

Antidiabetic bio-peptides of soft and hard wheat glutens

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ABSTRACT

The effects of various purification techniques on kiwifruit enzyme characteristics (protease activity, kinetic parameters, and protein patterns) and production of wheat gluten bio-active peptides were investigated. The enzyme extract purified by ammonium sulfate precipitation method exhibited the highest protease activity (26), K_m (0.04 ± 0.002 mM), K_{cat}/K_m (40), and yield (96%). Using actinidin, the hard and soft wheat gluten subunit proteins produced antidiabetic inhibitory (α -glucosidase and α -amylase) peptides. The smallest M_w fraction of soft wheat gliadin peptide (<1 kDa) showed the highest inhibitory capacity against α -glucosidase ($18.4 \pm 0.7\%$) and α -amylase ($53.3 \pm 1.9\%$). The presence of high levels of amino acids with hydroxyl groups and proline in P3 sub-fraction had a critical role on α -glucosidase (47.2%) and α -amylase (71.2%) inhibitory activities. In conclusion, wheat gluten subunit peptides showed significant metabolic effects relevant to glucose and insulin control in vitro.

1. Introduction

Bioactive peptides are fragments of proteins that mainly consist of 2–20 amino acids. These peptides possess health-promoting and disease risk-reducing effects (Karami & Akbari-adergani, 2019). In general, an individual amino acid is always inactive but upon the hydrolysis of the parent protein, either by digestive or microbial enzymes, or during processing, the peptides exert physiological, biological, and functional effects (Elnur et al., 2021). Depending on their structural characteristics (i.e., sequence and composition of amino acids), peptides can play a variety of roles including: heavy metals and minerals binding, opioids, regulating immune activity, antimicrobial and antioxidant or as cholesterol and blood pressure-lowering agents (Gao, Dong, Wang, Li, & Chen, 2018).

Wheat gluten is an inexpensive and readily available protein source having high thiol groups and excellent functional properties. It can be classified into two main sub-groups: gliadins (α -, β -, γ - and ω -gliadins) and glutenins (high molecular weight, HMW, and low molecular weight, LMW) (Wang, Zhao, Yang, & Jiang, 2006). It has been already confirmed that dietary peptides are much safer, softer, and more easily absorbable compared to synthetic drugs (Duez, Cariou, & Staels, 2012).

At present, different oral treatments of diabetes are available which essentially lower the blood glucose level but with unwanted side effects (Duez et al., 2012; Wang et al., 2019). Therefore, increasingly more interest has been targeted on cereal and pseudo-cereal based bio-active peptides (Karimi, Azizi, & Ahmadi Gavlighi, 2020). The IC_{50} (i.e., 50% of maximal inhibitory concentration) values of buckwheat-based peptides are usually higher than those of oat and barley peptides (Wang, Yu, Zhang, Zhang, & Fan, 2015). The tartary buckwheat protein hydrolysates, prepared using alcalase, also strongly reduced the activities of α -amylase and α -glucosidase by up to 79 and 90%, respectively (Tao, Pan, Zheng, & Ma, 2019). The released peptides of wheat gluten are known to have high biological activity (Mousavi, Azizi, & Abbasi, 2022). It is claimed that gluten hydrolysate contains relatively potent dipeptidyl peptidase-IV (DPP-IV) inhibitor peptides such as Val-Pro-Leu and may have anti-diabetic potential in humans (Nongonierma & FitzGerald, 2017). A similar study reported that ginger protease also efficiently hydrolyzed the gluten and the produced tripeptides were effective in inhibiting DPP-IV, therefore recommended as a functional food to treat diabetics (Taga, Hayashida, Kusubata, Ogata-Goto, & Hattori, 2017). All glutenin hydrolysates exhibited an anti-diabetic effect depending on the DH. The anti-diabetic activity of the hydrolysate increased with

Abbreviations: WGS, wheat gluten subunit hydrolysate; SG, hard wheat glutenin hydrolysate; WG, soft wheat glutenin hydrolysate; SGL, hard wheat gliadin hydrolysate; WGL, soft wheat gliadin hydrolysate; HMW, high molecular weight; LMW, low molecular weight; WGS, Wheat gluten subunit hydrolysate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; F1, (WGS<1kDa); F2, (WGS 1–10 kDa); F3, (WGS 10–100 kDa); F4, (WGS>100kDa); MWCOs, molecular weight cut-offs; UF, Ultrafiltration membranes; DPP-IV, dipeptidyl peptidase-4; PBS, phosphate buffer saline.

