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PII: S0308-8146(14)00839-5
DOI: http://dx.doi.org/10.1016/j.foodchem.2014.05.123
Reference: FOCH 15906

To appear in: *Food Chemistry*

Received Date: 25 March 2014
Revised Date: 13 May 2014
Accepted Date: 22 May 2014

Please cite this article as: Karnjanapratum, S., Benjakul, S., Glycyl endopeptidase from papaya latex: Partial purification and use for production of fish gelatin hydrolysate, *Food Chemistry* (2014), doi: http://dx.doi.org/10.1016/j.foodchem.2014.05.123

# Glycyl endopeptidase from papaya latex: Partial purification and use for production of fish gelatin hydrolysate

Running title: Papaya glycyl endopeptidase and its use

To be submitted to Food Chemistry

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**ABSTRACT**

An aqueous two-phase system (ATPS) in combination with ammonium sulfate ($(NH_4)_2SO_4$) precipitation was applied to fractionate glycyl endopeptidase from the papaya latex of Red Lady and Khack Dum cultivars. ATPS containing polyethylene glycol (PEG 2000 and 6000) and salts ($(NH_4)_2SO_4$ and $MgSO_4$) at different concentrations were used. Glycyl endopeptidase with high purity fold (PF) and yield was found in the salt-rich bottom phase of ATPS with 10% PEG 6000-10% $(NH_4)_2SO_4$. When ATPS fraction from Red Lady cultivar was further precipitated with 40–60% saturation of $(NH_4)_2SO_4$, PF of 2.1-fold with 80.23% yield was obtained. Almost all offensive odorous compounds, particularly benzyl isothiocyanate, were removed from partially purified glycyl endopeptidase (PPGE). The fish gelatin hydrolysates prepared using PPGE showed higher ABTS radical scavenging activity and less odour, compared with those of crude extract (CE). Thus antioxidative gelatin hydrolysate with negligible undesirable odour could be prepared with the aid of PPGE.

*Keywords*: ATPS; papaya latex; glycyl endopeptidase; odorous compounds; gelatin hydrolysates; antioxidative activity

## 1. Introduction

*Carica papaya* is widely cultivated in tropical and subtropical regions all around the world. Apart from the edible fruits, enzymes stored in its lactiferous cells can be produced and have found several applications (de Oliveira & Vitória, 2011). When these cells rupture, the coagulation of latex occurs. This represents an important defence mechanism of the plant against pathogens and other harmful attacks. In addition, the latex of *C. papaya* is a rich source of cysteine endopeptidases, including papain, glycyl endopeptidase, chymopapain and caricain, constituting more than 80% of total enzymes (Azarkan, El Moussaoui, Van Wuytswinkel, Dehon, & Looze, 2003). Papaya latex was used for preparing protein hydrolysates with bioactivities (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2012; Ngo, Rye, Vo, Himaya, Wijesekara, & Kim, 2011). Due to the abundance of glycine in gelatin molecules, glycyl endopeptidase, a major component which constitutes almost 30% of total protein in the latex of *C. papaya*, can serve as a potential protease, which preferably cleaves the peptide bonds in gelatin. However, undesirable off-odour of crude papaya latex leads to the offensive odour or flavour in the resulting gelatin hydrolysates, thereby causing consumer rejection.

The volatile compounds of various papaya cultivars have been extensively investigated (Pino, Almora, & Marbot, 2003). Twenty-five odorants were considered as odour-active compounds and contribute to the typical papaya aroma. The pungent-sour and green-note odours found in the green fruit are due to benzyl isothiocyanate and some C6 compounds (e.g., 1-hexen-3-one), respectively (Pino, 2014). Ulrich and Wijiya (2010) found that stinky and smokey odours were caused by butanoic acid and benzyl isothiocyanate. Therefore, the crude enzyme obtained from green fruit latex might contain those odorous

compounds. When papaya latex proteases were employed, compounds contributing to offensive off-odour were present in the resulting gelatin hydrolysate, thus obstructing the extensive utilisation and consumption of hydrolysate.

Several separation techniques have been applied for protein concentration and purification. Membrane-aided filtration and other techniques can be effective in protein separation. However, the adverse effects of operating conditions can be associated with enzyme denaturation (Nakkeeran & Subramanain, 2010; Krstiic, Antov, Pericin, Hoflinger, & Tekic, 2007). Aqueous-two-phase system (ATPS) is a good choice, which offers mild and non-disruptive purification conditions for biomolecules, especially enzymes (Prinz, Zeiner, Vössing, Schüttmann, Zorn, & Górak, 2012). ATPS has been widely employed as an effective and economical process for the separation, purification and concentration of enzymes (Subathra, Jeevitha, & Deepa, 2012; Rawdkuen, Pintathong, Chaiwut, & Benjakul, 2011; Ketnawa and Rawdkuen, 2011). ATPS can remove the undesirable compounds present in the system including unidentified polysaccharides, interfering protein and contaminants (Dubey & Jagannadham, 2003). Chaiwut, Kanasawud and Halling (2007) used ATPS followed by salt precipitation for isolation of glycyl endopeptidase from papaya latex. Therefore, ATPS can be used to fractionate glycyl endopeptidase in papaya latex and remove offensive odorous compounds under appropriate condition. As a consequence, a more active fraction without undesirable odour could be prepared from papaya latex and further used as a processing aid in production of gelatin hydrolysate. The aims of the present study were to fractionate glycyl endopeptidase from papaya latex of Red Lady and Khack Dum cultivars grown in Thailand, using ATPS and ammonium sulfate precipitation, and to determine odorous compounds in the obtained fraction. Additionally, antioxidative activities and odorous compounds of hydrolysate prepared using glycyl-endopeptidase-rich fraction were determined.

## 2. Material and methods

### 2.1. Chemicals

Fish skin gelatin from tilapia was purchased from Lapi Gelatine S.p.A (Empoli, Italy). Polyethylene glycol (PEG) was obtained from Fluka (Buchs, Switzerland). 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), sodium dodecyl sulfate (SDS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4´,4´´-disulfonic acid sodium salt (ferrozine) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Methanol and trichloroacetic acid (TCA), ferrous chloride and iron standard solution were obtained from Merck (Darmstadt, Germany). Ammonium thiocyanate was purchased from Lab-Scan (Bangkok, Thailand). Coomassie Blue R-250, and *N,N,N´,N´*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA). Boc-Ala-Ala-Gly-pNA was obtained from Bachem Inc. (Torrance, CA). Low molecular weight marker was purchased from GE Healthcare UK, Limited (Buckinghamshire, UK). All chemicals were of analytical grade.

### 2.2 Preparation of crude extract from papaya (*C. papaya*) latex

Fresh papaya latex was collected from two cultivars (Red Lady and Khack Dum) in Hat Yai, Thailand. Four to six longitudinal incisions were made on the green papaya fruit using a stainless steel knife. The exuded latex was collected using a receiving container. The latex was then transferred to a beaker and stored below 10 °C and used within 3 h.

