

Analysis of green kiwi fruit (*Actinidia deliciosa* cv. Hayward) proteinases by two-dimensional zymography and direct identification of zymographic spots by mass spectrometry

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Abstract

BACKGROUND: Proteinases present in kiwi fruits are potentially allergenic enzymes belonging to the papain family of cysteine proteinases. Actinidin is a prominent kiwi enzyme. The study of kiwi proteinases is important for the follow-up of fruit maturation, a deeper insight in the allergenic properties of individual proteins, and the application of kiwi proteinases for meat tenderisation and other industrial purposes.

RESULTS: Kiwi crude extracts were analysed by two-dimensional zymography on gelatin-containing gels. The digestion by the reactivated proteolytic enzymes after electrophoresis resulted in insights into kiwi proteinases. A mixture of several enzyme isotypes with the same pI but different molecular mass was observed. Clear spots, corresponding to the proteolytic activities, were excised, digested with trypsin, and submitted to MALDI-ToF mass spectrometry for protein identification. The most representative enzyme was actinidin.

CONCLUSIONS: The innovative achievements of the present study are the: (1) two-dimensional zymographic map of kiwi gelatinases without the need for extensive purification; and (2) direct identification of proteinase isotypes by means of direct MALDI-ToF MS analysis of the zymographic spots.

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Keywords: kiwi fruit; proteinases; actinidin; 2D zymography; MALDI-ToF mass spectrometry

INTRODUCTION

Kiwi fruit (*Actinidia deliciosa* and *Actinidia chinensis*), a sweet fruit cultivated in many countries, such as New Zealand, Italy, Chile, Japan, Greece and France, has significant effects on human health. It protects against oxidative DNA damage,¹ has cardiovascular protective properties,² is cytotoxic for tumour cell lines and has antimicrobial activities³ and laxation properties in elderly people.⁴ Actinidin preparations were also used in the food industry for tenderising meat.^{5,6}

Depending on study cohorts, it is assumed that kiwi fruit allergy is a relative risk,^{7–9} and some individuals have to avoid consumption of kiwi fruit.^{7,8} The most common symptoms include itching of the mouth and palate, as well as wheezing or collapsing. Twelve kiwi fruit allergens have been revealed, and only a few of these have been characterised. The major allergens of kiwi fruit are: actinidin,⁷ a thaumatin-like protein,¹⁰ and kiwellin,¹¹ designated Act d 1, Act d 2 and Act d 5, respectively.

Actinidin is a prominent proteolytic enzyme of kiwi fruit (EC 3.4.22.14). It belongs to the papain-like family of cysteine proteinases. Actinidin is secreted in an inactive form as a 39 kDa zymogen made of 380 amino acids. The mature enzyme, with a molecular mass of 30 kDa and pI 3.5,⁷ is obtained after cleavage of the peptide 1–126.

Many authors claim that actinidin is the major allergen in kiwi fruit,^{7,8,10} but some studies carried out in the United Kingdom on patients who are allergic to kiwi fruit have suggested that the major allergen in kiwi fruit is a 38 kDa protein and not actinidin.¹²

To gain a deeper insight into the properties of kiwi proteinases without the need to purify these, we applied the crude extracts of kiwi fruits to two-dimensional zymography (2D-Z). The corresponding enzymatic map was analysed by matrix-assisted laser desorption ionisation time-of-flight (MALDI-ToF) mass spectrometry (MS). Direct analysis of the clear, unstained, zymographic spots was achieved only in the case of the most prominent spots, where more enzyme was present and enzyme activity was higher.

In 2D-Z, proteinases are separated in the first dimension according to their pI and in the second dimension according to their Mr in a sodium dodecyl sulfate (SDS)-containing polyacrylamide gel co-polymerised with an appropriate protein

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substrate (here gelatin). Proteolytic enzymes, their isoforms and post-translational isomers present in the extract are detected as clear, unstained, spots on a dark blue background, after removal of SDS and reactivation. On these grounds, 2D-Z may represent a useful technique to detect the whole pattern of proteolytic enzymes present in the sample under study.

In the present work we have detected the presence of several actinidin isotypes in the kiwi fruit and have identified most of these without the need to have parallel 2D electrophoresis (2D-E) runs. This is a novel approach since in previous 2D-Z studies, proteolytic activities were identified by MS analysis only by using the corresponding 2D-E gels.^{13–19}

MATERIALS AND METHODS

Chemicals

All reagents used were of the highest grade and were purchased from Sigma-Aldrich (St Louis, MO, USA), Carlo Erba (Milan, Italy), Bio-Rad Laboratories (Segrate, Italy) and GE Healthcare (Uppsala, Sweden).

Enzyme extraction

Kiwi fruits (*Actinidia chinensis* var *deliciosa* cv. Hayward) used in this study were purchased in a local fruit market (Potenza, Italy). Three independent extracts were obtained from 80 g of kiwi fruits. Fresh fruits were homogenised (1:1 wt/vol) in 10 mmol L⁻¹ potassium phosphate buffer pH 7.0 in a cold Waring blender and the resulting homogenates were centrifuged for 20 min and 1 °C at 40 000 × g. A small aliquot of the supernatants was used for immediate enzyme activity measurements. The remaining samples were lyophilised and stored at -80 °C.