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increasing DH value (Bozkurt, Bekiroglu, Dogan, Karasu, & Sagdic, 2021). In a most recent report, wheat gluten protein hydrolysate displayed the DPP-IV inhibitory activity (IC_{50} of 1.25 ± 0.09 mg/mL). The peptide sequences of MPF and VAVPV exerted the highest DPP-IV inhibitory activity. It indicates that wheat gluten is a worthy source for DPP-IV inhibitory peptides, and wheat gluten-derived DPP-IV inhibitory peptides have potential as ingredients in functional foods. They are also medicinal candidates for the management of prediabetes and Type 2 diabetes mellitus (Ding et al., 2022). The main hypothesis of this study is that proline rich protein substrates, such as wheat gluten subunits (hard and soft wheats), upon hydrolysis with kiwifruit extract can generate potential bioactive peptides capable of inhibiting enzymes responsible for diabetes and obesity (α -amylase and α -glucosidase).

Therefore, this study aimed to evaluate the capability of the most active partially purified kiwifruit enzyme on the hydrolysis of wheat gluten (by-product of starch industry) subunits to obtain α -glucosidase and α -amylase inhibitory peptides. Several molecular weight cut-offs (MWCos) of ultrafiltration (UF) membranes were used to separate the peptides as well as the assessment of gluten subunit degradation by SDS-PAGE. The amino acid compositions of the most potent hydrolysates were also determined. The findings of this study not only open new horizons on the application of starch industry by-products (i.e., wheat protein) but could also provide new insights into the utilization of gluten subunit hydrolysates as antidiabetic agents.

2. Materials and methods

2.1. Materials

The hard (Chamran) and soft (Sepahan) wheats were purchased from Shahdineh Flour Company (Esfahan, Iran). The approved (Department of Botany, Guilan University, Iran) variety of kiwifruits (*Actinidia deliciosa* var. Hayward) were collected (May to June 2020) from local farms (Rasht city, Guilan province, Iran). Chemicals namely C_2H_5OH , C_3OH_6 , NaOH, K_2HPO_4 , KH_2PO_4 , $(NH_4)_2SO_4$, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), L-cysteine, porcine pancreatic α -amylase (Cat no. A3176), casein, trichloroacetic acid (TCA), PNPG (4-nitrophenyl α -D-glucopyranoside), tyrosine, bovine serum albumin (BSA), polyacrylamide, high-performance liquid chromatography (HPLC) grade water and acetonitrile (ACN), trifluoroacetic acid (TFA), ferrozine [3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt], rat intestinal α -glucosidase (Cat no. I1630), DNS (3,5-Dinitrosalicylic acid), potassium sodium tartrate, dialysis membrane (cut-off 14 kDa) and color indicators (bromophenol blue and coomassie brilliant blue G) were all purchased from Merck Chemicals Co (Darmstadt, Germany). The DNS reagent was prepared by adding 20 ml DNS (96 mM) in 8 ml sodium potassium tartrate solution (5.3 M) and 8 ml sodium hydroxide (2 M) then stirred on a hot plate. Upon the complete dissolution of components, the solutions were filtered and stored in dark glass bottles. All other chemicals were of analytical grade.

2.2. Extraction, purification, and concentration of kiwifruit enzyme

The fresh kiwifruits were peeled and pureed. To prevent any oxidation of natural enzymes, the phosphate buffer (0.05 M, pH 6.5, including 0.4 M $Na_2S_2O_5$) was immediately mixed with the kiwifruit puree (180 ml/kg kiwifruit puree). The pulp was centrifuged (15000g for 30 min at 4 °C, Sigma 3–30 K, Hamburg, Germany) following filtration through a muslin tissue. For protein and enzyme activity analyses, the supernatant was promptly kept at 4 °C (Kaur et al., 2010; Mousavi et al., 2022).

The clear supernatant was then subjected to solvent precipitation, ammonium sulfate precipitation, concentration, and dialysis as described in the following paragraphs.

Regarding solvent precipitation, the pre-cooled solvents (acetone and ethanol), four folds of the volume of clear supernatant, were added

and the mixture of supernatant and solvents were vortexed, incubated (60 min, -20 °C), and centrifuged (12000g, 15 min). The solvents of the precipitates were evaporated at room temperature. Then, phosphate buffer (0.05 M, pH 7.0) was added, and tubes vortexed thoroughly to dissolve the protein pellet (Michail, Vasiliadou, & Zotos, 2006).