To prepare crude extract, the latex was mixed with cold distilled water (≤4 °C) with a latex to water ratio of 1:1 (w/v). The mixture was gently stirred at 4 °C for 1 h. Then, the mixture was centrifuged at 9,000 *g* at 4 °C for 20 min using a refrigerated centrifuge model

Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA). The supernatant was filtered using a Whatman No.1 filter paper, followed by freeze-drying (Kittiphattanabawon et al., 2012). These crude extract powders from papaya latex of Red Lady and Khack Dum cultivars, referred to as "CE-RL" and "CE-KD", respectively, were kept at –40 °C until use.

2.3 Fractionation of glycyl endopeptidase using aqueous two-phase system (ATPS)

ATPS was prepared in a 10-ml centrifuge tube according to the method of Nitsawang, Hatti-Kaul and Kanasawud (2006), and Rawdkuen et al. (2011). Crude extract powder (1 g) was dissolved in 8 ml of distilled water. The pH of solution was adjusted to 6.0 using 6 M HCl and the volume was made up to 10 ml with distilled water, to obtain a concentration of 100 mg/ml prior to fractionation using ATPS.

2.3.1. Effect of salts on fractionation of glycyl endopeptidase from papaya latex

To study the effect of salts on the partitioning of glycyl endopeptidase from papaya latex using ATPS, $(NH_4)_2SO_4$ or $MgSO_4$ at different concentrations (10, 15, 20 and 25% w/w) was added in conjunction with 10% PEG (2000 and 6000 Da) in an aqueous system. Crude extract solution (100 mg/ml) was added into the system to obtain 20% (w/w). Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 15 min using a Vortex mixer (Vortex Genie 2, G-560E; Merck). Phase separation was achieved by centrifuging the mixture at 9,000 *g* for 20 min at 4 °C. The salt-rich bottom phase was carefully separated using a pipette. Volumes of both top and bottom phases were measured and recorded. The enzyme activity was evaluated and protein content was determined in both phases using the Bradford method (Bradford, 1976). The phase composition giving the highest yield and purification was chosen for further study.

2.3.2 Effect of PEG on fractionation of glycyl endopeptidase from papaya latex

$(NH_4)_2SO_4$ at 10% was used in the system. PEG (2000 and 6000 Da) at different concentrations (10, 15 and 20%, w/w) was used. The biphasic systems were generated after addition of crude extract and distilled water as described previously.

2.3.3 Calculation of ATPS parameters

Top and bottom phases from all tested ATPS were subjected to calculation of ATPS parameters. Yield, specific activity (*SA*), purification fold (*PF*), partition coefficient of protein concentration (*KP*) and volume ratio (*VR*) were calculated as follows:

$$\text{Yield}(\%)=\frac{A_T}{A_i}\times 100$$

where $A_T$ is total glycyl endopeptidase activity in the protease rich phase and $A_i$ is the initial glycyl endopeptidase activity of the crude extract before being partitioned.

$$\text{SA(unit/mg protein)}=\frac{\text{Gglycyl endopeptidase activity}}{\text{protein concentration}}$$

$$\text{PF}=\frac{SA_e}{SA_i}$$

where $SA_e$ is the *SA* of each phase and $SA_i$ is the initial *SA* of the crude extract before being partitioned.

$$\text{KP}=\frac{C_T}{C_B}$$

where $C_T$ and $C_B$ are concentrations of protein in top and bottom phase, respectively.

$$\text{VR}=\frac{V_T}{V_B}$$

where $V_T$ and $V_B$ are top and bottom phase volume, respectively.

Based on purity and recovery yield, the ATPS containing PEG at the concentration rendering the most effective partitioning was chosen for further study.

173 2.4 Ammonium sulfate precipitation

174 Glycyl endopeptidase was further precipitated from the selected ATPS fraction by
175 ammonium sulfate at different saturations (28–80% saturation). After centrifugation at 9,000
176 *g* at 4 °C for 20 min, the pellet was re-dissolved in distilled water and dialysed against 20
177 volumes of distilled water 6 times. After lyophilisation, enzyme powder was stored at –40
178 °C until use. The partially purified glycyl endopeptidase from papaya latex of Red Lady and
179 Khack Dum cultivars referred to as "PPGE-RL" and "PPGE-KD", respectively, were
180 subjected to characterisation.

181

182 2.5 Assay for glycyl endopeptidase activity

183 Activity of glycyl endopeptidase was determined following the method of Buttle
184 (1994). The enzyme solution (200 µl) was mixed with 500 µl of activating agent (40 mM
185 cysteine/20 mM $Na_2$·EDTA in 0.5 M phosphate buffer, pH 7.5). The phosphate buffer (275
186 µl, pH 7.5) was added and the mixture was incubated in a water bath (Model W350;
187 Memmert, Schwabach, Germany) at 40 °C for 5 min. The reaction was then started by adding
188 25 µl of substrate solution (50 mM Boc-Ala-Ala- Gly-pNA in dimethylsulfoxide). After 8
189 min, 1 ml of stopping reagent (50% TCA, w/v) was added. The reaction mixture was
190 centrifuged at 5,000 *g* for 10 min, and the absorbance of the supernatant containing the
191 released *p*-nitroaniline was measured at 410 nm. Blank was prepared in the same manner,
192 except the substrate was added after addition of stopping reagent. One unit of enzyme activity
193 was defined as the amount of enzyme causing an increase of 0.1 in absorbance per min under
194 the assayed condition.

195

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198 2.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

199 2.6.1 Protein staining

200 SDS-PAGE of crude extract and partially purified glycyl endopeptidase from both
201 cultivars was performed according to the Laemmli method (Laemmli, 1970). Protein
202 solutions were mixed at a 1:1 (v/v) ratio with the sample buffer (0.125M Tris–HCl, pH 6.8,
203 4% SDS, 20% glycerol). The mixture was boiled for 3 min. The samples (15 µg protein) were
204 loaded onto the gel made of 4% stacking and 15% separating gels. They were subjected to an
205 electrophoresis set at a constant current of 15 mA per gel using a Mini Protean Tetra Cell unit
206 (Bio-Rad Laboratories, Richmond, CA). After electrophoresis, the gel was stained overnight
207 with staining solution (0.02% (w/v) Coomassie Brilliant Blue R-250) in 50% (v/v) methanol,
208 and 7.5% (v/v) acetic acid. Protein patterns were then visualised after destaining with 30%
209 methanol and 10% acetic acid until a clear background was obtained.

210 2.6.2 Protease activity staining

211 The protease separated using SDS-PAGE was subjected to activity staining as per the
212 method of Garcia-Carreno et al. (1993). Sample was mixed with sample buffer as mentioned
213 previously. However, the mixture was not boiled prior to loading onto the gel (4% stacking
214 and 15% separating gel). After electrophoresis, the gel (3 µg protein each lane) was washed
215 in 2.5% Triton X-100 at 4 °C for 15 min to remove SDS and renature the proteins. The gels
216 were then washed again with distilled water and incubated with 2% casein in 50 mM
217 phosphate buffer pH 7.5, containing 40 mM cysteine in a water bath for 1 h at 40 °C. The
218 gels were washed again with distilled water, fixed, stained and destained as described above.
219 The appearance of a clear zone on the dark background indicated protease activity. The bands
220 with protease activity were calculated for their molecular weights.

