



Cite this: *Anal. Methods*, 2022, 14, 3552

Actinidin diversity: discovery of common and selective substrates for actinidin isoforms and *Actinidia* cultivars

Harry Martin,^{ID}*^a Robert M. Simpson,^a Alan Seal,^b Ronan Chen^{ID}^a and Duncan Hedderley^a

The actinidin proteinase family has a striking sequence diversity; isoelectric points range from 3.9 to 9.3. The biological drive for this variation is thought to be actinidin's role as a defense-related protein. In this study we map mutations in the primary sequence onto the 3D structure of the protein and show that the region with the highest diversity is close to the substrate binding groove. Non-conservative substitutions in the active site determine substrate preference and therefore create problems for quantification of actinidin activity. Here we use a peptide substrate library to compare two actinidin isoforms, one from the kiwiberry cultivar 'Hortgem Tahi' (*Actinidia arguta*), and the other from the familiar kiwifruit cultivar 'Hayward' (*Actinidia chinensis* var. *deliciosa*). Among 360 octamer substrates we find one substrate (RVAAGSPI) with the useful property of being readily cleaved by all the functionally active actinidins in a set of *A. arguta* and *A. chinensis* var. *deliciosa* isoforms. In addition, we find that two substrates (LPPKSQPP & ILRDKDNT) have the ability to differentiate different isoforms from a single fruit. We compare actinidins from 'Hayward' and *A. arguta* for their ability to digest the allergenic gluten peptide (PFPQQLPY) but find the peptide to be indigestible by all sources of actinidin. The ability to inactivate salivary amylase is shown to be a common trait in *Actinidia* cultivars due to proteolysis by actinidin and is particularly strong in 'Hortgem Tahi'. A mixture of 10% 'Hortgem Tahi' extract with 90% saliva inactivates 100% of amylase activity within 5 minutes. Conceivably, 'Hortgem Tahi' might lower the glycaemic response in a meal rich in cooked starch.

Received 22nd June 2022
Accepted 22nd August 2022

DOI: 10.1039/d2ay01007k

rsc.li/methods

1. Introduction

The cysteine proteinase actinidin is expressed in kiwifruit at concentrations of up to 10 mg ml⁻¹ and is the most abundant protein in the fruit.¹ It functions as a pathogenesis-related protein that confers protection from insect pests. For example, cysteine proteinase expression in the leaves of maize plants was observed to reduce insect larval mass by 50% and to delay insect development by several days.^{2,3}

Kiwifruit actinidin is often thought of as a single cysteine proteinase but the actinidins are a diverse family of proteinases whose isoelectric points range from 3.9 to 9.3⁴. The variation in isoforms is usually ignored in methods that attempt to compare actinidins from different cultivars using a single substrate, or by immunological methods using an antiserum raised to a single isoform. Typically casein is used as a substrate for quantifying actinidin,⁵ despite the fact that casein is barely soluble at pH 4.5, the pH optimum of actinidin.⁶⁻⁸ Casein substrates for

actinidin are normally labelled with a coloured (sulfanilamide)⁹ or fluorescent (bodipy)^{10,11} dye. Neither of these substrates is ideal for measuring actinidin function because the substrates are not chemically defined, the rate of signal generation is non-linear throughout the digestion process and signal generated is likely to vary with the particular actinidin isoform used.

The critical effect of substrate choice was demonstrated using zFRAMC (benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin) and Bz-Arg-AMC (nalpha-benzoyl-L-arginine-7-amido-4-methylcoumarin) substrates tested against a set of 15 *Actinidia* extracts.¹² In several cases, if one substrate was cleaved by the extract then the alternative substrate was not. Using 3D modelling, the authors showed that the likely explanation was a steric clash between the phenylalanine residue on one substrate and the lysine residue in the actinidin binding site of a particular actinidin isoform.¹²

Although the main commercial application of actinidin has been as a meat tenderiser,¹³ the highly diverse nature of the actinidin family suggests many potential additional applications. For example, the actinidin in a whole extract of *Actinidia chinensis* var. *deliciosa* 'Hayward' kiwifruit was found to rapidly cleave and inactivate human salivary amylase *in vitro*.¹⁴ Salivary amylase is estimated to be responsible for the digestion of 80% of cooked

^aThe New Zealand Institute for Plant and Food Research Limited, Batchelar Road, Palmerston North 4410, New Zealand. E-mail: Harry.Martin@plantandfood.co.nz

^bKiwifruit Breeding Centre (previously The New Zealand Institute for Plant and Food Research Limited), Te Puke, New Zealand

starch¹⁵ and for the high glycaemic response to starch-rich food.¹⁶ Furthermore, the acidity of lemon juice has been shown to reduce the glycaemic response to bread consumption by its inhibitory action on salivary amylase.¹⁷ Gluten is a dietary protein of considerable bio-medical and commercial interest and it has been suggested recently that the glutamine- and proline-rich peptides in gluten that resist digestion by the human digestive enzymes are nonetheless susceptible to digestion with actinidin,¹⁸ and it is possible that some actinidin isoforms would be active against this peptide.

The first aim of this study was to discover a substrate that would (a) be commercially available, (b) be chemically defined, (c) be suitable for high throughput (automated) assay, (d) be cleaved by various actinidins, and (e) give a linear output of signal as digestion proceeds. To achieve this, we tested two actinidin isoforms from 'Hayward' kiwifruit and *Actinidia arguta* 'Hortgem Tahi' kiwiberry against a commercially available fluorescent substrate library of 360 octamer peptide substrates, labelled with the EDANS-DABCYL donor-quencher pair. The second aim was to explore alternative applications of actinidin by looking at (a) its action on the proteinase-resistant and allergenic gluten nonamer peptide¹⁹ and (b) the effect on salivary amylase.

2. Materials and methods

2.1 Plant material

Actinidia genotypes used included the cultivars 'Hayward' (*A. chinensis* var. *deliciosa*), 'Zesy002' (commonly known as Gold3, *A. chinensis* var. *chinensis*), 'Hortgem Tahi', 'Hortgem Rua', and 'Hortgem Toru' (*A. arguta*), and seedlings of *A. arguta* var. *purpurea*, *A. melanandra*, *A. valvata*.

2.2 Protein data

Extant actinidin protein sequences were imported from UniProt, yielding 10 sequences: ACT1A (P00785) (*deliciosa*), ACT1B (A5HII2) (*arguta*), ACT1C (A5HII3) (*eriantha*), ACT2A (A5HII4) (*deliciosa*), ACT2B (A5HII5) (*eriantha*), ACT2C (A5HII6) (*arguta*), ACT2D (A5HII7) (*eriantha*), ACT3A (A5HII8) (*eriantha*), ACT4A (A5HII9) (*eriantha*) and ACT4B (A5HIJ0) (*eriantha*). The original isoform protein sequences and species they were sourced from were reported by Nieuwenhuizen.⁴

Extinction coefficients were predicted from the amino acid sequence using the EXPASY online 'ProtParam' tool: <https://web.expasy.org/protparam/>

The extinction coefficient of ACT1A ('Hayward' peak 27.4) is $57215 \text{ M}^{-1} \text{ cm}^{-1}$. The extinction coefficients for the 10 published actinidin sequences ranges from $44265 \text{ M}^{-1} \text{ cm}^{-1}$ to $64205 \text{ M}^{-1} \text{ cm}^{-1}$. Where the full sequence of an actinidin was unknown, the average of the 10 known sequences, namely $51349 \text{ M}^{-1} \text{ cm}^{-1}$ was used.

