activity (against this substrate) combined with the lower enzyme concentration translates into an apparent 6-fold lower proteinase activity of gold in comparison to green kiwifruit. Very similar results were obtained using bodipy-casein as substrate (data not shown). Conceivably the use of Bz-Phe-Arg-AMC and bodipy-casein at pH 4.5 are sub-optimal conditions for measuring gold kiwifruit actinidin and in different circumstances gold kiwifruit actinidin might perform better. In Figure 10 the rates were estimated using a simple linear plot in excel, without

recourse to Origin® software because the weak activity of the gold sample generated data that was clearly linear. Using the first 10 min of the green kiwifruit data where the dose response was also linear allowed the whole analysis to be performed using excel software. This affected the final result by less than 2% when compared with MonoMolecular curve fit data (not shown). Finally, a surprising finding is that the nature and depth of the microplate used in the assays has a noticeable effect on the fluorescent output (data not shown). Low volume white 384 well plates and assay volumes of 20 -30 µl are recommended for use with bodipy-casein in favour of 96 well plates because the greater sensitivity of white plates and the increased linearity of the kinetic data as the assay progresses. Possibly an inner filter effect is responsible to the flattened dose-response curves seen using 96-well microplates.

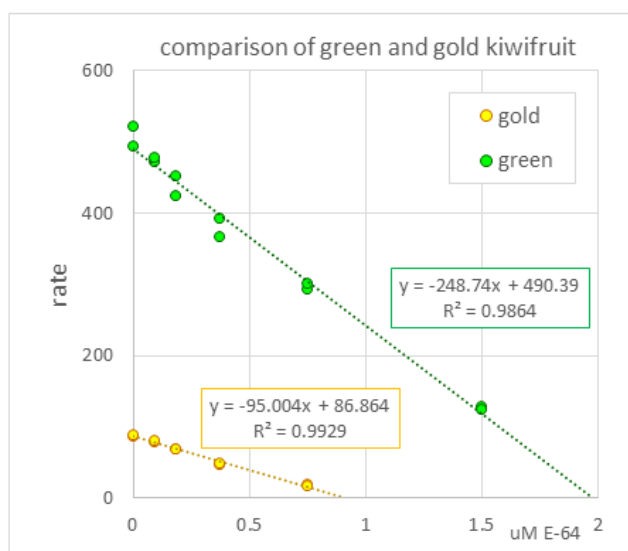


Figure 10. Initial rates of gold and green kiwifruit extracts after inhibition with E-64

4. Conclusions

Kiwifruit actinidin can be quantified by titration against the selective, cysteine proteinase inhibitor E-64. Either fluorescent bodipy-casein or Bz-Phe-Arg amino methyl coumarin may be used as a substrate. The casein substrate reports a 15% higher amount of actinidin. Although the commercial sources of bodipy-casein are usable, lighter labelling of bovine β -casein with a Fluor: protein coupling ratio of 5:1 yields three-fold improvement in signal: noise ratio. A simple linear plot of enzyme rate against inhibitor concentration generates the molar concentration of inhibitor (and therefore enzyme) in the sample while the Y-axis shows the shows that actinidin from different Actinidia species differ quantitatively in their activity. The use of E-64 should allow the standardization of actinidin data in future reports.

Acknowledgment

The author wishes to thank William Laing, Alistair Hall and Duncan Hedderley of Plant & Food Research for

helpful advice on enzymology, graphical analysis and statistics.

List of Nonstandard Abbreviations

AMC: aminomethylcoumarin; CAPS: cyclohexyl-3-aminopropanesulfonic acid; LT: LifeTechnologies; PBS: phosphate buffered saline; PFR: Plant and Food Research

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