221

222

223 2.7 Preparation of gelatin hydrolysate using crude extract and partially purified glycyl
224 endopeptidase from papaya latex

225 2.7.1 Determination of protease activity

226 Both crude extract and partially purified glycyl endopeptidase (1.0 mg/ml) were
227 determined for protease activity as per the method of Vallés, Furtado and Cantera (2007) with
228 a slight modification. The enzyme solution (0.1 ml) was mixed with 1.1 ml of 1% (w/v)
229 casein in 0.1M Tris–HCl, pH 7.0 containing 12 mM cysteine. The mixture was incubated at
230 37 °C for 20 min. The reaction was stopped by adding 1.8 ml of 5% TCA. After
231 centrifugation at 3,000 *g* for 15 min, the absorbance of the supernatant was measured at 280
232 nm. One caseinolytic unit was defined as the amount of enzyme causing an increase of 1.0
233 absorbance unit per min under the assayed condition (Vallés et al., 2007).

234 2.7.2 Comparative study on gelatin hydrolysis

235 Crude extract or partially purified glycyl endopeptidase from both cultivars was added
236 to the commercial fish skin gelatin solution (3%, w/v) at a level of 40 units/g protein. During
237 hydrolysis at 40 °C, the sample was taken every 10 min for 2 h, followed by enzyme
238 inactivation by heating at 90 °C for 15 min in a temperature-controlled water bath. The
239 mixture was then centrifuged at 5,000 *g* for 10 min. The supernatant was determined for α-
240 amino group content and ABTS radical-scavenging activity. Crude extract and partially
241 purified glycyl endopeptidase from the cultivar yielding the highest hydrolysis were selected.
242 The hydrolysis time providing the highest α-amino group content within the range of initial
243 velocity was chosen for further study.

244 2.7.3 Production of gelatin hydrolysate with different degrees of hydrolysis (DH)

245 The crude extract and partially purified glycyl endopeptidase from Red Lady cultivar
246 papaya latex were used to produce gelatin hydrolysate with different DHs (10, 15, 20 and
247 25% DH) as per the method of Benjakul and Morrissey (1997). Fish gelatin solution (3%,

248 w/v) was added with enzyme at different concentrations (40, 80, 160, 320 and 640 unit/g
249 protein). The mixture was incubated at 40 °C for 1 h and the enzyme was then inactivated by
250 heating at 90 °C for 15 min in a temperature-controlled water bath. DH of the gelatin
251 hydrolysates was measured. $Log_{10}$ (enzyme concentration) *vs*. DH was plotted and enzyme
252 concentrations required to hydrolyse fish gelatin solution to obtain the desired DHs were
253 calculated from the regression equation

254 After enzyme inactivation by heating at 90 °C for 15 min, the resulting gelatin
255 hydrolysate was centrifuged at 9,000 *g* at 4 °C for 20 min. The supernatant was collected and
256 freeze-dried. The gelatin hydrolysate powder was placed in polyethylene bag and stored at
257 –40 °C. Hydrolysate powder was also determined for antioxidative activities.

258 2.7.4 Determination of α-amino group content

259 The α-amino group content was determined according to the method of Benjakul et al.
260 (1997). To diluted samples (125 µl), 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of
261 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a
262 temperature-controlled water bath at 50 °C for 30 min in the dark. The reaction was
263 terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixture was cooled at room
264 temperature for 15 min. L-Leucine standard solutions with concentrations ranging from 0.5 to
265 5.0 mM were used. The absorbance was read at 420 nm and α–amino group content was
266 expressed in terms of L-leucine.

267 2.7.5 Estimation of degree of hydrolysis (*DH*)

268 The obtained hydrolysates were subjected to the determination of DH according to
269 method of Benjakul et al. (1997). *DH* was calculated using the following equation:

270 $$\mathbf{DH} - \frac{(L_t - L_0)}{(L_{max} - L_0)} \times 100$$

where $L_t$ corresponds to the amount of α-amino acid released at time *t*. *Lo* is the amount of α–amino acid in original sample. $L_{max}$ is the maximum amount of α–amino acid in sample obtained after acid hydrolysis (6 N HCl for 12 h at 100 °C).

2.8 Determination of antioxidative activities

2.8.1 ABTS radical-scavenging activity

ABTS radical-scavenging activity of gelatin hydrolysates was determined as described by Binsan, Benjakul, Visessanguan, Roytrakul, Tanaka and Kishimura (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities. The mixture was allowed to react for 12 h at room temperature in the dark. The solution obtained (1 ml) was then diluted with 50 ml distilled water, in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Fresh ABTS solution was prepared for each assay. Sample (150 µl) was mixed with 2850 µl of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/g protein.

2.8.2 Ferrous chelating activity

Chelating activity of gelatin hydrolysates towards ferrous ion ($Fe^{2+}$) was measured by the method of Thiansilakul, Benjagul and Shahidi (2007) with a slight modification. Sample (200 µl) was mixed with 800 µl of distilled water. Thereafter, 0.1 ml of 2.0 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0–1.0

296 mM) was prepared. The control was prepared in the same manner except that distilled water
297 was used instead of the sample. Ferrous chelating activity was expressed as μmol EDTA
298 equivalents (EE)/g protein.

299 2.8.3 Hydrogen peroxide scavenging activity

300 Hydrogen peroxide scavenging activity was assayed according to the method of
301 Wettasinghe and Shahidi (2000) with a slight modification. Briefly, the sample (1 ml) was
302 mixed with 83 μl of 100 mM hydrogen peroxide (prepared in 0.1 M phosphate buffer, pH
303 7.4). The mixture was allowed to react for 40 min at room temperature. The absorbance at
304 230 nm of the reaction mixture was read and the blank (devoid of hydrogen peroxide) was
305 used for background subtraction. Trolox (0–10 mM) was used as the standard. The hydrogen
306 peroxide scavenging activity was expressed as μmol TE/g protein.

307

308 2.9 Measurement of volatile compounds

309 The volatile compounds in crude extract and partially purified glycyl endopeptidase as
310 well as their corresponding gelatin hydrolysates with 25% DH were determined using solid-
311 phase microextraction followed by gas chromatography-mass spectrometry (SPME/GC-MS)
312 following the method of Pino (2014) with slight modification.

313 2.9.1 Extraction of volatile compounds by SPME fibre.

314 To extract volatile compounds, 80 mg of sample were mixed with 4 ml of deionised
315 water and stirred continuously to dissolve the sample. The mixture was heated at 60 ºC in a
316 20-ml headspace vial with equilibration time of 10 h. The SPME fibre (50/30 μm
317 DVB/Carboxen™/ PDMS StableFlex™; Supelco, Bellefonte, PA) was conditioned at 270 ºC
318 for 15 min before use and then exposed to the headspace. The 20 ml-vial (Agilent
319 Technologies, Palo Alto, CA, USA) containing the sample extract and the volatile

compounds were allowed to absorb into the SPME fibre at 60 ºC for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC.