Assessment of proteolytic activity

Protein concentration was determined according to the method of Bradford²⁰ and the Bio-Rad reagent using bovin albumin as standard protein. Total proteolytic activity was assessed by using the spectrophotometric method based on azocasein. Briefly, 50 µL of the enzymatic extracts were added to 0.5 mL of 1.5 g L⁻¹ azocasein (Sigma), 2 mmol L⁻¹ DTT, 5 mmol L⁻¹ cysteine, in 40 mmol L⁻¹ phosphate buffer pH 6.8 at 30 °C. The reaction was stopped after 15 min by the addition of 0.55 mL 100 g L⁻¹ trichloroacetic acid and centrifuged for 6 min at 8000 rpm (Amicon microcentrifuge MC-13; Amicon, Beverly, MA, USA). The supernatants were separated from the undigested substrate and the absorbance at 440 nm of the released dye was recorded. One unit of total proteolytic activity (U) was defined as the amount of enzyme yielding 0.1 unit of absorbance per min at 440 nm under the assay conditions. The assay included an appropriate blank, in which TCA was added before the substrate. The proteolytic specific activity was reported as U mg⁻¹ protein.

To determine the effects of specific proteinases inhibitors (Sigma) on total proteinase activity, the extracts were incubated with *trans*-epoxysuccinyl-leucylamide-4-guanidinyl-butane (E-64) (200 µmol L⁻¹ in ethanol) or iodoacetamide (200 mmol L⁻¹ in water) for cysteine proteinases; pepstatin A (200 µmol L⁻¹ in methanol) for aspartic proteinases; phenylmethylsulfonyl fluoride (PMSF) (20 mmol L⁻¹ in ethanol) for serine proteinases and 1,10-phenanthroline (200 mmol L⁻¹ in ethanol) for metallo-proteinases. Aliquots of 5 µL of each inhibitor stock solution were mixed separately with 45 µL of enzyme extract, incubated for 1 h at 25 °C and then added to azocasein. Final concentrations of the

inhibitors were 20 µmol L⁻¹ E-64, 20 mmol L⁻¹ iodoacetamide, 20 µmol L⁻¹ pepstatin, 2 mmol L⁻¹ PMSF and 20 mmol L⁻¹ 1,10-phenanthroline, respectively. Control assays contained only the solvent used to dissolve the inhibitor. Residual activity was calculated in relation to uninhibited activity.

One-way ANOVA was carried out to test for significant differences, results were considered to be statistically significant at $P < 0.05$.

SDS-PAGE and one-dimensional gelatin zymography analysis

SDS-PAGE was carried out using a 4% stacking gel and 12% running gel under reducing and non-reducing conditions. Aliquots of kiwi fruit extracts containing 15 µg of proteins were mixed with the sample buffer (40 g L⁻¹ SDS, 120 mL L⁻¹ glycerol, 0.1 g L⁻¹ bromophenol blue, 50 mmol L⁻¹ Tris-HCl at pH 6.8) containing 20 mL L⁻¹ β-mercaptoethanol or not. Electrophoresis was performed at 4 °C at 150 V constant using a mini-Protean II apparatus (Bio-Rad Laboratories). The molecular mass of proteinases in kiwi fruit extract was estimated by comparison with the low range standard protein kit of Bio-Rad. The protein bands were visualised by staining with 0.1% Coomassie brilliant blue R-250 and the gel was scanned using a ScanMaker 9800 XL-Microtek (Hsinchu, Taiwan) in a grey scale.

Proteolytic activities in kiwi fruit extract were detected by gelatin gel zymography. Aliquots of extract containing 8 µg of proteins were supplemented with 15 µL of electrophoresis non-reducing sample buffer. Samples were then separated under non-reducing conditions in a 120 g L⁻¹ polyacrylamide gel co-polymerised with 1 g L⁻¹ gelatin. Stacking gels contained 40 g L⁻¹ polyacrylamide. Electrophoresis was carried out in the same conditions described before for SDS-PAGE. Low range prestained standard proteins (Bio-Rad) were used as molecular weight markers. After electrophoresis, the gels were washed (2 × 40 min) in 25 g L⁻¹ Triton X-100, 40 mmol L⁻¹ phosphate buffer pH 6.0 (washing buffer) in order to remove SDS, then incubated for 14 h at room temperature in developing buffer: 10 g L⁻¹ Triton X-100, 40 mmol L⁻¹ phosphate buffer pH 6.0, 4 mmol L⁻¹ DTT, 10 mmol L⁻¹ cysteine, in the absence and presence of specific inhibitors (20 µmol L⁻¹ for E-64 and 20 mmol L⁻¹ for iodoacetamide, respectively). For the development of enzyme activity, the gels were stained with Coomassie brilliant blue R-250, destained in methanol-acetic acid-H₂O and scanned using an ImageMaster DTS (Pharmacia Biotech, Uppsala, Sweden) scanner. Proteolytic activities were detected as clear, unstained bands on a blue background.