2.3 Chemicals

The proteinase substrate Z-Phe-Arg-AMC (zFRAMC-AMC) was supplied by Bachem AG, Switzerland. The amylase substrate 2-chloro-4-nitrophenyl α -maltotrioxide (CNP3) was supplied by

Merck & Co, Readington Township, NJ, USA. Four *N*-[4-[(dimethylamino)phenyl]azo]benzoyl]-5-((2-aminoethyl)amino) naphthalene-1-sulfonic acid (DABCYL-EDANS) octamer substrates were synthesised by AnaSpec, Fremont, CA, USA: DABCYL-ILRDKDNT-Glu (EDANS); DABCYL-RVAAGSPI-Glu (EDANS); DABCYL-LPPKSQPP-Glu (EDANS); DABCYL-PFPQQLPY-Glu (EDANS). A DABCYL-EDANS octamer peptide library was supplied by JPT Peptide Technologies GmbH, Germany. Crude amylase was collected as the saliva of the researchers and co-workers and used unprocessed. A rabbit polyclonal antiserum to 'Hayward' actinidin was supplied by Thermo Fisher Scientific, Waltham, Massachusetts, USA as a commercial service.

2.4 Assays and protein detection

Fluorescence and spectrometry measurements were taken using a Tecan Spark®, microplate reader (Tecan, Männedorf, Switzerland). Actinidin activity was determined using $100 \mu\text{M}$ zFRAMC-AMC dissolved in 100 mM acetate pH 4.5, 0.1% 2-mercaptoethanol, 0.05% IGEPAL CA-630 buffer, detected using excitation and emission wavelengths of 351 nm and 430 nm , respectively. The same buffer was used for analysis of $5 \mu\text{M}$ DABCYL-EDANS labelled peptides, with detection using excitation and emission wavelengths of 340 nm and 490 nm . For screening the peptide library, protein MonoQ peaks were diluted to achieve a final concentration of $10 \mu\text{g ml}^{-1}$ in a $20 \mu\text{l}$ volume in the 384 well microplate. Fluorescence data was collected at 20 second intervals for 60 minutes. All rates were represented as a fraction of the maximum rate. Functional actinidin concentration in 'Hayward' and 'Hortgem Tahi' extracts were determined using the E-64 titration method.¹¹

Amylase activity was measured spectrophotometrically at 405 nm with 1.25 mM CNPG3 in a 20 mM Bis-Tris buffer pH6.0, containing 50 mM NaCl, 5 mM CaCl_2 , 0.1% Igepal CA-630.²⁰ The effect of avidin on amylase activity was determined by mixing *Actinidia* extract with saliva in equal volumes at 37°C and incubated for 5, 15 and 45 minutes. $100 \mu\text{M}$ E-64 was added to immediately inactivate the actinidin and the samples were then tested for residual amylase activity.

Proteins were analysed by SDS-polyacrylamide gel electrophoresis on 10/15/20% step gradient gels. Proteins were visualised by incorporation of trichloroethanol in the gels followed by exposure of the gels to UV light²¹ in a Biorad Geldoc System (Hercules, CA, USA). For immunoblotting analysis, proteins were transferred onto nitrocellulose in a pre-chilled 10 mM *N*-cyclohexyl-3-aminopropane-sulfonic acid buffer pH 11 containing 10% methanol. Transfer was performed for 1 hour at 100 volts. Nitrocellulose was blocked by incubation in non-fat milk protein in phosphate buffered saline. Actinidin was detected using rabbit antiserum raised to 'Hayward' actinidin and a goat anti-rabbit antibody labelled with the fluor Alexa 488. Fluorescent bands on the nitrocellulose were visualised using a Fujifilm FLA-5100 fluorescent image analyser (Minato City, Tokyo, Japan). N-terminal sequencing was carried on a commercial basis by Alphalyse, Odense, Denmark.

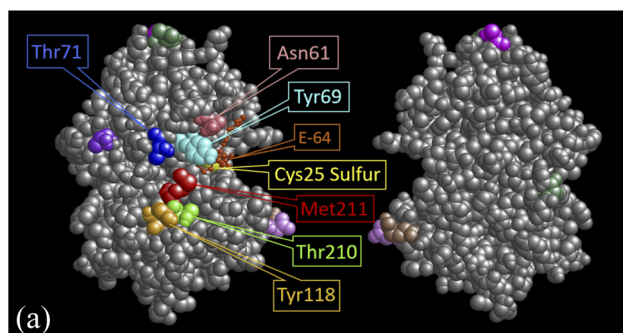
2.5 Protein purification

Kiwifruit flesh was homogenised in a 7 ml Dounce homogeniser at 4 °C. After addition of 2-mercaptoethanol to a final concentration of 0.1%, the homogenate was centrifuged at 19 000g to remove debris. Protein in the supernatant was precipitated with saturated ammonium sulfate (2 parts homogenate: 3 parts ammonium sulfate). The ammonium sulfate pellet was dissolved in 20 mM Bis-Tris pH 6.0 containing 0.1% 2-mercaptoethanol and the protein desalted on a PD10 column equilibrated with the same buffer. Actinidin isoforms were separated on a 1 ml MonoQ HR 5/5 anion exchange column equilibrated in the same buffer using an Äkta Explorer (Chicago, Illinois, USA). Protein elution was achieved with a segmented 1 M NaCl gradient, going to 15% in 5 column volumes, then 40% in 20 column volumes and to 100% in a final 10 column volumes. Protein was detected at 280 nm and fractions were collected in 0.2 ml volumes in 96 well microplates.

3. Results and discussion

3.1 Active site structure and sequence analysis

Although the high diversity of actinidins has been reported,⁴ the locations of the most variable amino acids have not yet been



Sequence identifier	61	69	70	71	118	210	211	Amino acid position
ACT4A	P	T	L	S	S	R	A	
ACT4B	P	S	R	A	R	T	S	
ACT3A	I	F	M	D	P	S	Y	
ACT1C	S	Y	M	T	Y	T	M	
ACT1A	N	Y	I	T	Y	T	M	
ACT1B	N	S	I	T	Y	T	K	
ACT2D	R	Y	M	N	A	M	V	
ACT2C	S	Y	M	T	S	R	M	
ACT2A	I	L	M	T	S	K	M	
ACT2B	R	L	M	T	S	R	M	
(b)	5	5	5	5	5	5	6	No. of distinct residues

Fig. 1 (A) 3D structure of actinidin ACT1A. Amino acids with five or more residues at a single location are shown in colour. The sulfur atom of the active site cysteine is shown in yellow and the covalent inhibitor E-64 is in orange. The right hand side shows the same protein after rotation by 180°. (B) Alignment of variable amino acids identified in (A) showing the non-conservative nature of many substitutions.

mapped onto the 3D structure of actinidin. Fig. 1A shows the 3D structure of actinidin. The 10 primary actinidin sequences available from the protein sequence database were aligned and positions where five or more amino acids occur at the same location were identified. Highly variable amino acids are shown in colour. It is evident that the substrate binding groove was the most variable region in the protein. This is consistent with the hypothesis that actinidin is a pathogenesis-related protein.³

The non-conservative nature of many of these substitutions is shown in Fig. 1B. Small polar amino acids are sometimes replaced with large hydrophobic residues, or small hydrophobic amino acids are sometimes replaced with large charged residues, etc. The chaotic nature of the substrate binding groove of actinidin implies that the use of proteolytic activity as a measure of actinidin concentration is unwise without first calibrating the particular substrate against a defined and purified actinidin standard.

3.2 Purification of isoforms from cultivars

Desalted ammonium sulfate precipitates from *Actinidia* were applied to a 1 ml MonoQ column and eluted with a gradient of 1 M NaCl. Fig. 2A shows the A280 nm absorbance and actinidin activity profiles of the *A. arguta* var. *purpurea* fractions. Fig. 2B shows the SDS-PAGE protein profile of selected fractions and Fig. 2C shows the western blot image of the same gel. The major protein in the whole protein preparation was actinidin. Three major peaks were visible in the absorbance profile but only two are detectable as proteinase activity. The active peaks were labelled 'Pu' for 'purpurea' and given a number that corresponds to the percent 1 M salt at the peak maximum. The immunoblot (C) confirmed the identity of actinidin bands. The fluorescent antibody in Fig. 2C was a molecular size marker and was not detected antigenically.