2.9.2 GC–MS analysis

GC–MS analysis was performed using a HP 5890 series II gas chromatograph (GC) coupled with HP 5972 mass-selective detector equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA). Compounds were separated on an HP-Innowax capillary column (30 m ± 0.25 mm ID, with film thickness of 0.25 µm; Agilent Technologies, Santa Clara, CA). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 15 ºC/min to a final temperature of 250 ºC and holding for 10 min. Helium was employed as a carrier gas with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 260 ºC. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 ºC. Full-scan-mode spectra were acquired over the mass range *m/z* 25–500 and scan rate of 0.220 s/scan. All analyses were performed with ionisation energy of 70 eV, filament emission current at 150 µA, and the electron multiplier voltage at 500 V.

2.9.3 Analyses of volatile compounds

Identification of the compounds was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds was performed, based on the retention time and mass spectra in comparison with those of standards from ChemStation Library Search (Wiley 275.L). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing 3 replicates of each sample. The identified volatile compounds were presented in terms of abundance of each identified compound.

2.10 Statistical analysis

All experiments were run in triplicate using three different lots of latex. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (Steel & Torrie, 1980). Statistical analysis was performed using SPSS for Windows (SPSS Inc., Chicago, IL). Data with $p < 0.05$ were considered to be statistically significant.

## 3. Results and discussion

3.1 Effect of ATPS and ammonium sulfate precipitation on fractionation of glycyl endopeptidase from papaya latex

3.1.1 Effect of ATPS

To fractionate glycyl endopeptidase from papaya latex of Red Lady and Khack Dum cultivars, several ATPS comprising PEG (2000 and 6000) at 10% in the presence of $(NH_4)_2SO_4$ and $MgSO_4$ at various concentrations (10, 15, 20 and 25% (w/w)) were used (Table 1 and 2). It was found that ATPS containing 10% PEG 2000 and 10% $(NH_4)_2SO_4$ or 10-15% $MgSO_4$ had no phase separation. Both salts in the range used could not generate the two-phase formation. The mechanism of biphasic generation in PEG-salt system is dependent on balancing of enthalpic and entropic forces involved in the aqueous hydration of the solutes (Huddleston, Veide, Kohlez, Flanagan, Enfors, & Lyddiatt, 1991). The partitioning of proteolytic enzyme is also dependent on "volume exclusion effect" of the polymer and "salting-out effect" of salts (Huddleston et al., 1991).

In the present study, the partitioning of glycyl endopeptidase from papaya latex was strongly dependent on the type and concentration of salts. The increase in salt concentration from 10 to 25% resulted in the decreases in most partition parameters, except for *KP*. Increasing salt concentration led to the higher proportion of salt-rich bottom phase as

370 indicated by the decreased *VR*. The distribution of the protein in ATPS was indicated by *KP*.
371 The ATPS containing 10% PEG 6000-25% $(NH_4)_2SO_4$ provided the highest *KP* of 5.00 and
372 3.03 for latex from Red Lady and Khack Dum cultivars, respectively. These results suggested
373 that most of the proteins from latex were preferably partitioned to the top phase under such a
374 condition. In contrast, the ATPS having 10% PEG 6000-10% $(NH_4)_2SO_4$ showed lower *KP*
375 (0.48–0.76) with higher *SA*, *PF* and yield ($p < 0.05$), indicating that most of the target
376 protease, glycyl endopeptidase, shifted to the bottom phase. The *SA*, *PF* and yield were
377 generally decreased, when salt concentration increased. Increase in salt concentration
378 provided the salting-out effect (Rawdkuen et al., 2011). The phase system containing
379 $(NH_4)_2SO_4$ generally showed superior partitioning efficiency to those containing $MgSO_4$.
380 Huddleston et al. (1991) concluded that the effectiveness of various salts in promoting phase
381 separation reflects the lyotropic series (a classification of ions based upon salting-out ability).
382 For the latex of Red Lady cultivar, the highest *SA* (2525 unit/mg protein) and *PF* (2.04-fold)
383 were obtained from ATPS containing 10% PEG 6000-15% $(NH_4)_2SO_4$, whilst the highest
384 recovery was obtained in ATPS having 10% PEG 6000-10% $(NH_4)_2SO_4$, in which *SA* of 2443
385 unit/mg protein and *PF* of 1.97-fold were obtained. For Khack Dum cultivar (Table 2), a
386 phase system containing 10% PEG 6000 and 10% $(NH_4)_2SO_4$ gave the highest *SA* (1325
387 unit/mg protein) and *PF* (1.30-fold) with the highest yield (99.90%). This result was in
388 agreement with Chaiwut et al. (2007), who reported that glycyl endopeptidase from papaya
389 latex was successfully fractionated using ATPS comprising PEG 6000 (6%, w/w) and
390 $(NH_4)_2SO_4$ (15%, w/w). Due to the high *SA*, *PF* and yield, $(NH_4)_2SO_4$ was found to be
391 appropriate for ATPS containing PEG.

392 Influences of PEG with different molecular weights (2000 and 6000) and
393 concentrations (10, 15 and 20% (w/w)) on partitioning of glycyl endopeptidase from papaya
394 latex of Red Lady and Khack Dum cultivars were also studied. The highest *SA* (1325–2525

395 units/mg protein) and yield (98.73–99.90) could be obtained from the ATPS with PEG 6000,
396 regardless of salt and papaya cultivar, compared with those found in ATPS containing PEG
397 2000. These results were in accordance with Subathra et al. (2012), who reported that the best
398 ATPS for separation of protease from Neem leaves was found in the system having PEG with
399 higher MW (8000). However, Rawdkuen et al. (2011) found that when protease from the
400 latex of *Calotropic procera* was partitioned by ATPS, PEG 1000 gave a higher yield than
401 PEG 2000 and 3000. PEG concentration (10, 15 and 20%, w/w) had no significant effect on
402 protease partition (data not shown). Moreover, the lower yield was obtained from the ATPS
403 with higher PEG concentration (20%, w/w). A similar trend was observed for the
404 fractionation of papain from papaya latex using ATPS (Nitsawang et al., 2006). Due to the
405 high viscosity of mixtures containing high PEG concentration, a lower yield was obtained
406 (Nitsawang et al., 2006).

407 Amongst all ATPS tested, the system comprising 10% PEG 6000-10% $(NH_4)_2SO_4$
408 effectively partitioned glycyl endopeptidase to the salt-rich bottom phase and undesired
409 proteins to the PEG-rich top phase. Under this condition, the resulting glycyl endopeptidase
410 fraction from papaya latex of Red Lady and Khack Dum cultivars had *SA* of 1325–2443
411 units/mg protein, *PF* of 1.30 to 1.97-fold and yield of 98.97–99.90%.