Gelatin two-dimensional zymography

Proteinase activities present in kiwi fruit extracts were detected by gelatin 2D zymography. Samples were applied under non-reducing conditions. Aliquots of kiwi extracts (45 µg of proteins) were mixed with the rehydration solution containing 7 mol L⁻¹ urea, 2 mol L⁻¹ thiourea, 20 g L⁻¹ CHAPS, 5 mL L⁻¹ Immobilized pH Gradient (IPG) buffer, plus a trace of bromophenol blue, to a final volume of 450 µL. Isoelectrofocussing (IEF) was performed on IPG Dry-Strips of 24 cm in a non-linear pH gradient of 3–11 (pH 3–11 NL) (GE Healthcare). IPG Dry-Strips were rehydrated with a sample-containing rehydration solution for 12 h at 20 °C. IEF was run using an IPGphor unit (Amersham Biosciences, Uppsala, Sweden) at 20 °C for a total of 37 900 V h (200 V for 1 h, 500 V for 2 h, 1000 V for 3 h, 1000–8000 V linear gradient in 30 min, and 8000 V for 4 h).

After IEF, IPG strips were equilibrated for 20 min by gentle shaking in equilibration buffer: 6 mol L⁻¹ urea, 300 mL L⁻¹

glycerol, 20 g L⁻¹ SDS, 50 mmol L⁻¹ Tris-HCl, pH 8.8. In the second dimension, proteins were separated in a 120 g L⁻¹ polyacrylamide gel co-polymerised with 1 g L⁻¹ gelatin. The separation was carried out on the Ettan DALT II system (Amersham Biosciences) at 15 °C first for 30 min at 240 V and then for 5 h at 350 V. Prestained low-range standard proteins (Bio-Rad) were used as molecular weight markers. After electrophoresis, gels were washed twice (2 × 40 min) in washing buffer: 25 g L⁻¹ Triton X-100, 40 mmol L⁻¹ phosphate buffer pH 6.0 in order to remove SDS, then incubated for 14 h at room temperature in developing buffer: 10 g L⁻¹ Triton X-100, 40 mmol L⁻¹ phosphate buffer pH 6.0, 4 mmol L⁻¹ DTT, 10 mmol L⁻¹ cysteine in the absence and presence of specific inhibitors E-64. For the development of enzymatic activities, the gels were stained with Coomassie brilliant blue R-250 (for 30 min at room temperature) and destained in methanol–acetic acid–H₂O. Proteolytic activity was detected as a clear, unstained, spot on a blue background.

Gels were scanned using a ScanMaker 9800 XL-Microtek in a grey scale. The images were digitally inverted and the digested spots were reported as positive values.

Image analysis for the determination of apparent Mr and pI, was carried out using the ImageMaster 2D Elite V.2002.01 software (Amersham Biosciences). Proteolytic activity was detected as clear, unstained spots on a blue background and was quantified by densitometric analysis using the ImageMaster 2D Elite software. Proteolytic activity of the digested spots was expressed as relative activity (reported as the mean of three gels). The individual digested spot volumes were normalised as a percentage of the total volume in all of the digested spots present in the gel. Gels were matched and spots present in at least two zymograms of the three gels were selected for protein identification by MALDI-ToF MS analysis.

Non-reducing two-dimensional electrophoresis

Samples preparation for 2D-E analysis of kiwi extracts was carried out as follows: 800 µL of methanol were added to 200 µL of kiwi juice (240 µg of proteins) and mixed for 1 min, successively 200 µL of chloroform and 600 µL of deionised water were added, respectively; the mixture was vortex for 2 min and centrifuged for 2 min at 13 000 × g (Amicon microcentrifuge MC-13). The upper phase was discarded, then 800 µL of methanol were added and after mixing the mixture was centrifuged. The resulting pellet was washed with 400 µL of deionised water and with 1.6 mL of ice-cold methanol and centrifuged for 2 min at 13 000 × g. The obtained pellet was dried speed-vac for 15 min and redissolved in 450 µL of non-reducing rehydration solution.

Two-dimensional electrophoresis analysis was carried out in the same conditions as described for gelatin two-dimensional zymography. Gels were stained with colloidal Coomassie G-250, destained and digitised as 300 dpi, 16 bit TIFF images and imported to the ImageMaster 2D Elite software (Amersham Biosciences).

Direct analysis of zymographic spots by MALDI-ToF MS

After staining with Coomassie blue and the development of the clear, unstained spots, corresponding to the kiwi proteinases activity, these spots were excised from 2D gelatin zymography and sonicated in 50 µL 25 mmol L⁻¹ NH₄HCO₃ for 5 min in a cold water-bath. Then, the ammonium bicarbonate was discarded and the clear spots were sonicated once again in 50 µL 25 mmol L⁻¹ NH₄HCO₃. Subsequently, the spots were washed with 25 mmol L⁻¹ NH₄HCO₃–acetonitrile (1 : 1) followed