As shown in Fig. 2A–C, most of the protein in *A. arguta* var. *purpurea* was actinidin. Some lower molecular weight antigen was evident in the immunoblot, probably due to autocatalysis, which was observed if actinidin was maintained under active conditions. The actinidin in Fraction 36 had less antigenic reactivity to the antiserum (raised against 'Hayward' actinidin) relative to the intensity of the trichloroethanol stain. This was more likely due to large sequence variation between different isoforms of actinidin than the slight variation in tryptophan content of the actinidins.⁴ Double bands of actinidin were also evident in the same fractions (fraction 36 and fraction 80). The size of the separation was equivalent to at least 2 kDa. This was larger than the maximum difference between all of the actinidin sequences identified so far, whose masses differ by a maximum of 0.7 kDa. This size difference might be the result of N or C terminal truncation due to autolysis, as occurs during purification of the plant cysteine proteinase ficin.²² Although the protein peak heights were roughly similar there were striking differences between the activities of the peaks as determined by the commonly used substrate zFRAMC. Isoforms that are unable to cleave this substrate have been previously reported.⁴

These data emphasise the risks of using antigenic methods and activity measurements for quantifying the amount of

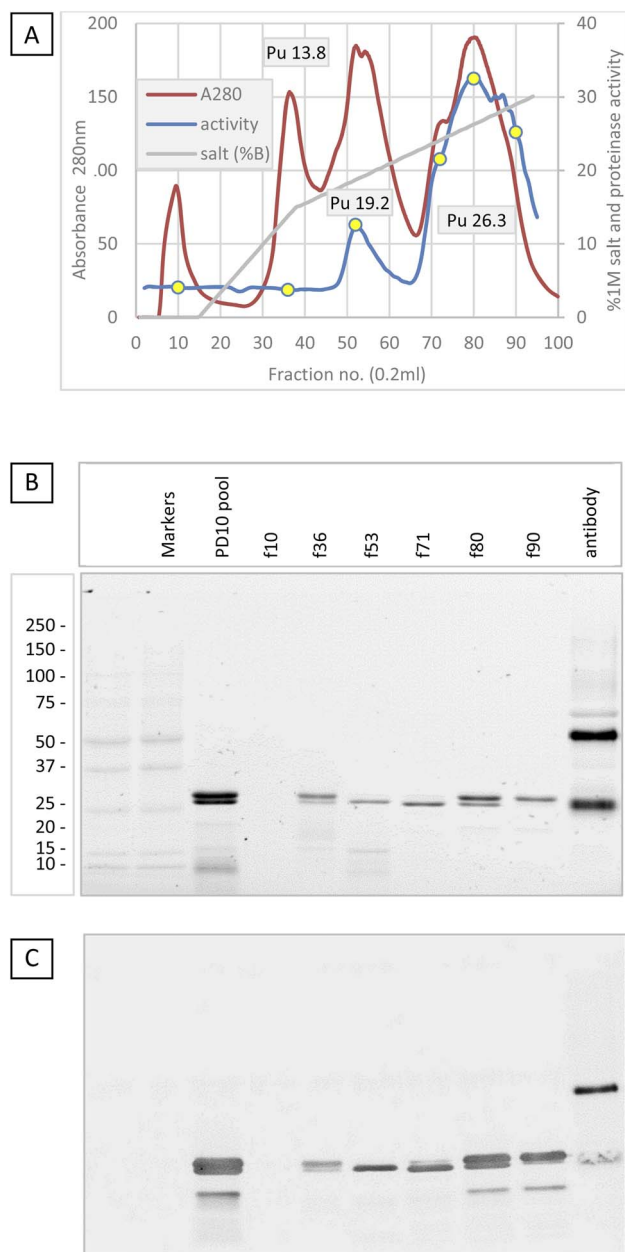


Fig. 2 (A) *Actinidia arguta* var. *purpurea* MonoQ elution profile; absorbance at 280 nm, actinidin activity (measured using zFRAMC-AMC substrate) and salt concentration. (B) SDS-PAGE protein stain (trichloroethanol detection of tryptophan) and (C) actinidin immunoblot of fractions selected from (A).

actinidin in a sample. In addition, the data suggested that any application of actinidin from amylase digestion or gluten digestion may be greatly influenced by the particular isoforms used. A further set of *Actinidia* samples were analysed using the same procedures (MonoQ chromatography, activity analyses and SDS-PAGE/western blotting of the fractions). Fig. 3 summarises the number of antigenic peaks resolved on the MonoQ ion-exchange column, the absorbance at 280 nm of these peaks and the relative activity against zFRAMC of each peak compared to maximum activity in the peaks. Gold3 had

a single antigenic peak and a single activity peak whereas all of the Kiwiberry cultivars have three antigenic peaks. 'Hayward' had only two antigenic peaks, both of which were active against zFRAMC. While 'Hortgem Tahí' and 'Hortgem Toru' had three peaks all active against zFRAMC, *A. purpurea*, *A. melanandra* and *A. valvata* had antigenic peaks with zero, or almost zero, activity against zFRAMC. *A. valvata* was unusual because the great majority of its actinidin was inactive against zFRAMC.

3.3 Identification of selected 'Hortgem Tahí', Gold3 and 'Hayward' peaks by Edman degradation

From the 7 genotypes tested, 18 activity peaks were generated on the MonoQ column. Since 'Hayward' and Gold3 are commercially the most significant cultivars, the three peaks from these two cultivars were chosen for N-terminal sequencing. In addition, 'Hortgem Tahí' peak 1 (Ta 17.6) was also analysed by N-terminal sequencing because its elution position was different from the 'Hayward', and Gold3 peaks and the 'Hortgem Tahí' substrate preference was reported to be different from that of 'Hayward'.¹²

Table 1 compares the known sequences of 10 actinidin isoforms with the N-terminal sequences of these 4 MonoQ peaks. The two actinidin peaks in 'Hayward' have the same N-terminal sequence which is also identical to the actinidin sequence of the single peak in Gold3. The N-terminal sequence of the first eluted 'Hortgem Tahí' peak (Ta 17.6) is a new isoform not yet described in the literature. The 'Hayward' peak 2 (Ha 24.9) and Ta 17.6 were chosen to explore a substrate library to discover substrates that may be cleaved by most or all isoforms and substrates that differentiate between isoforms. The octamer sequences of the DABCYL-EDANS labelled library are given in Table 2.

Testing the 'Hayward' and 'Hortgem Tahí' peaks against the octamer substrate library, produced three substrates of particular interest; RVAAGSPI stood out clearly as the best substrate for both 'Hayward' Peak 1 (Ha 24.9) and 'Hortgem Tahí' (Ta 17.6) proteinases. On the other hand, LPPKSQPP was rapidly cleaved by Ta 17.6 but not by Ha 24.9. Conversely, ILRDKDNT was an excellent substrate for the Ha 24.9 yet very poor for Ta 17.6 (Fig. 4). The locations of these three octamers in the library are highlighted in Table 2. These three substrates were synthesised commercially and used to probe the purified *Actinidia* MonoQ peaks (Fig. 5). The single Gold3 peak was not included in the study due to its similarity to Ha 24.9 in salt elution and N-terminal sequence.