412 3.1.2 Effect of ammonium sulfate precipitation

413 The selected ATPS fractions (10% PEG 6000-10% $(NH_4)_2SO_4$) of Red Lady and Khack
414 Dum latex were subjected to ammonium sulfate precipitation at different % saturations
415 (Table 3). The ATPS fraction from latex of Red Lady cultivar obtained from ammonium
416 sulfate precipitation using 50–60% saturation showed the highest *SA* (2806 units/mg protein)
417 and *PF* (2.26-fold) with 55.33% yield. However, the sufficient yield is the one factor
418 considered for enzyme fractionation. The fraction with 40–50% saturation also had high yield
419 (24.90%) with slightly lower specific activity and purity. Therefore, the glycyl endopeptidase

from Red Lady cultivar was precipitated using ammonium sulfate (40–60% saturation) to obtain the higher yield (80.23%) with the promising *SA* (2647 units/mg protein) and *PF* (2.14-fold). For Khack Dum cultivar, the increases in *SA* (1892 units/mg protein) and *PF* (1.86-fold) with 73.08% yield were obtained when ammonium sulfate at 60–70% saturation was used. Ammonium sulfate precipitation is widely used to isolate water-soluble proteins of either plant or animal origin (Brovko & Zagranichnaya, 1998). Ammonium sulfate precipitation is rapid, inexpensive and convenient for protein separation (Eursakun, Simsiriwong, & Ratanabanangkoon, 2012). Ammonium sulfate precipitation separated different protein components with diverse properties and characteristics (Achouri & Boye, 2013). Different amounts of ammonium sulfate can precipitate proteins with different properties, in which the protein with similar properties and characteristics could be concentrated and pooled at the same ammonium sulfate saturation (Achouri et al., 2013). Due to the high *SA*, *PF* and yield, ammonium sulfate saturation of 40–60% and 60–70% were shown to be the optimum range for the recovery of the glycyl endopeptidase from ATPS fraction of papaya latex from Red Lady and Khack Dum cultivars, respectively. Therefore, these conditions were selected for preparing the partially purified glycyl endopeptidase.

### 3.2 Protein pattern and activity staining of crude extract and partially purified glycyl endopeptidase from papaya latex

Protein pattern and activity staining of crude extract (CE) and partially purified glycyl endopeptidase (PPGE) are shown in Fig. 1 (A) and (B). The crude extract obtained from papaya latex of Red Lady (CE-RL) and Khack Dum (CE-KD) cultivars showed the major protein bands with MW between 33 and 66 kDa (Fig. 1 (A)). Bands with MW below 14 kDa were also observed. This was in agreement with Azarkan et al. (2003) who reported the pattern of the whole protein fraction from papaya latex using SDS-PAGE. After partial

purification using ATPS-ammonium sulfate precipitation, the proteins with MW lower than 14 kDa were mostly eliminated. For activity staining (Fig 1 (B)), protein bands with proteolytic activities were observed with MW around 23 kDa and higher. There were several proteases in papaya latex (Azarkan et al., 2003). It was noted that CE obtained from papaya latex with different cultivars showed the different activity bands, indicating the presence of varying proteases with different MWs. CE-RL had the higher intensity of protease bands with MW >45 kDa, compared with those of CE-KD. On the other hand, PPGE-RL showed the less protease bands, compared with those of CE-RL. The result suggested that some proteases might be removed during partitioning using ATPS or ammonium sulfate precipitation. Four distinctive protease bands with MW of 23, 33, 40 and 50 kDa were obtained for PPGE-RL. PPGE-KD possessed increasing numbers of protease bands, especially with MW >30 kDa, compared with those of CE-KD. The results suggested that other proteases might co-migrate along with glycyl endopeptidase to the bottom phase. These results were in agreement with their protein pattern as shown in Fig 1. (A). Zerhouni et al. (1998) studied the protein pattern of papaya cysteine proteases using SDS-PAGE. Glycyl endopeptidase band was observed at MW of 23 kDa, whereas papain and chymopapain had MW lower than 14 kDa (Zerhouni et al., 1998). In the present study, activity bands with MW less than 14 kDa were observed as smear bands, suggesting that papain might be constituted to some extent in the fraction. In addition, Chaiwut et al. (2007) used an ATPS of 6% PEG 6000-15% $(NH_4)_2SO_4$ for removing papain from crude papaya latex to the PEG-rich top phase. Chymopapain was also separated from the salt-rich bottom phase using ammonium sulfate precipitation. From Fig. 1 (A) and (B), high intensity of glycyl endopeptidase band with MW of 23 kDa was observed in PPGE-RL, compared with PPGE-KD. This was in agreement with the higher specific activity (Table 3). The PPGE-RL (2647 units/mg protein) showed higher specific enzyme activity ($p < 0.05$) than those of PPGE-KD (1892 units/mg protein). Thus, the fractionation used in the present

study could be used for partitioning glycyl endopeptidase, a target enzyme, and also removed undesired proteins or enzyme contaminants.

### 3.3 Fish skin gelatin hydrolysates prepared using crude extract and partially purified glycyl endopeptidase and their antioxidative activities

The comparative study of crude extract (CE-RL) and partially purified glycyl endopeptidase (PPGE-RL) obtained from papaya latex of Red Lady cultivar on gelatin hydrolysis was conducted. Based on the α-amino group content of resulting gelatin hydrolysates, CE-RL and PPGE-RL showed the highest hydrolysis toward gelatin, in comparison with those of Khack Dum cultivar. Additionally, the hydrolysates prepared by protease from Red Lady cultivar exhibited the higher ABTS radical-scavenging activity ($p < 0.05$). Therefore, CE-RL and PPGE-RL were selected and used for preparing gelatin hydrolysate with different DHs. Gelatin hydrolysates were also determined for antioxidative activities.

ABTS radical-scavenging activity of gelatin hydrolysates with different DHs obtained from CE-RL and PPGE-RL is shown in Fig. 2 (A). In general, the increases in radical-scavenging activity were found in hydrolysate, compared with gelatin ($p < 0.05$). The results indicated that antioxidative peptides were produced during the hydrolysis. ABTS radical-scavenging activity gradually increased as *%DH* increased up to 25% ($p < 0.05$), especially those prepared using PPGE-RL. However, ABTS radical-scavenging activity of gelatin hydrolysate obtained from CE-RL with 20*%DH* and 25*%DH* was not different ($p > 0.05$). When comparing the ABTS radical-scavenging activity of gelatin hydrolysate prepared using CE-RL and PPGE-RL at the same *%DH*, gelatin hydrolysate prepared using the latter showed the higher activity ($p < 0.05$). Due to the different proteases between CE-RL and PPGE-RL, as shown in Fig. 1, the resulting gelatin hydrolysates containing different antioxidative

peptides could be obtained. In general, the peptides exhibit different physicochemical properties and biological activities, depending on their molecular weight and amino acid sequence, mainly determined by proteases used (Kim & Wijeselara, 2010). It was found that PPGE-RL had higher glycyl endopeptidase (Table 1). As a result, peptide bonds with Gly at $P_1$ (Buttle, Ritonja, Pearl, Turk, & Barrett, 1990) were more cleaved. Therefore, the resulting gelatin hydrolysates prepared using PPGE-RL probably contained more Gly residue at the *C*-terminus, comparing with those prepared using the crude extract. Antioxidative peptides isolated from Alaska pollock skin contained a Gly residue at the *C*-terminus (Kim, Kim, Byun, Nam, Joo, & Shahidi, 2001).