by dehydration with acetonitrile. Proteins were reduced in 10 mmol L⁻¹ DTT–25 mmol L⁻¹ NH₄HCO₃ for 45 min at 55 °C, and alkylated in 55 mmol L⁻¹ iodoacetamide–25 mmol L⁻¹ NH₄HCO₃ for 30 min at room temperature (in the dark). The gel slices were washed with 25 mmol L⁻¹ NH₄HCO₃, dehydrated with acetonitrile, and digested with trypsin (Promega, Madison, WI, USA) overnight at 37 °C. For each spot, three digested supernatants were pooled (30 µL each), dried speed-vac, resuspended with 5 µL of 1 mL L⁻¹ trifluoroacetic acid and then concentrated on µZipTips (Millipore, Bedford, MA, USA), according to the manufacturer's instructions. The peptide mixtures were eluted with 0.8 µL of matrix solution: 10 g L⁻¹ α-cyano-4-hydroxy-cinnamic acid in acetonitrile–water (1 : 1 v/v) acidified with 5 mL L⁻¹ trifluoroacetic acid and applied in duplicate directly onto target, allowed to air dry, and analysed by MALDI-ToF MS. Mass spectra were acquired in positive reflectron mode at 20 kV using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences) equipped with a UV–nitrogen laser (337 nm) with delayed extraction mode and low mass rejection. For each spectrum 256 single shots were accumulated. Peptide spectra were calibrated using two standard peptides (ile-7AngIII, M+H 897.531, monoisotopic, and hACTH 18–39, M+H 2465.191, monoisotopic). Protein identification was performed by the MASCOT search engine (<http://www.matrixscience.com>) against the NCBI nr protein and Swiss Prot databases using peptide mass fingerprinting (PMF). The following parameters were used for database search: (1) taxonomy group: Viridiplantae (green plants); (2) mass tolerance of 0.2 Da, (3) one missed tryptic cleavage allowed, (4) carboamidomethylation of cysteine (as a fixed modification) and (5) oxidation of methionine (as a variable modification). Proteins were identified by MASCOT using the probability based MOWSE score, equal to $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Protein scores of >53 were considered statistically significant ($P < 0.05$) under the selected variables.

Protein bands and spots were excised from the SDS-PAGE and 2D-E gels, respectively, washed twice with 25 mmol L⁻¹ NH₄HCO₃–acetonitrile (1 : 1) and dehydrated with acetonitrile. Successively, the protein spots were reduced, alkylated and digested in-gel with trypsin. Two microlitres of each generated peptide mixture was mixed with 2 µL of matrix solution. Subsequently, 0.3 µL of each matrix–peptide mixture, was applied in duplicate to a target disk and allowed to air dry and analysed by MALDI-ToF MS.

RESULTS

Assessment of total proteolytic activity extracted from kiwi fruits and its inhibition

Results obtained by measuring total proteolytic activity with azocasein are shown in Table 1. Studies carried out in the presence of specific inhibitors did not show any inhibition by pepstatin, PMSF and phenanthroline, specific inhibitors of aspartic, serine and metallo-proteinases, respectively, whereas the inhibitors of cysteine proteinases E-64 and iodoacetamide were effective. E-64 was much more effective (92.9% ± 4.9%) than iodoacetamide (71.1% ± 6.6%).

SDS-PAGE and one-dimensional zymographic analysis

SDS-PAGE analysis of the kiwi fruit extracts was performed under both reducing and non-reducing conditions (Fig. 1). Clearly, the apparent molecular masses of proteins can be determined by

Table 1. Inhibition of proteolytic activity extracted from kiwi fruit

Total proteolytic activity ^a (U mg ⁻¹)	Inhibitor	Inhibition (%) ^b
–	E-64	92.9 ± 4.9*
2.5 ± 0.2	Iodoacetamide	71.1 ± 6.6*
–	Pepstatin	ND
–	PMSF	ND
–	1,10-Phenanthroline	ND

^a Total proteolytic activity was expressed in U mg⁻¹ of proteins, where U corresponds to the amount of enzyme yielding 0.1 unit of absorbance at 440 nm per min (pH 6.8, temperature of 25 °C), proteolytic activity was assessed on azocasein in solution.

^b Effect of specific inhibitors on total proteolytic activity. Residual activity was calculated in relation to uninhibited activity (mean ± SD, *n* = 6).

* Indicates *P* < 0.05.

ND, not detected.

Table 2. Densitometric analysis of the digested spots detected by 2D zymography

Spot number	Relative activity ^a (normalised volume)	pI _{Exp} /MW _{Exp}	pI _{Theor} ^b /MW _{Theor}
1	21.439	3.92/33.83	–
2	53.457	3.92/32.20	4.37/27.40
3	25.104	3.92/31.42	–

^a Proteolytic activity of the digested spots was expressed as relative activity.

Relative activity is intended as the ratio, expressed as a percentage, between the intensity of each digested spot and the sum of all digested spots detected in the gel. Since the spot intensities are calculated by the software in terms of volumes, relative activities can be expressed also as normalised volumes. Values are reported as mean of three independent experiments. Image analysis was carried out using the ImageMaster 2D Elite V. 2002.01 software (Amersham Biosciences).

^b The computation (pI/MW) has been carried out on the mature chain sequences at SwissProt.