With the exception of *A. arguta* var. *purpurea* peak Pu 13.8, all *Actinidia* MonoQ peaks cleave the RVAAGSPI substrate, whereas substrate LPPKSQPP is cleaved only by isoforms eluting below 0.2 M salt. Conversely substrate ILRDKDNT was preferentially cleaved by acidic actinidins eluting above 0.2 M salt. The N-terminal sequences of 'Hayward' actinidins identify them as Act1a, with an isoelectric point of 4.16. These acidic proteins would be expected to elute late from the MonoQ column whereas the basic actinidins should elute early. Based on N-terminal sequence data (Table 1), the 'Hortgem Tahí' sequence we have discovered does not match any known

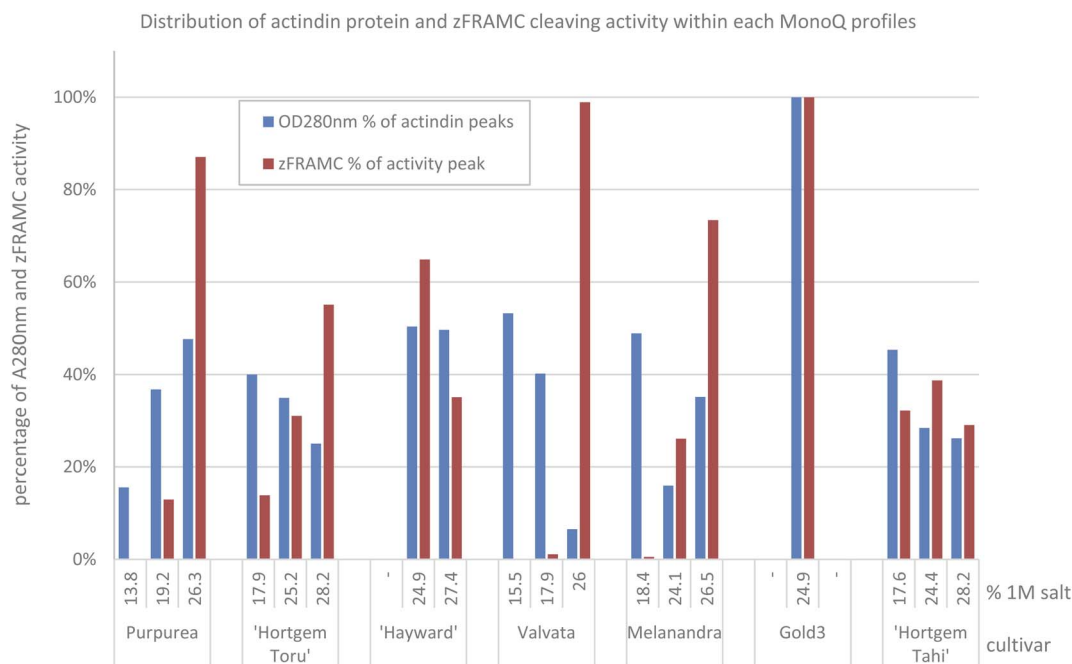


Fig. 3 Actinidin antigen peaks in *Actinidia* genotypes are labelled with the salt concentration at which the peaks eluted from the MonoQ column. Absorbance and activity in each peak is shown as a percentage of total.

actinidin sequences and is therefore a new isoform. We would expect it to be a basic actinidin, due to its early elution from the MonoQ column. The reason for the elution of two separate 'Hayward' actinidin isoforms is unknown. Their activity profiles against all substrates was very similar. A distinct actinidin isoform (ACT2A) has been described in 'Hayward' expressed at very low concentrations but its N-terminal sequence is distinct from Act1a and it is a basic isoform.⁴ We found no Act2a activity in our MonoQ profile in these preparations. From its isoelectric-point (IEP), this isoform is unlikely to have bound to the MonoQ, but no activity was observed in the flow through.

The *A. valvata* isoforms are also functionally distinct from the other isoforms since they readily digest RVAAGSPI but are

very poor at digesting the other substrates. The *A. arguta* var. *purpurea* peak Pu 13.8 elutes from the MonoQ very early and clearly has antigenic activity. Its activity is undetectable using the conventional zFRAMC-AMC substrate, and only a trace of activity is detectable using the RVAAGSPI substrate (Fig. 5). Conceivably, screening Pu 13.8 against the substrate library might reveal better substrates for this isoform. In addition, evidence that the Pu13.8 peak is functionally active could be obtained by observing whether it consumes the E-64 inhibitor in a mixed assay with an active actinidin.

Summarising the SDS-gel, N-terminal sequence data (Ta17.6, Table 1) and octamer substrate activity data (Fig. 5), it is clear that even within a single genotype there are usually three

Table 1 Comparison of selected N-terminal actinidin amino acid sequences with published sequences

UniProt ID	Gene name	IEP	MonoQ peak	N-terminal 10 amino acids	<i>Actinidia</i> species	Common name
A5HII9	ACT4A	9.12		LPDSVDWRKK	<i>eriantha</i>	
A5HIJ0	ACT4B	8.63		LPNSIDWRKK	<i>eriantha</i>	
A5HII8	ACT3A	4.29		LPDYVDWRTT	<i>eriantha</i>	
A5HII3	ACT1C	4.11		LPDYVDWRSE	<i>eriantha</i>	
P00785	ACT1A	4.16		LPSYVDWRSR	<i>chinensis</i>	
A5HII2	ACT1B	4.17		LPDYVDWRSR	<i>arguta</i>	
A5HII7	ACT2D	5.04		LPNYVDWRTV	<i>eriantha</i>	
A5HII6	ACT2C	5.39		LPDYVDWRSE	<i>arguta</i>	
A5HII4	ACT2A	8.62		LPDYVDWRTV	<i>chinensis</i>	
A5HII5	ACT2B	8.43		LPDYVDWRTV	<i>eriantha</i>	
			Ha 24.9	LPSYVD-RSA	<i>chinensis</i>	'Hayward'
			Ha 27.4	LPSYVD-RSA	<i>chinensis</i>	'Hayward'
			Go 24.9	LPSYVD-RSA	<i>chinensis</i>	Gold3
			Ta 17.6	LPNYVD-TSV	<i>arguta</i>	'Hortgem Tah'

Table 2 Commercial fluorogenic octamer library substrates in 384 well microplate layout (text-wrapped)^a