Ferrous chelating activity of gelatin hydrolysates was also investigated. Ferrous ion ($Fe^{2+}$) is a pro-oxidant and can interact with hydrogen peroxide in a Fenton reaction to produce reactive oxygen species and hydroxyl ($OH^{\bullet}$). All gelatin hydrolysates prepared using CE-RL and PPGE-RL had no ability in complexing with $Fe^{2+}$, regardless of DHs (data not shown).

$H_2O_2$ scavenging activity of gelatin hydrolysates with different DHs is shown in Fig 2 (B). Commercial fish gelatin had $H_2O_2$ scavenging activity of 744 μmol TE/g protein. The gelatin hydrolysates with 20-25% *DH* had a decrease in $H_2O_2$ scavenging activity ($p < 0.05$). It was noted that gelatin hydrolysate prepared using PPGE-RL had lower activity, compared with those using CE-RL at the same *DH* tested ($p < 0.05$). Therefore, it was possible that short peptide chain obtained from hydrolysis had a low ability in scavenging $H_2O_2$. Wu, Chen and Shiau (2003) found that size, level and composition of free amino acids of peptides affected the antioxidative activity. Peptides generated, when PPGE-RL was used, might show lower potential in binding $H_2O_2$, compared with those prepared using CE-RL. Nevertheless, no changes in $H_2O_2$ scavenging activity were obtained in the hydrolysate prepared using CE-RL with DHs of 10% and 15%, in comparison with gelatin.

The results suggested that gelatin hydrolysates contained peptides or proteins, which served as hydrogen or electron donors, which could convert the radicals to more stable forms. Thus, they could reduce and retard the oxidation, mainly *via* their radical-scavenging activity. Moreover, the partially purified glycyl endopeptidase fractionated from Red Lady cultivar papaya latex had the potential to produce antioxidative gelatin hydrolysates, especially at 25% *DH*.

### 3.4 Effect of partitioning on removal of odorous compounds in papaya latex and gelatin hydrolysate

Crude extract (CE-RL) and partial purified glycyl endopeptidase (PPGE-RL) from papaya latex of Red Lady cultivar were determined for odorous compounds (Table 4). Benzyl isothiocyanate (42% abundance) was the major odorous compound in CE-RL, followed by benzeneacetonitrile and ethyl hexadecanoate. Benzyl isothiocyanate at high amount is associated with the pungent-sour odour in the green papaya fruit (Fischer, 1996), and defined as an important odorant in papaya odour (Pino, 2014). Jirovetz, Buchbauer and Shahabi (2003) reported that the green-notes of green papaya were due to some C6 compounds (e.g., (E)-3-hexen-1-ol). In the present study, 2-ethyl-1-hexanol, was found in CE-RL. Benzeneacetonitrile, hexadecanoic acid and ethyl hexadecanoate were also isolated and quantified from fresh papaya (Pino, 2014). In general, all compounds detected in PPGE-RL were markedly lower in abundance than those of CE-RL, especially 2-ethyl-1-hexanol, which was not found in PPGE-RL. These results indicated the potential of ATPS and ammonium sulfate precipitation in removal of odorous compounds in papaya latex.

The corresponding gelatin hydrolysates produced using CE-RL showed a high content of odorous compounds, which was in accordance with those found in CE-RL. Benzyl isothiocyanate was found as the major odorous compound (15% abundance) in gelatin

hydrolysate with a small amount of ethyl hexadecanoate and methyl hexadecanoate. On the other hand, the gelatin hydrolysate prepared using PPGE-RL had lower levels of odorous compounds. These results suggested that use of PPGE-RL yielded gelatin hydrolysate with negligible off-odour compounds.

## 4. Conclusion

The glycyl endopeptidase from papaya latex was partitioned using aqueous two-phase (10% PEG 6000-10% $(NH_4)_2SO_4$) in combination with ammonium sulfate precipitation (40-60% saturation). The partially purified glycyl endopeptidase showed the potential in production of antioxidative gelatin hydrolysates. The enzyme fraction contained lower odorous compounds in papaya latex. The gelatin hydrolysate produced using the selected fraction had negligible odorous compounds. This could increase the exploitation of papaya latex for production of antioxidative gelatin hydrolysate.

## Acknowledgment

The authors would like to express their sincere thanks to Thailand Research Fund under the Royal Golden Jubilee Ph.D. Program to Supatra Karnjanapratum (PHD/0019/2554) and the Grant-in-Aid for dissertation from Graduate School, Prince of Songkla University, Thailand for financial support. The TRF senior research scholar program was also acknowledged.

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665

**Figure Legends**

Fig. 1. SDS-PAGE patterns (A) and activity staining (B) of crude extract and partially purified glycyl endopeptidase from papaya latex. MW: molecular weight marker; CE-RL: Crude extract-Red Lady; PPGE-RL: Partially purified glycyl endopeptidase-Red Lady; CE-KD: Crude extract-Khack Dum and PPGE-KD: Partially purified glycyl endopeptidase-Khack Dum

Fig. 2. ABTS radical scavenging activity (A) and $H_2O_2$ scavenging activity (B) of gelatin and gelatin hydrolysates prepared using crude extract (CE-RL) and partially purified glycyl endopeptidase (PPGE-RL) from papaya latex of Red Lady cultivar with different DHs. Different lowercase letters on the bars within the same DH indicate significant difference ($p < 0.05$). Different upper case letters on the bars indicate significant difference ($p < 0.05$).

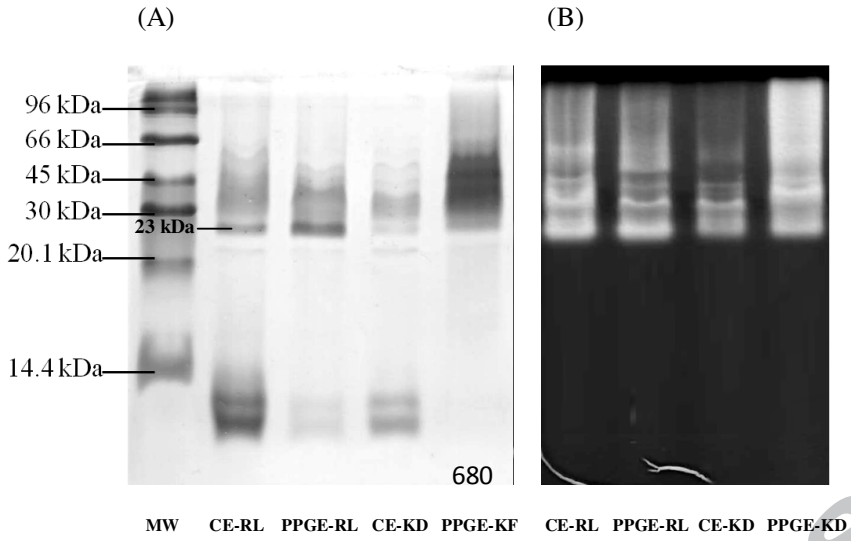
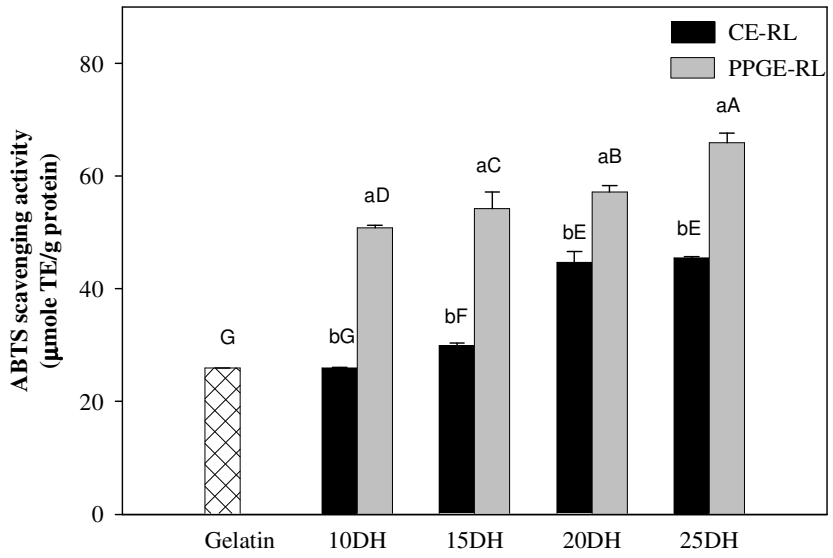