In another set of experiments, the clear supernatant phase was mixed with ammonium sulfate (65% saturation) to precipitate the protease. To do this, ammonium sulfate was first slowly added to the supernatant in the presence of ice, dissolved well (65% saturation) and allowed to stir (3 h, 4 °C), then centrifuged (12000 g, 15 min, 4 °C) and the precipitate was dialyzed (cut off 14,000 Daltons) against phosphate buffer (pH 7, 4 °C) for 24 h (Michail et al., 2006).

The clear supernatant phase was also concentrated by an Amicon ultrafiltration cell (model 8200, Millipore, MA, USA) using polyethersulfone membranes (cut-off 10,000 Da, Biomax, Millipore, MA USA). The ultrafiltration was carried out under constant inert gas flow (13.5 ml/h, 2.5 bar) and gentle magnetic stirring (30 rpm) at ambient (21 °C) temperature (Duez et al., 2012).

The clear supernatant of crude extract was also dialyzed (cut off 14000 Da) with buffer phosphate (0.05 M, pH 7.0) for 24 h using magnetic stirrer under constant stirring (4 °C). The buffer was renewed every 2 h (Mousavi et al., 2022).

2.3. Determination of enzymatic activity

Protease activity of various kiwifruit extracts was tested at room temperature in phosphate buffer (50 mM containing 5 mM DTT, 2 mM EDTA, and 5 mM L-cysteine, pH = 6.5) using casein as substrate. First, the enzyme extract (50 μ L) was mixed with buffer (350 μ L), then casein (400 μ L of 1% w/v) was added and the reaction was performed (35 °C for 60 min) at 10 min intervals. The reaction was then stopped by trichloroacetic acid solution (10%, 800 μ L). The sample was stored (30 min) at room temperature, centrifuged (12000g, 10 min), and the absorbance of the supernatant was recorded ($\lambda = 280$ nm). One unit of enzyme activity (U) equals that much of enzyme which can convert protein (e.g., casein) to the equivalent products (e.g., tyrosine amino acid) and expressed as U/ml. Various concentrations (0.1–1 mM) of tyrosine amino acid were also used to plot the standard curve (Homaei & Etemadipour, 2015). The quantity of soluble protein was determined using the Bradford procedure at 595 nm with bovine serum albumin (BSA, 0.1–1 mg/ml) as a standard protein. Specific activity, fold purification, and yield at each method were determined using Eqs. (1), (2), and (3), respectively, and all experiments were performed with three replicates.

$$\text{Specific activity (U/mg protein)} = \frac{\text{Total activity}}{\text{Total protein}} \quad (1)$$

$$\text{Purification fold} = \frac{\text{Specific activity at a particular method}}{\text{Specific activity of crude extract}} \quad (2)$$

$$\text{Yield (\%)} = \frac{\text{Total activity of a particular method}}{\text{Total activity of crude extract}} \quad (3)$$

2.4. Determination of kinetic parameters

To determine the kinetic parameters, the enzyme activity was measured at increasing concentrations of casein (0–20 mg/mL). The initial rates of reaction were plotted against different concentrations of casein and then Michaelis constant (K_m , mM), maximum velocity (V_{max} , mM/h), and catalytic constant (K_{cat}) were determined by using the Michaelis-Menten equation and Lineweaver-Burk plots in GraphPad Prism software version 5.04 (Anitha & Palanivelu, 2013).

2.5. Fractionation of wheat flour proteins by Osborne method

Kernels of 'Chamran' (hard wheat with $32.4 \pm 1.2\%$ wet gluten) and 'Sepahan' (soft wheat with $19.8 \pm 0.87\%$ wet gluten) wheat cultivars were milled using a lab-scale mill (Brabender, Duisburg, Germany) and the flours (ash content 0.55%, particle size < 0.2 mm) were collected and used for fractionation. The major wheat protein fractions (i.e., gliadin and glutenin) were extracted from defatted flour according to the sequential procedure described by Singh, Donovan, Batey, and MacRitchie (1990) and finally, dried by vacuum drying (Christ -Alpha 1-2 LD-Plus, Osterode am Harz, Germany). The protein powders were stored in the refrigerator until further processing (Mousavi et al., 2022).