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	WQGG RRKF	NVRH LVQP	PKDK RYGG	RGAR ARLG	SYRK VLGQ	DSRR AQDF	LGKR RSGP	QLQK RFFG	RMRR YADA	SAAR PAPP	VARR KLPT	ARRH SDGT	GKRS EQDA	ELQR SANS	SDKP DMAE	LQKR GIVE	AEVD GDDD	KTRR EAED	GEDD RDSA	GGKR AMSD	RSKR CGNL	GKKR DMSS	KGAN KKGL	NPRK YVMG
B	EGKR SYSM	KPVG KKRR	EFKR ELTG	DDDK SLQE	QTVW IVGG	PRED NKPT	LAKR RATW	FMKK YGGF	MEKR DAEE	AAGV YGGF	MGKK APLS	PRGR SLEP	AVPL STGE	MGKK PAED	GEDA SKPD	EAYP RQRY	LVDA QRYG	QRYG KRDG	PQRF GRNT	LSPR AGEG	APQR FGKK	RVKR YRQS	HPSA FSAR	
C	GPQG LLGA	GPAG LSVL	GPAG IVTK	SDSD MGFG	PPGA YHGA	LRAY LLPA	GPYE LKAL	GHAR LVHV	KPQQ FFGL	QPVG INTS	RPAV MTSP	HVLN LRST	DPQS IRSQ	DPLE FKSH	RPIP ITAS	RVLG LKAH	KVLN LTDN	IVAM LRAP	FPAD EANS	MTAK QFRK	AAAL IGDE	GMAG SGRR	RDAQ AHPG	LGAR GHRP
D	FSAR GHRP	IDAR AHRP	ANAR IKLP	TVAD GLKK	ALAD SLGK	ALAE AMKK	EEAD SMKS	GLAR SNLD	HLAR NNHN	KPAP SPAA	HLAD SPAV	IMAE NRKS	RVAAGSPI GATA	LLDR SANG	DEDD SMPK	TVDA IMEK	QIDR HRLG	ELDY GYFV	PEDD EEDL	LDED TFGL	FNEK QIGH	SLEK DKEE	GKEN GKGY	REEM GKGY
E	DEED ILSH	LTED HLDL	DEED LQRA	EGED DRDS	DEED DSGK	LLER GVTA	TEED GVPS	TQFD AAHP	VYFQ GKKK	SPFR SSRI	SPFR SVQV	LAGE GALA	EAGR NAQV	DTGD EDSA	LNGG AFSW	DAGD VGAA	MTGR TGHG	IGGL IGAN	RLGR EGVQ	QLGR LHMK	SLGR KIQI	LRGG AINW	DEGD SLDG	SKGR SLIG
F	SGGF MLTP	LHGG ALGW	LAGE LMVG	TSGP NQEQ	MYGG ARRL	IEGR TSED	PQGR IVGG	IDGR VEG	LAGE AWIN	TGGR LYGG	DRHD SGLD	DEHD EHDE	QHHV VQDM	DYHD EYHG	FWHR GVTK	DEID HAEM	FLIE GEEP	LTIK SFTK	LPIK TFRG	LGIR VEFN	RAID NGKQ	GKIG ALRE	VGIP GLKG	
G	DNID NLSP	IDIR GPRL	ADID GQYA	TEID GRSI	DQID DTVE	AEIR ATSE	RTKR EAGA	VSKR SADD	EVKM DAEF	LFKR TEVL	YVKA SEHP	ERKK SEHP	PVKH GGEQ	QQRK KIVL	RNKR SKSN	LLKD FLKK	RSKR ALKQ	DLKD HMRE	LEKK SPLG	WEKL IQDQ	KPKD SSVD	RKKR STSA	ISKR STEE	RRKR STRE
H	RKKR STKK	RAKR SVHF	RNKR AVQG	YLKA SVQG	YVKA SVSP	RVKR RVKR	RVKR QVRS	RTKR DYDS	FKLE VAQE	GPLG IAGI	RRLR RAIP	HDLR QAFR	EGLD AAAS	EPLD LASY	HGLL KDFL	SALD GDQM	NGLR ADPM	TKLR MELQ	SLLR SEET	RHLL GFSA	RHLL GFSE	VRLQ AGDE	KELG LGRH	SYLD SGIH
I	DLLD DGEI	DELD YHRG	DELD SHTM	VKLE GKSK	PQLR MKNM	ISLH GKGS	EDLD GLKG	RGLT SLAD	DGLP GLKG	SLLD ELES	ETLD MMKK	KALK LNFA	WYLR SNNG	AKLN ANMW	PGLK IPKE	MELD GPKG	ISLR GPRL	EDLK TPIN	HMLG LPST	DGLD GPTY	DSLQ LQPI	ISLM GRSL	SSLD KRPP	AREV AREV
J	SELD ASKT	RKLD NTKF	PQLH STGG	FGLN VTGK	RGLT TTSL	IVLD GTDN	AQLG TTSS	LQMD YATN	RSML EAES	RYMR ADEA	VKMD AEFR	DEMMD EKSE	VEMD SLSE	DLMD GLPP	EEMD FRSG	SPMG ESAV	FKNE VARE	FANE FAEG	LVNA SCEP	DIND GHCQ	LRNR AQSG	SSNQ LQRR	LVNA SSAA	DLND GTQA
K	IVPD IATG	DLPD CEAD	DAVD TGIS	QSPR SFQK	LDPR SFLH	LSPR TFHP	IEPR SFSQ	FNPR TFGS	PGPK RGTE	AEPD YGAL	VIPR SGGS	VDPK LIDG	RRPS FIAI	SQPP EKTE	TEPK VKLP	FPSS MKET	TEPK LTLT	TEPK ALQK	VSPE SPQA	VSPE GPEE	VSPE APVF	DGPD SPVF	PRPP APLK	SEPD IPGQ
L	DEPD SPPV	ELPD GQVI	SSPD GQLM	LPPK SQPP	NSPD AQDF	LIPE AQGR	DEPD SRAG	MTPR SRGS	ELPT VSQE	PPPP LSGG	DYPD SSVS	MMPD GTLG	IQPF ATNG	ASPR ITSI	IKPR IVGG	KTPR VVG	LGPR VVG	VVPR IVNG	FRGP SVVV	PAPR GYPG	FIQR FAGD	YLQA SEKF	RCQA GFPS	PVQP IPGQ
M	VNQF SIEG	EKQR IIGG	QRQR IIGG	RHQR DISL	RHQR EITR	RHQR ELSA	GLQR ALEI	GAQM GQPW	GSQH IRAE	LAQA VRSS	ESQD VSGS	GPQR FSGV	HHQK LVFF	DDRD SANG	VMRD PASK	RSRR EADK	RARR DAGP	VHRD MAAR	DPRL IDGK	YYRA DDAN	ILRD KDND	EIRA GKGR	RNRK AFQG	AQRD SHLG
N	LDRR GIQR	MLRR EIKS	AKRR TKRD	DLRD DKDT	RARR ELAP	RRRR ELDP	DPRI VNGT	RKRR APLA	YMRA DQAA	FYRA DQPR	VKRR VQDV	AKRA ASQI	VYRD GTGV	EARP IVVG	LDRK EVLG	RVRR DVEQ	RKRR AVLT	LERK EVLV	DTRD NVYY	GIRS LYGS	DDSD AATF	DYSL VAPP	EESD EDMG	LLSR SEED
O	DSSD SELE	LESQ YFGK	LKSN EIPD	LSSD APGV	DPSS STDS	EPSS LOES	TYSR SRYL	EISS KRRS	LPSR SSPT	KESD LSHV	APQY TYAQ	LASR IVGG	YSSR IVGG	KPTP IQLN	AETD GQAS	AATR RQAV	TSTR IVGG	SHVD GAAK	SEVK MDAE	DTVD GKEI	DGVD LKTO	GGVR GPRV	KNVA MOGM	DEVN MOGM

^a Highlighted cells correspond to the 3 octamer substrates selected for their useful properties; cell D13 (green) contains RVAAGSPI (cleaved by almost all isoforms), cell L4 (blue) contains LPPKSQPP (preferentially cleaved by more basic isoforms eluting at lower salt concentrations), cell M21 (red) contains ILRDKDNT which is preferentially cleaved by more acidic isoforms.

discernible antigenic peaks. It is likely that there are several new actinidin isoforms within these *A. arguta* MonoQ peaks for the following reasons; (a) Ta 17.6 has a new N-terminal sequence not described in the protein sequence database, (b) Va 15.5 has the unique property of cleaving RVAAGSPI but neither of the

other two octamers, (c) Pu 13.8 has almost zero activity against any of the substrates tested, (d) Me 24.1 cleaves RVAAGSPI and ILRDKDNT at the same rate whereas To 25.2 has clearly cleaves RVAAGSPI at almost twice the rate that it cleaves ILRDKDNT. Thus we would anticipate that the image of the highly variable