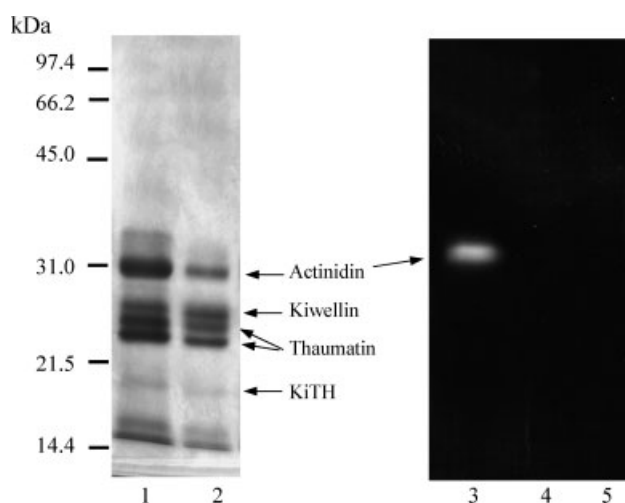


Figure 1. 1D SDS polyacrylamide gel electrophoresis and 1D zymography of kiwi fruit extract. Lanes 1–2: SDS-PAGE (120 g L⁻¹ polyacrylamide) of kiwi extract under reducing and non-reducing conditions, respectively; 15 µg of proteins were applied to each lane. Lanes 3–5: gelatin zymograms of kiwi fruit extract, 8 µg of proteins were applied to each lane. Zymograms were carried out under non-reducing conditions on 120 g L⁻¹ polyacrylamide co-polymerised with 1 g L⁻¹ gelatin, in the absence (lane 3) and the presence of specific inhibitors: 20 µmol L⁻¹ for E-64 (lane 4) and 20 mmol L⁻¹ for iodoacetamide (lane 5), respectively.

SDS-PAGE only under reducing conditions. The protein pattern of the kiwi fruit extracts were similar in the presence (lane 1) of β-mercaptoethanol, a reducing agent, or in its absence (lane 2). In the sample analysed in reducing conditions (lane 1), an additional band with a molecular mass above 30 kDa was observed. In both runs, the presence of six protein bands in the range of 30–17 kDa was observed. Bands obtained in reducing conditions showed a lower electrophoretic migration than those separated in non-reducing conditions. Both runs showed a major band at 30 kDa that was identified by MALDI-ToF MS analysis as actinidin (AC P00785), the most abundant protein present in the edible part of kiwi fruit. Three additional protein bands were clearly resolved in the range 26.5–24 kDa. The upper band of 26 kDa was identified as kiwellin (AC P84527) while the bands of 25 and 24 kDa were found to correspond to the thaumatin-like protein (AC P81370).

A band of 20 kDa was identified as KiTH,²¹ a protein obtained by *in vivo* proteolytic cleavage of kiwellin by the actinidin. The lower band of 17 kDa could correspond to the propeptide of actinidin.⁷

Analysis by mono-dimensional gelatin zymography revealed the presence in the extract (lane 3) of one band with apparent molecular mass of 33.5 kDa corresponding to the actinidin. This band disappeared in presence of the specific inhibitor of cysteine proteinases E-64 and iodoacetamide, as shown in lanes 4 and 5, respectively.

Two-dimensional zymographic analysis

To detect all the proteolytic enzymes extracted from the kiwi fruit, non-reducing 2D gelatin zymography was performed. The crude extracts were analysed in the first dimension (IEF) according to their pI in non-linear pH gradient of 3–11 and in the second dimension by their Mr in a 120 g L⁻¹ polyacrylamide gel (SDS-PAGE) co-polymerised with 1 g L⁻¹ gelatin.

In preliminary runs, the IEF performed in the presence of 4 mol L⁻¹ urea and 2 mol L⁻¹ thiourea gave only poor resolved spots after digestion of the substrate (Fig. 2A) in the acidic region of the gel at pI = 4. To improve resolution of the digested spots, IEF was performed using 7 mol L⁻¹ urea and 2 mol L⁻¹ thiourea in the rehydration solution.

Analysis of the 2D gelatin zymogram revealed the presence of three digested spots with apparent molecular masses of 33.8, 32.2 and 31.4 kDa, respectively, and a pI of 3.92 (Fig. 2B). The highest activity was detected in spot 2 (Table 2). All spots disappeared in the presence of the specific inhibitor E-64 in developing buffer (Fig. 2C), indicating the presence of cysteine proteases in kiwi extracts.

A non-reducing 2D-E was run in parallel (Fig. 2D). The spots detected by 2D-E were excised from the gel and identified by MALDI-ToF MS analysis.

Numbered spots were identified as actinidin isoforms. In particular spots 1–3 with molecular masses of 31.2, 30.3 and 29.3 kDa, respectively, and a pI of 3.95 were found to correspond to the acidic actinidin (AC P00785), while spot 4 with Mr of 23.9 kDa and pI of 9.54 was identified as the basic actinidin Act2a (AC ABQ10192). Boxed and circled spots were identified as kiwellin and thaumatin-like proteins isoforms, respectively.