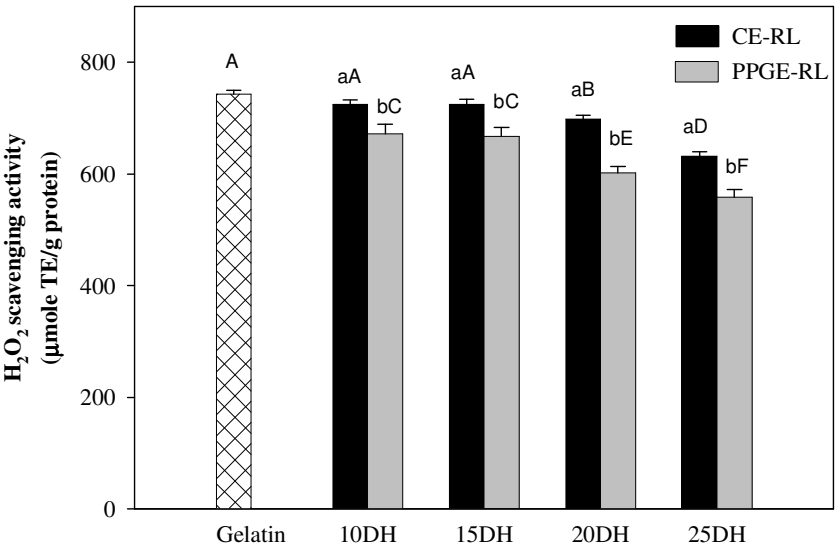

Fig. 1.

699 (A)




700

701 (B)




702

703 Fig. 2.

704

705

Table 1 Effect of phase composition in PEG-salts ATPS on partitioning of glycyl endopeptidase from papaya latex of Red Lady cultivar.

| Phase composition (%, w/w) | VR | KP | SA | PF | Yield (%) |
|---|---|---|---|---|---|
| 10%PEG 2000–10% $(NH_4)_2SO_4$ | ns | ns | ns | ns | ns |
| 10%PEG 2000–15% $(NH_4)_2SO_4$ | 0.32 ± 0.04e | 0.67 ± 0.02f | 1965 ± 26.41d | 1.58 ± 0.02d | 96.60 ± 1.30bc |
| 10%PEG 2000–20% $(NH_4)_2SO_4$ | 0.25 ± 0.04f | 1.36 ± 0.13d | 1534 ± 7.46f | 1.24 ± 0.01f | 40.72 ± 0.20f |
| 10%PEG 2000–25% $(NH_4)_2SO_4$ | 0.21 ± 0.06f | 4.94 ± 0.46b | 261 ± 1.58i | 0.21 ± 0.00g | 1.61 ± 0.01hi |
| 10%PEG 6000–10% $(NH_4)_2SO_4$ | 0.67 ± 0.06c | 0.76 ± 0.01e | 2443 ± 38.91b | 1.97 ± 0.03b | 98.97 ± 1.58a |
| 10%PEG 6000–15% $(NH_4)_2SO_4$ | 0.56 ± 0.00d | 1.01 ± 0.14d | 2525 ± 48.39a | 2.04 ± 0.04a | 90.29 ± 1.73e |
| 10%PEG 6000–20% $(NH_4)_2SO_4$ | 0.32 ± 0.03e | 2.93 ± 0.06c | 909 ± 10.68g | 0.73 ± 0.01h | 10.87 ± 0.13g |
| 10%PEG 6000–25% $(NH_4)_2SO_4$ | 0.32 ± 0.00e | 5.00 ± 0.28a | 495 ± 10.33h | 0.40 ± 0.01i | 2.93 ± 0.06h |
| 10%PEG 2000–10% $MgSO_4$ | ns | ns | ns | ns | ns |
| 10%PEG 2000–15% $MgSO_4$ | ns | ns | ns | ns | ns |
| 10%PEG 2000–20% $MgSO_4$ | 0.88 ± 0.07a | 0.55 ± 0.01g | 1836 ± 7.87e | 1.48 ± 0.01e | 92.29 ± 0.39d |
| 10%PEG 2000–25% $MgSO_4$ | 0.78 ± 0.00b | 0.52 ± 0.01g | 1820 ± 0.88e | 1.47 ± 0.00e | 95.17 ± 0.04c |
| 10%PEG 6000–10% $MgSO_4$ | ns | ns | ns | ns | ns |
| 10%PEG 6000–15% $MgSO_4$ | 0.88 ± 0.07a | 0.56 ± 0.00g | 2029 ± 0.99c | 1.64 ± 0.00c | 99.32 ± 0.05a |
| 10%PEG 6000–20% $MgSO_4$ | 0.78 ± 0.00b | 0.49 ± 0.01g | 1801 ± 4.18e | 1.45 ± 0.00e | 98.79 ± 0.23a |
| 10%PEG 6000–25% $MgSO_4$ | 0.67 ± 0.00c | 0.50 ± 0.02g | 1817 ± 41.19e | 1.46 ± 0.03e | 98.73 ± 2.23ab |

VR: volume ratio (top/bottom); KP: partition coefficient of protein in the top phase; KE: partition coefficient of enzyme in the top phase SA: specific activity (unit/mg protein) of the top phase; PF: purification factor; yield: activity recovery; ns: no phase separation. Different lower case letters in the same column indicate significant differences ($p < 0.05$).