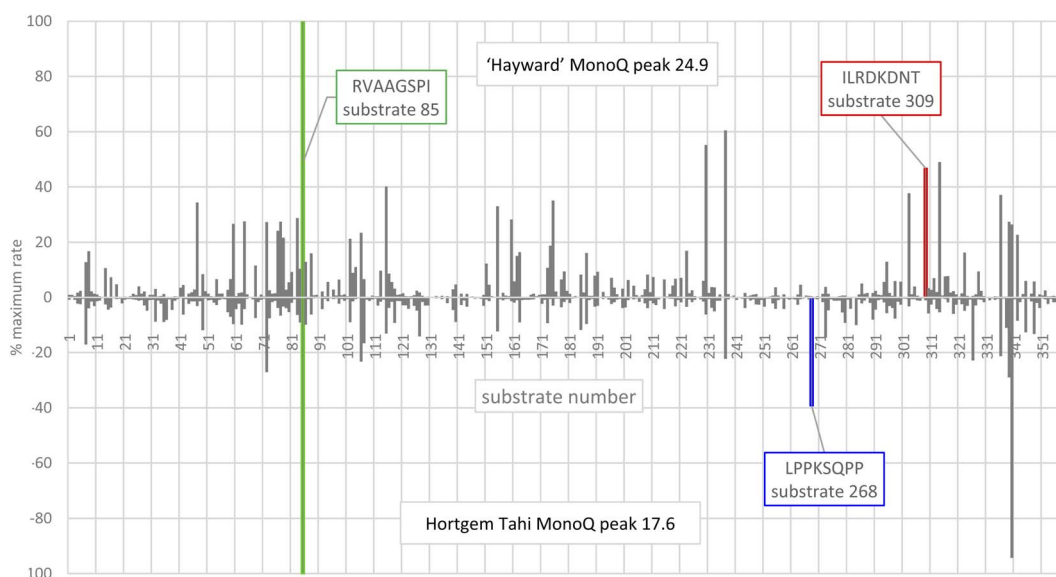


Fig. 4 Relative rates of cleavage of substrate library fluorogenic octamers (shown in Table 2). The octamer RVAAGSPI, the most rapidly cleaved substrate for both isoforms, is shown in green. The octamer ILRDKDNT, preferentially cleaved by 'Hayward' is shown in red, while the LPPKSQPP octamer is preferentially cleaved by the 'Hortem Tahi' isoform (blue).

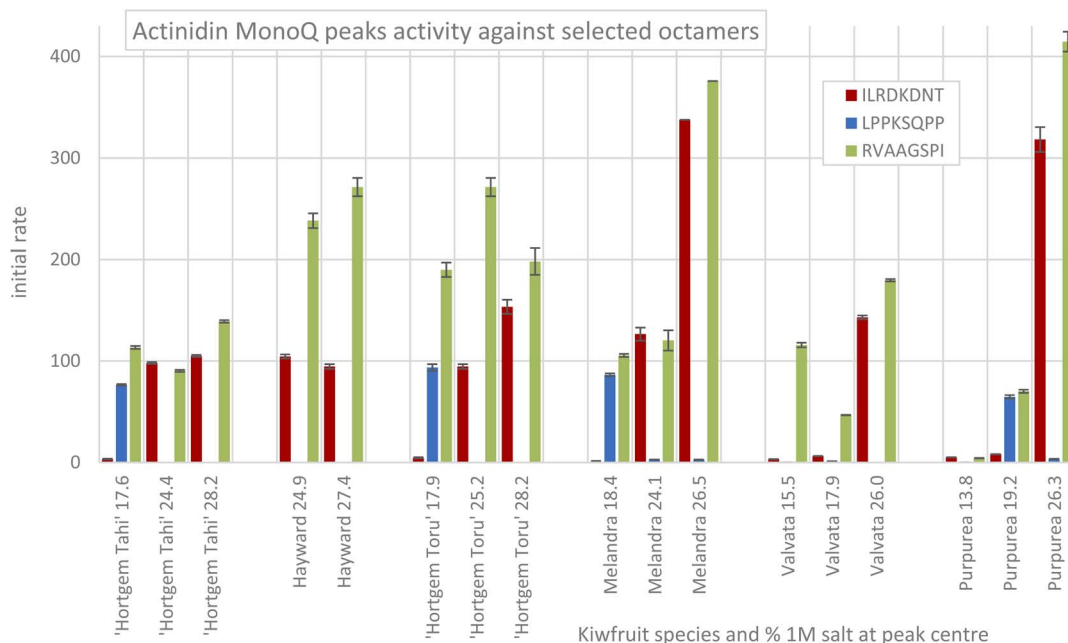


Fig. 5 Cleavage of three DABCYL-EDANS octamer substrates by actinidin isoforms (actinidin balanced assuming the same average A280 nm absorbance for all peaks except 'Hayward'). Seventeen MonoQ peaks containing actinidin from 6 *Actinidia* species were tested against the 3 library octamers selected from Fig. 5.

actinidin active site conformations (Fig. 1) would become still more varied once these new isoforms are sequenced and included in the comparison.

Salivary amylase is responsible for the majority of starch digestion when bread is consumed.¹⁵ The actinidin in the 'Hayward' kiwifruit cultivar digested human salivary amylase to the extent that 85% of amylase activity was lost within 5 minutes at a ratio of 1 part kiwifruit extract to 3 parts saliva. The fragments produced by actinidin digestion of salivary amylase were large and stable as seen by SDS-PAGE, suggesting that a single cleavage site may be responsible for this actinidin effect on amylase.¹⁴ The specific nature of the amylase polypeptide cleavage raises the question of whether amylase inactivation is a general characteristic of actinidins or limited to the 'Hayward' isoform. We compared various kiwifruit, as whole extracts, for their ability to inactivate amylase function. The results (Fig. 6) showed that amylase inactivation was a common feature of actinidin from different genotypes. Only the *A. valvata* cultivar did not substantially inactivate amylase. One-hundred percent loss of amylase activity occurred within 5 minutes of incubation with 'Hortgem Tahi' extract. In order to better quantify the 'Hortgem Tahi' effect, 'Hortgem Tahi' extract was mixed with saliva at different concentrations from 50% to 5% for 5 minutes at 37 °C. Ten percent 'Hortgem Tahi' extract was sufficient to inactivate 100% of the amylase activity within 5 minutes (Fig. 6). The reason for the sudden loss of amylase digesting activity as the 'Hortgem Tahi' extract dropped from 10% to 5% is not clear. This may be due the combined effect of (a) a two-fold dilution in proteinase, (b) an increase in pH from 5.3 to 6 (the pH optimum of actinidin is around pH 4.5 and activity declines sharply between pH 5 and pH 6 (ref. 6)) or (c) loss of anti-oxidant activity

in the assay buffer. To mimic physiological conditions, no antioxidants were added to the saliva or the kiwifruit extract to protect the actinidin active site sulfhydryl group. Although *Actinidia* have a high vitamin C content, a solution of only 5% extract may be unable to maintain the reducing environment preferred by cysteine proteinases, especially as the pH increases. Unfortunately, we did not have sufficient stocks to include 'Hortgem Toru' in the amylase study, so the ability of 'Hortgem Toru' to inactivate salivary amylase remains unknown at present. The remarkable ability of 'Hortgem Tahi' to fully inactivate salivary amylase within 5 minutes even at a concentration of only 10% extract, raises the possibility that 'Hortgem Tahi' might reduce the glycaemic response that follows consumption of cooked starch if it is consumed as part of the meal.

The functional concentration for the 'Hayward' and 'Hortgem Tahi' extracts were 0.71 and 4.7 mg ml⁻¹, respectively. These are whole crude extracts with mixed isoforms. Thus, it may be that 'Hortgem Tahi' owes its amylase inhibitory activity to the quantity of actinidin rather than to its isoform composition. Comparing the three active actinidin-MonoQ peaks of 'Hortgem Tahi' (Fig. 3) for amylase cleavage will be part of a later study.