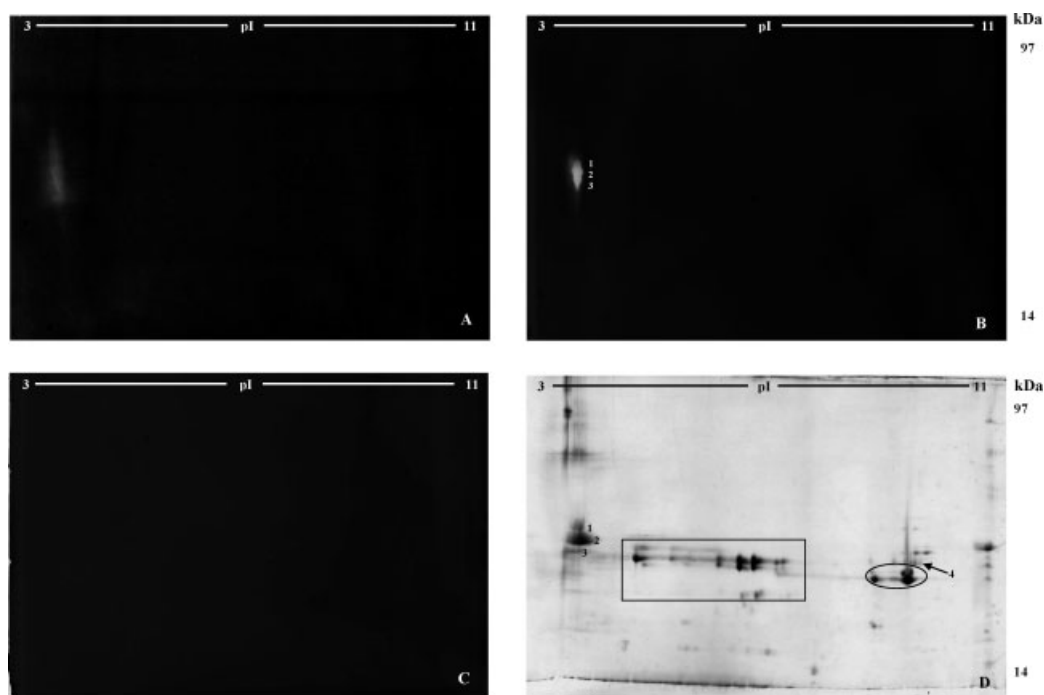


Figure 2. Detection by two-dimensional zymography (2D-Z) of the proteolytic activities extracted from kiwi fruit and comparison with 2D-E analysis. Proteolytic activities were detected by 2D zymography on gels co-polymerised with gelatin (A–C). In (D), 2D-E shows the corresponding protein pattern in absence of the substrate in the gel. Gels were stained with Coomassie blue. IPG-strips before IEF were rehydrated in the presence of 4 mol L^{-1} urea and 2 mol L^{-1} thiourea (gel A) or in presence of 7 mol L^{-1} urea and 2 mol L^{-1} thiourea (gel B and gel C). In (C) gel was incubated with the specific inhibitor E-64. Numbers (gel B) indicate the proteinases identified by MALDI-ToF MS, as listed in Table 3. In gel D, numbered spots correspond to the actinidin isoforms while boxed and circled spots correspond to kiwelin and thaumatin-like proteins isoforms, respectively.

Enzyme identification by MALDI-ToF MS

To identify the proteolytic activities observed in 2D zymograms, the spots were digested with trypsin and applied to MALDI-ToF mass spectrometry. Each spot was present at least in two zymograms of three independent gels of 2D gelatin zymography.

By applying the MASCOT search engine, all the spots detected in the 2D gelatin zymography (Fig. 2B) were found to correspond to different forms of actinidin, the major enzyme in kiwi fruit.

Spots were identified with an average sequence coverage of 27.0% of the mature chain amino acid sequence. The 1035 Da peptide, corresponding to the N-terminal fragment 1–8 (LPSYVDWR) of actinidin, was found in all spots. Matched peptides with corresponding molecular masses are listed in Table 3.

The digested spots detected in 2D gelatin zymograms showed a molecular mass higher than the corresponding spots in 2D-E. These results are in agreement with those obtained by 1D gelatin zymography in which the proteolytic band corresponding to actinidin showed a molecular mass of 33 kDa as compared with the protein band (30 kDa) detected by SDS-PAGE.

The over-estimation of the molecular masses observed when gelatin was incorporated into the gel might be due to the interaction of actinidin with gelatin during the electrophoretic run and to the consequent reduction of the migration rate of proteins. This hypothesis is in accord with reports by other authors indicating that the inclusion of gelatin in polyacrylamide gels reduces the migration rate of proteins by 15–20%.²²

In the 2D zymography any digested spot corresponding to the basic actinidin was detected. This could be ascribed to its low expression level. Indeed, as clearly shown in 2D-E gel (Fig. 2D), the spot corresponding to basic actinidin (spot 4) is present at very low level with respect to the acidic actinidins (spots 1–3).

The identification by MALDI-ToF MS of the digested spots was initially not satisfactory due to the low amount of peptides in the trypsin digested supernatants and to the presence of interfering peptides in the spectra derived from the protein substrate in the resolving gel. To overcome this problem, each digested spot was excised in triplicate from three independent gels and the corresponding trypsin-digested supernatants were pooled and concentrated as described in the materials and methods section. In the meantime, the amount of interfering gelatin peptides was reduced by applying two sonication steps to the spot before trypsin digestion.