Table 2 Effect of phase composition in PEG-salts ATPS on partitioning of glycyl endopeptidase from papaya latex of Khack Dum cultivar.

| Phase composition (%, w/w) | VR | KP | SA | PF | Yield (%) |
|---|---|---|---|---|---|
| 10%PEG 2000–10% $(NH_4)_2SO_4$ | ns | ns | ns | ns | ns |
| 10%PEG 2000–15% $(NH_4)_2SO_4$ | 0.98 ± 0.17c | 0.43 ± 0.01e | 1194 ± 0.52b | 1.17 ± 0.00b | 99.97 ± 0.04a |
| 10%PEG 2000–20% $(NH_4)_2SO_4$ | 0.88 ± 0.00c | 0.60 ± 0.01d | 694 ± 13.0563i | 0.68 ± 0.01i | 48.94 ± 0.92f |
| 10%PEG 2000–25% $(NH_4)_2SO_4$ | 0.88 ± 0.08c | 3.09 ± 0.26a | 283 ± 16.02j | 0.28 ± 0.01j | 3.81 ± 0.21h |
| 10%PEG 6000–10% $(NH_4)_2SO_4$ | 1.50 ± 0.00ab | 0.48 ± 0.03e | 1325 ± 0.96a | 1.30 ± 0.00a | 99.90 ± 0.07a |
| 10%PEG 6000–15% $(NH_4)_2SO_4$ | 0.92 ± 0.00c | 0.49 ± 0.01e | 1143 ± 0.64c | 1.12 ± 0.00c | 99.40 ± 0.05a |
| 10%PEG 6000–20% $(NH_4)_2SO_4$ | 0.92 ± 0.00c | 1.87 ± 0.06c | 948 ± 10.57d | 0.93 ± 0.01d | 21.06 ± 0.23g |
| 10%PEG 6000–25% $(NH_4)_2SO_4$ | 0.92 ± 0.00c | 3.03 ± 0.06b | 299 ± 4.73j | 0.29 ± 0.00j | 4.10 ± 0.06h |
| 10%PEG 2000–10% $MgSO_4$ | ns | ns | ns | ns | ns |
| 10%PEG 2000–15% $MgSO_4$ | ns | ns | ns | ns | ns |
| 10%PEG 2000–20% $MgSO_4$ | 1.11 ± 0.28bc | 0.35 ± 0.01f | 835 ± 1.23g | 0.83 ± 0.00g | 66.82 ± 0.10d |
| 10%PEG 2000–25% $MgSO_4$ | 1.04 ± 0.00bc | 0.32 ± 0.00f | 907 ± 19.53e | 0.89 ± 0.02e | 73.62 ± 1.58b |
| 10%PEG 6000–10% $MgSO_4$ | ns | ns | ns | ns | ns |
| 10%PEG 6000–15% $MgSO_4$ | 1.63 ± 0.49a | 0.34 ± 0.00f | 864 ± 10.54f | 0.84 ± 0.01f | 69.74 ± 0.85c |
| 10%PEG 6000–20% $MgSO_4$ | 1.38 ± 0.00b | 0.34 ± 0.00f | 792 ± 10.70h | 0.78 ± 0.01h | 62.69 ± 0.85e |
| 10%PEG 6000–25% $MgSO_4$ | 1.27 ± 0.00b | 0.32 ± 0.00f | 847 ± 1.35f | 0.83 ± 0.00f | 70.46 ± 0.11c |

VR: volume ratio (top/bottom); KP: partition coefficient of protein in the top phase; KE: partition coefficient of enzyme in the top phase SA: specific activity (unit/mg protein) of the top phase; PF: purification factor; yield: activity recovery; ns: no phase separation. Different lower case letters in the same column indicate significant differences ($p < 0.05$).

Table 3 Ammonium sulfate precipitation of glycyl endopeptidase from 10% PEG 6000-10% $(NH_4)_2SO_4$ ATPS fraction.

| ammonium sulphate (% saturation) | specific activity (units/mg protein) | purity (fold) | yield (%) |
|---|---|---|---|
| Red Lady Cultivar | | | |
| ATPS: 10%PEG 6000–10% $(NH_4)_2SO_4$ | 2443 ± 38.9b | 1.97 ± 0.03b | 98.97 ± 1.58a |
| 28-40% | nd | nd | nd |
| 40-50% | 2487 ± 21.4b | 2.01 ± 0.02b | 24.90 ± 0.32c |
| 50-60% | 2806 ± 27.5a | 2.26 ± 0.02a | 55.33 ± 0.77b |
| 60-70% | 1427 ± 31.6c | 1.15 ± 0.02c | 1.82 ± 0.10d |
| 70-80% | 1097 ± 26.2d | 0.88 ± 0.02d | 0.36 ± 0.03d |
| >80% | 1138 ± 6.47d | 0.92 ± 0.00d | 0.49 ± 0.01d |
| Khack Dum Cultivar | | | |
| ATPS: 10%PEG 6000–10% $(NH_4)_2SO_4$ | 1325 ± 0.96c | 1.30 ± 0.00c | 99.90 ± 0.07a |
| 28-40% | nd | nd | nd |
| 40-50% | nd | nd | nd |
| 50-60% | 1463 ± 44.7b | 1.44 ± 0.04b | 7.79 ± 0.26c |
| 60-70% | 1892 ± 25.7a | 1.86 ± 0.02a | 73.08 ± 1.07b |
| 70-80% | 992 ± 11.2d | 0.97 ± 0.01d | 6.37 ± 0.08c |
| >80% | 396 ± 2.86e | 0.39 ± 0.00e | 2.55 ± 0.03d |

nd: no detected.

Different lower case letters in the same column within the same cultivar indicate significant differences ($p < 0.05$).

Table 4 Odorous compounds in crude extract, partial purified glycyl endopeptidase and their corresponding gelatin hydrolysates.

| compounds | peak area (abundance) × $10^7$ | | | |
|---|---|---|---|---|
| | enzymes | | corresponding gelatin hydrolysates | |
| | CE-RL | PPGE-RL | CE-RL | PPGE-RL |
| 2-ethyl-1-hexanol | 3.1 | nd | 3.7 | nd |
| benzeneacetonitrile | 285.8 | 0.4 | 4.0 | nd |
| benzyl isothiocyanate | 533.1 | 1.1 | 43.1 | 8.2 |
| methyl hexadecanoate | 39.9 | 0.9 | 23.7 | 7.5 |
| ethyl hexadecanoate | 84.2 | 1.1 | 38.5 | 11.6 |
| 1,2-benzenedicarboxylic acid, dibutyl ester | 37.2 | 0.7 | 35.8 | 29.6 |
| hexadecanoic acid | 17.6 | 0.5 | 28.5 | 20.3 |
| 1,2-benzenedicarboxylic acid | 45.3 | 7.4 | 5.7 | 5.6 |

nd: no detected.

706 **Highlight**

707 • Glycyl endopeptidase was fractionated from papaya latex.

708 • ATPS (PEG-$(NH_4)_2SO_4$) in combination with $(NH_4)_2SO_4$ precipitation was applied.

709 • Benzyl isothiocyanate was the major off-odour compound in papaya latex.

710 • Partial purified glycyl endopeptidase (PPGE) contained odorous compounds.

711 • Antioxidative gelatin hydrolysates with negligible off-odour could be produced by

712 PPGE.

713