In addition to salivary amylase, another important protein target, namely gluten, has been proposed as a target for actinidin.¹⁸ Resistance of dietary protein to gastric digestion with pepsin, and intestinal digestion with trypsin, chymotrypsin and pancreatic elastase leaves behind immunogenic polypeptides.²³ The indigestible nature of gluten polypeptides is the major cause of their allergenicity. Around 25 proline/glutamine-rich gluten polypeptides were identified by their resistance to

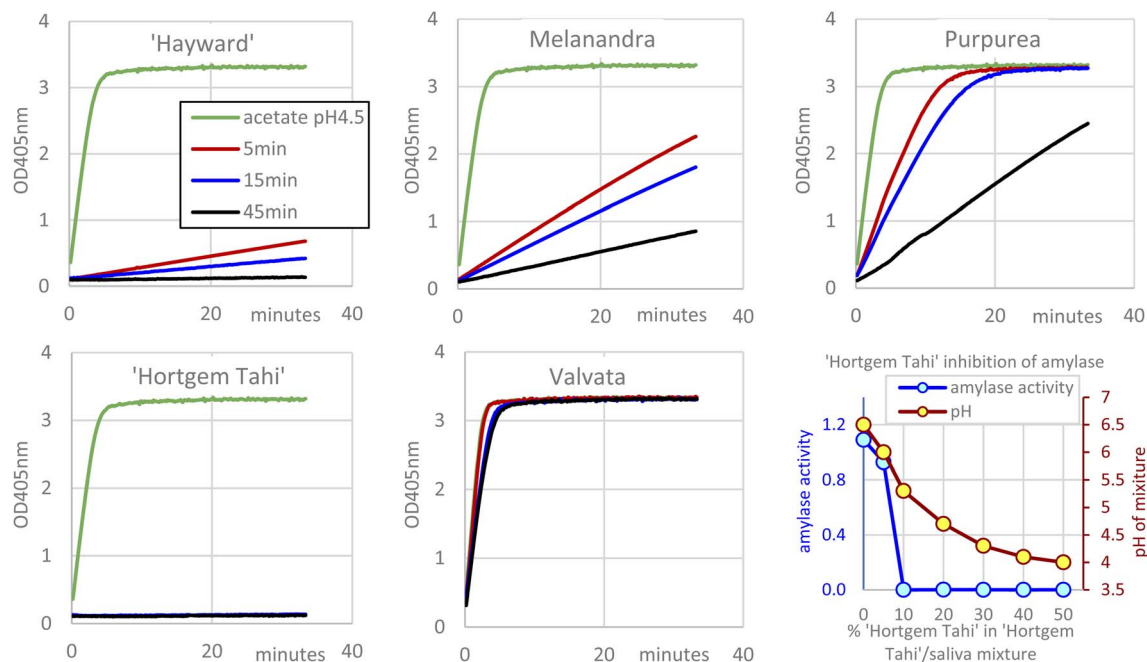


Fig. 6 Loss of salivary amylase activity following addition of *Actinidia* extracts (50% saliva + 50% extract). Whole extracts of 5 *Actinidia* species were compared for their ability to inhibit amylase activity in human saliva. Following incubation of saliva and *Actinidia* extracts at 37 °C, 0.1 mM E-64 was added to inactivate actinidin. The sample was then diluted $\times 300$ into amylase assay buffer and assayed against chromogenic substrate CNPG3.

gastric and intestinal proteinases including brush-border membrane vesicle endopeptidases.^{19,24} In our study, the gliadin peptide PFPQQLPY was selected for digestion with our

Actinidia extracts because it was one of the more stable gluten-derived allergenic peptides. The rates of cleavage of the gliadin substrate were compared with the cleavage rates of the actinidin

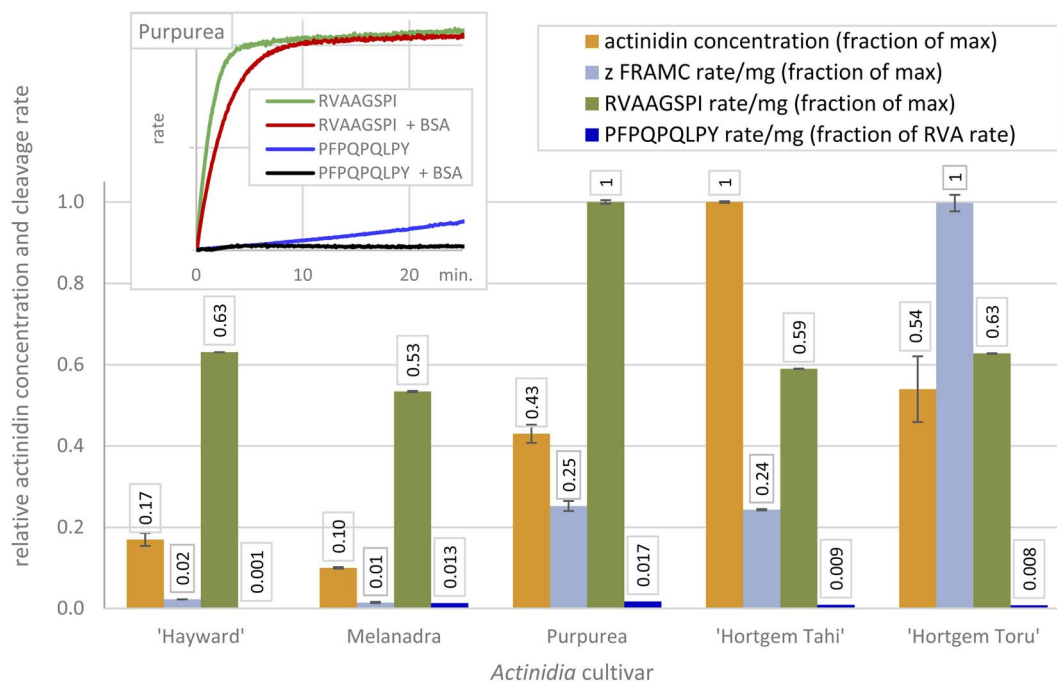


Fig. 7 Cleavage of zFRAMC, and DABCYL-EDANS peptides RVAAGSPI & PFPQQLPY by whole *Actinidia* extracts. The rate of gliadin peptide cleavage is shown as a fraction of the RVAAGSPI cleavage rate for the same kiwifruit extract. The maximum concentration of actinidin was 4.7 mg ml⁻¹ (in 'Hortgem Tahi').

substrate RVAAGSPI against a panel of whole *Actinidia* extracts (Fig. 7).

Both octamer peptides were assayed for 25 minutes at 37 °C at a concentration of 5 µM. The actinidin extracts were assayed at a final dilution of 1/200 in the main figure. The cleavage rate of the RVAAGSPI octamer was not measurable at higher enzyme concentrations because full substrate digestion occurred almost instantly (see inset in Fig. 7). In contrast, the gluten peptide was resistant to cleavage by all of the five whole *Actinidia* extracts.

Due to the variation in concentration of actinidin in the extracts, the data were presented as a rate of cleavage per mg of actinidin in the sample. The highest concentration of actinidin was found in 'Hortgem Tah' (4.7 mg ml⁻¹) and the lowest in *A. melanandra* (0.1 mg ml⁻¹). Almost no digestion of the gliadin peptide occurred under these conditions. The concentration of actinidin in the 'Hortgem Tah' digestion was around 1 µM, 20% of the substrate concentration of 5 µM. When bovine serum albumin was added as a competitor substrate to a concentration of 5 mg ml⁻¹, the digestion rate of RVAAGSPI slowed by 50% (inset, Fig. 7) and the rate of digestion of the gluten peptide dropped to zero. The concentration of protein in milk is around 30 mg ml⁻¹. With about 10 g of gluten in 100 g of bread this implies that the contribution of 100 g of 'Hortgem Tah' kiwiberry containing 5 g of actinidin (at the higher end of the *Actinidia* concentration scale) will have little effect on digestion of this nonamer gliadin epitope. It is possible that other proline- and glutamine-rich peptides might be susceptible to actinidin digestion, yet the report suggesting that actinidin digests gliadin, identifies several cleavage sites within the nonamer gliadin peptide that are purportedly cleaved by actinidin.¹⁸

4. Conclusions

In this study we have shown that the actinidin active site is characterised by particularly high rates of amino acid substitution. Using a peptide library we have discovered that the octamer substrate RVAAGSPI has considerable advantages over conventional peptide and protein substrates, such as zFRAMC or dye-labelled-casein for actinidin assay; it is chemically defined, commercially available, cleaved by all actinidins known to be active and gives a linear dose-response curve. Conversely, the octamers ILRDKDNT and LPPKSQPP differentiate between isoforms; the former is a preferred substrate for more basic isoforms whereas the latter is preferentially cleaved by more acidic isoforms in *A. arguta* and *A. chinensis*. The allergenic gluten sequence PFPQPQLPY resists digestion by seven different *Actinidia* extracts implying that actinidin may be of little value in reducing gluten intolerance. Salivary amylase digestion is a common feature of *Actinidia*; 'Hortgem Tah' is particularly effective in this regard raising the possibility that certain *Actinidia* may reduce the glycaemic response when consumed with a meal high in cooked starch.