Figure 3A, B and C shows the MALDI-ToF mass spectra of the three spots identified in 2D gelatin zymography (Fig. 2B) as actinidin. Peaks with an arrow correspond to the identified peptides, whereas those with SP superscripts correspond to the standard peptides ile-7AngIII and hACTH 18–39 with molecular masses of 897.531 and 2465.191 Da, respectively.

DISCUSSION

The aim of this paper was to develop a procedure based on 2D zymography and mass spectrometry to achieve a complete map of the proteolytic enzymes present in kiwi fruits and their direct identification from zymographic gels. Our results show that actinidin is actually a mixture of several isotypes with the same pI but different molecular mass.

It is possible to formulate various hypotheses to explain the presence of multiple spots identified as actinidin in the 2D-Z gel (Fig. 2B). The observed spots could be due to alternative splicing or to a combination of post-translational modifications. Alternative splicing forms of kiwi actinidin mRNAs encoding for proteins of

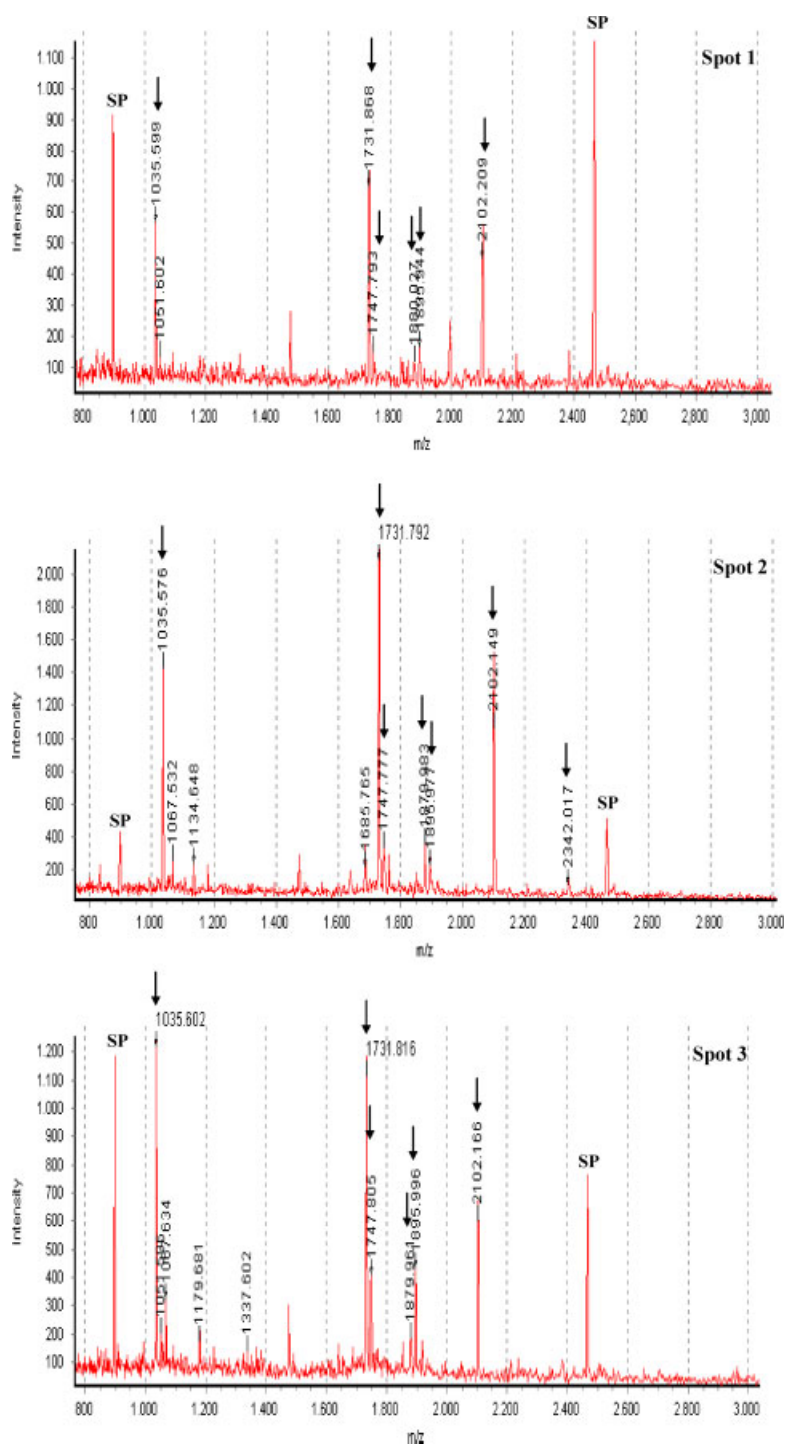


Figure 3. MALDI-ToF MS spectra of the peptides obtained by digestion of zymographic spots. MALDI-ToF mass spectra of spots 1–3 detected in 2D-Z gelatin. Spots were excised from the gel in triplicate, subjected to two sonication steps and digested with trypsin. The digested supernatants were pooled, concentrated and then analysed by MS. Arrows: kiwi fruit actinidin matching peptides. SP, standard peptides.

different molecular weight are not described in the literature. On the contrary, ten different actinidin mRNAs were identified encoding mature proteins with similar molecular mass (about 24 kDa), but with different pI's ranging from 3.9 to 9.3.²³

Changes in the molecular mass could be due to glycosylation or proteolytic breakdown. However, since actinidin appears to be an unglycosylated protein,⁷ the existence of multiple spots with

the same pI could be ascribed to autodigestion and formation of actinidin fragments.