Author contributions

Harry Martin: SDS-PAGE, immunoblotting and enzyme assays. Robert Simpson: ion-exchange chromatography. Alan Seal:

provision of *Actinidia* germplasm. Ronan Chen: automated assays for actinidin. Duncan Hedderley: data analyses.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We wish to thank Ross Atkinson and Dave Brummel for helpful discussion and provision of anti-actinidin antiserum. Funded by Plant and Food Research 'Blue Skies' project P/200222/83.

References

- 1 P. Latocha, The Nutritional and Health Benefits of Kiwiberry (*Actinidia arguta*) – a Review, *Plant Foods Hum. Nutr.*, 2017, 72, 325–334.
- 2 T. Pechan, L. J. Ye, Y. M. Chang, A. Mitra, L. Lin, F. M. Davis, W. P. Williams and D. S. Luthe, A unique 33-kD cysteine proteinase accumulates in response to larval feeding in maize genotypes resistant to fall armyworm and other lepidoptera, *Plant Cell*, 2000, 12, 1031–1040.
- 3 L. A. Malone, J. H. Todd, E. P. J. Burgess, B. A. Philip and J. T. Christeller, Effects of kiwifruit (*Actinidia deliciosa*) cysteine protease on growth and survival of *Spodoptera litura* larvae (Lepidoptera : Noctuidae) fed with control or transgenic avidin-expressing tobacco, *N. Z. J. Crop Hortic. Sci.*, 2005, 33, 99–105.
- 4 N. J. Nieuwenhuizen, L. L. Beuning, P. W. Sutherland, N. N. Sharma, J. M. Cooney, L. R. F. Bielecki, R. Schrodera, E. A. MacRae and R. G. Atkinson, Identification and characterisation of acidic and novel basic forms of actinidin, the highly abundant cysteine protease from kiwifruit, *Funct. Plant Biol.*, 2007, 34, 946–961.
- 5 A. Wallace, S. Eady, L. Drummond, D. Hedderley, J. Ansell and R. Gearry, A Pilot Randomized Cross-Over Trial to Examine the Effect of Kiwifruit on Satiety and Measures of Gastric Comfort in Healthy Adult Males, *Nutrients*, 2017, 9(7), 639–649.
- 6 H. Martin, Proteinase Activities of Kiwifruit, Pineapple and Papaya Using Ovalbumin, Soy Protein, Casein and Bovine Serum Albumin as Substrates, *J. Food Nutr. Res.*, 2017, 5, 214–225.
- 7 A. C. Arcus, Proteolytic enzyme of *Actinidia chinensis*, *Biochim. Biophys. Acta*, 1959, 33, 242–244.
- 8 I. Nishiyama, Fruits of the actinidia genus, *Adv. Food Nutr. Res.*, 2007, 52, 293–324.
- 9 J. Charney and R. M. Tomarelli, A Colorimetric Method for the Determination of the Proteolytic Activity of Duodenal Juice, *J. Biol. Chem.*, 1947, 171, 501–505.
- 10 L. J. Jones, R. H. Upson, R. P. Haugland, N. PanchukVoloshina, M. J. Zhou and R. P. Haugland, Quenched BODIPY dye-labeled casein substrates for the assay of protease activity by direct fluorescence measurement, *Anal. Biochem.*, 1997, 251, 144–152.

- 11 H. Martin, Quantification of Functional Actinidin in Whole Kiwifruit Extract Using the Selective Cysteine Proteinase Inhibitor E-64, *J. Food Nutr. Res.*, 2016, **4**, 243–250.
- 12 R. Maddumage, N. J. Nieuwenhuizen, S. M. Bulley, J. M. Cooney, S. A. Green and R. G. Atkinson, Diversity and Relative Levels of Actinidin, Kiwellin, and Thaumatin-Like Allergens in 15 Varieties of Kiwifruit (Actinidia), *J. Agric. Food Chem.*, 2013, **61**, 728–739.
- 13 M. Ha, A. E. D. Bekhit, A. Carne and D. L. Hopkins, Characterisation of commercial papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat proteins, *Food Chem.*, 2012, **134**, 95–105.
- 14 H. Martin, S. B. Cordiner and T. K. McGhie, Kiwifruit actinidin digests salivary amylase but not gastric lipase, *Food Funct.*, 2017, **8**, 3339–3345.
- 15 D. Freitas, S. Le Feunteun, M. Panouille and I. Souchon, The important role of salivary alpha-amylase in the gastric digestion of wheat bread starch, *Food Funct.*, 2018, **9**, 200–208.
- 16 F. S. Atkinson, D. Hancock, P. Petocz and J. C. Brand-Miller, The physiologic and phenotypic significance of variation in human amylase gene copy number, *Am. J. Clin. Nutr.*, 2018, **108**, 737–748.
- 17 D. Freitas, F. Boue, M. Benallaoua, G. Airinei, R. Benamouzig and S. Le Feunteun, Lemon juice, but not tea, reduces the glycemic response to bread in healthy volunteers: a randomized crossover trial, *Eur. J. Nutr.*, 2021, **60**, 113–122.
- 18 I. A. Jayawardana, M. J. Boland, K. Higgs, M. Zou, T. Loo, W. C. McNabb and C. A. Montoya, The kiwifruit enzyme actinidin enhances the hydrolysis of gluten proteins during simulated gastrointestinal digestion, *Food Chem.*, 2021, 341.
- 19 M. R. Perez-Gregorio, R. Dias, N. Mateus and V. de Feitas, Identification and characterization of proteolytically resistant gluten-derived peptides, *Food Funct.*, 2018, **9**, 1726–1735.
- 20 T. Suganuma, Y. Maeda, K. Kitahara and T. Nagahama, Study of the action of human salivary alpha-amylase on 2-chloro-4-nitrophenyl alpha-maltotriose in the presence of potassium thiocyanate, *Carbohydr. Res.*, 1997, **303**, 219–227.
- 21 C. L. Ladner, J. Yang, R. J. Turner and R. A. Edwards, Visible fluorescent detection of proteins in polyacrylamide gels without staining, *Anal. Biochem.*, 2004, **326**, 13–20.
- 22 H. Zare, A. A. Moosavi-Movahedi, M. Salami, M. Mirzaei, A. A. Saboury and N. Sheibani, Purification and autolysis of the ficin isoforms from fig (*Ficus carica* cv. Sabz) latex, *Phytochemistry*, 2013, **87**, 16–22.
- 23 E. Untersmayr, The influence of gastric digestion on the development of food allergy, *Rev. Fr. Allergol.*, 2015, **55**, 444–447.
- 24 K. Schalk, C. Lang, H. Wieser, P. Koehler and K. A. Scherf, Quantitation of the immunodominant 33-mer peptide from alpha-gliadin in wheat flours by liquid chromatography tandem mass spectrometry, *Sci. Rep.*, 2017, **7**, 1–12.