An important aspect to be taken in consideration in the present work is the demonstration that 2D zymography can be directly coupled with MALDI-ToF mass spectrometry, assuming that the enzyme content is not too low.

Table 3. Identification of cysteine proteinases extracted from kiwi fruit and detected by 2D gelatin zymography^a

Spot number	Protein name	Accession number	Peptides matched	Fragment	Identified peptides ^b	(%)Cov ^c	Mascot score	Observed	Mr(expt)	Mr(calc)	Delta
1	Actinidin	P00785	6	1–8	LPSYVDWR	24	77	1035.5990	1034.5917	1034.5185	0.0732
				40–58	ITGVLSLSEQLIDCGR			2102.2090	2101.2017	2101.1089	0.0928
				182–195	NSWDTTWGEEGYMR			1731.8680	1730.8607	1730.6995	0.1612
				182–195	NSWDTTWGEEGYMR (Ox. Met)			1747.7930	1746.7857	1746.6944	0.0913
				199–217	NVGGAGTCGIATMPSYPVK			1880.0270	1879.0197	1878.8968	0.129
				199–217	NVGGAGTCGIATMPSYPVK (Ox. Met)			1895.9440	1894.9367	1894.8917	0.0450
2	Actinidin	P00785	7	1–8	LPSYVDWR	33	86	1035.5760	1034.5687	1034.5185	0.0502
				18–39	SQEGCGCWAFSAITVEGINK			2342.0170	2341.0097	2341.0467	–0.0370
				40–58	ITGVLSLSEQLIDCGR			2102.1490	2101.1417	2101.1089	0.0328
				182–195	NSWDTTWGEEGYMR			1731.7920	1730.7847	1730.6995	0.0852
				182–195	NSWDTTWGEEGYMR (Ox. Met)			1747.7770	1746.7697	1746.6944	0.0753
				199–217	NVGGAGTCGIATMPSYPVK			1879.9830	1878.9757	1878.8968	0.0789
3	Actinidin	P00785	6	1–8	NVGGAGTCGIATMPSYPVK (Ox. Met)	24	59	1895.9770	1894.9697	1894.8917	0.0780
				1–8	LPSYVDWR			1035.6020	1034.5947	1034.5185	0.0762
				40–58	ITGVLSLSEQLIDCGR			2102.1660	2101.1587	2101.1089	0.0498
				182–195	NSWDTTWGEEGYMR			1731.8160	1730.8087	1730.6995	0.1092
				182–195	NSWDTTWGEEGYMR (Ox. Met)			1747.8050	1746.7977	1746.6944	0.1033
				199–217	NVGGAGTCGIATMPSYPVK			1879.9610	1878.9537	1878.8968	0.0569
				199–217	NVGGAGTCGIATMPSYPVK (Ox. Met)			1895.9960	1894.9887	1894.8917	0.0970

^a Clear spots excised from 2D zymography were digested with trypsin and identified by MALDI-ToF MS analysis.^b Proteins were identified by MASCOT using the probability based MOWSE score (scores greater than 53 are significant at $P < 0.05$).^c Coverage was calculated respect to the mature chain sequence: f127–380.

At present, the application of 2D-Z has been limited to the investigation of the proteolytic enzymes present in human pure pancreatic juice,¹³ colon carcinoma,¹⁴ viperid snake venoms,¹⁵ plants,¹⁶ bacteria¹⁷ and marine organisms.^{18,19} In these papers, the identification of proteolytic enzymes was never achieved directly from the 2D zymographic spots but by comparison with classical 2D-E and the analysis of the corresponding 2D-E spots. Enzyme identification was based on the direct analysis of the enzymatic spots only in one case.²⁴ However, the study was not really based on a zymographic run; proteolytic enzymes were separated by two-dimensional electrophoresis and enzyme detection was performed with fluorogenic substrates, which were not co-polymerised with the gel.

The real innovation of the present study is to reveal all proteolytic activities by means of direct MALDI-ToF MS analysis of zymographic spots. This is an important step towards better 2D-E analysis of extracts obtained from a vegetal matrix, since plant extracts may contain several interfering substances such as polyphenols, carbohydrates and pigments that could make the analysis difficult.

Some limitations of 2D-Z may derive from: (1) under-estimation of the number of proteinases due to the poor degradation of substrate or to the failure to reactivate the enzymes after SDS-zymography; or (2) the irreversible enzyme inactivation by urea. However, this should not be the case of kiwi proteinases since enzymes of the papain superfamily show high stability in concentrated urea solution.²⁵ Finally, as clearly shown in Fig. 2B, actinidin isotypes were even better focused after increasing the concentration of urea in the rehydration buffer.

In conclusion, 2D-Z can offer not only the complete map of the enzymes present in a biological sample or in a food matrix, but it can allow the direct identification of the enzymes including their isotypes.

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