2.6. The hydrolysis of gluten subunits and separation of peptides via ultrafiltration

The hydrolysis process of wheat proteins was performed (pH 6.5, 35°C) using the most active purified kiwifruit extracts. In a reaction container equipped with a stirrer, wheat gluten subunits (WGS) powder was dispersed (5% w/v) in distilled water and pretreated (85°C , 10 min), then the pH (6.5) (Metrohm780, Herisau, Switzerland) and temperature (37°C) were regulated. Subsequently, the enzyme (substrate: enzyme ratio, 1:10 based on WGS protein content) was added and the enzymatic digestion process continued (6 h, under the same constant conditions). The pH of the mixture was fixed by the pH-State method (NaOH, 1 N). Upon the completion of the digestion, the excess enzyme was inactivated by immersing the reaction chamber in a hot water bath (90°C for 10 min), then immediately cooled down in the ice pool. The insoluble components were allowed to sediment (8000 g, 4°C , 15 min), the supernatant was filtered and isolated using three membranes (MW cut-offs, 100, 10, and 1 kDa). Four fractions including F1 (WGS < 1 kDa), F2 (WGS 1–10 kDa), F3 (WGS 10–100 kDa), and F4 (WGS > 100 kDa) were collected and vacuum dried. They were stored in the refrigerator before running *in vitro* inhibitory assays by using α -glucosidase and α -amylase (Wang et al., 2006).

2.7. SDS-PAGE electrophoresis

The gels were prepared with a 5% stacking (pH 6.8) and 15% polyacrylamide resolving (pH 8.8) gels. Prior to load, the samples were dissolved in buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS, 5% β -mercaptoethanol, and 0.02% bromophenol blue). Using methanol (40% v/v) and acetic acid (7% v/v) solution, the gels were stained with Coomassie brilliant blue R-250 (0.125% w/v), then de-stained with the same solution without color indicator (Laemmli, 1970).

2.8. Inhibitory assays

2.8.1. Alpha-glucosidase

The inhibitory activity of WGS against α -glucosidase was evaluated according to Liu, Ai, Qu, Chen, & Ni, 2017 with minor modifications. Briefly, alpha-glucosidase solution (50 μL , 0.5 U/mL) was mixed with sample (100 μL , 30 mg/ml PBS, pH 6.8, 0.1 M) and incubated (37°C , 10 min). PNPG (100 μL , 3 mM) was then added and the mixture was incubated (37°C , 15 min). The reaction was terminated by Na_2CO_3 (750 μL , 0.1 M). The absorbance of the mixture was recorded by spectrophotometer ($\lambda = 405$ nm, Agilent-Carry 60, California, USA). The positive control was acarbose.

The α -glucosidase inhibitory activity was calculated by:

$$\alpha - \text{glucosidase inhibition}(\%) = \frac{A - B}{A} \times 100 \quad (4)$$

where A denotes the absorbance of the control and B expresses the absorbance of the sample mixture.

2.8.2. Porcine alpha-amylase

The inhibitory activity of the porcine α -amylase was evaluated according to Connolly, Piggott, and FitzGerald (2014) with minor modifications. First, the enzyme solution (3.75 U/ml, 100 μL) and sample (100 μL , 30 mg/ml) mixture was incubated (37°C , 10 min) then, the starch solution (0.5% w/v, 100 μL) was added. DNS solution (200 μL) was used to terminate the reaction. The mixtures were then immersed in boiling water (5 min) and immediately placed in an ice pool and diluted with distilled water (500 μL). The absorbance of the mixture was recorded ($\lambda = 540$ nm) and acarbose was used as positive control.

The α -amylase inhibitory activity was calculated by:

$$\alpha - \text{amylase inhibition}(\%) = \frac{A - B}{A} \times 100 \quad (5)$$

where A and B denote the absorbance of the control and sample mixture, respectively.

2.9. Chromatographic analysis (RP-HPLC)

The fraction with the highest inhibitory effect was lyophilized and resuspended in ultrapure water (10%, w/v). A semi-preparative RP-HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a fraction collector was used for sample fractionation. Samples (20 μL) were then loaded onto a C_{18} column (Agilent ZORBAX SB-C18, 5 μm , 4.6 mm \times 250 mm). With a slight modification of Zhang, Sun, Liu, Li, and Jiang (2017), the elution protocol was performed using 0.1% (v/v) TFA in ultrapure water (A) and 0.1% TFA in acetonitrile (ACN) (B). Elution was conducted using the following sequence: 1–5 min, 20% B; 5–35 min, 20–45% B; 35–45 min, 45–90% B then back to 20% B for 5 min. The absorbance of the fraction was monitored ($\lambda = 280$ nm) under a constant flow rate (1 ml/min) and the fractions were collected and lyophilized.

2.10. Amino acid analysis

The amino acid composition of the hydrolysates was determined using an automatic amino acid analyzer (Hitachi High-Technologies Co., Japan, L-8900) equipped with a Hitachi ion exchange resin column (60 mm \times 4.6 mm, id = 1 μm) and ninhydrin was used as the reactive reagent for amino acids detection. Briefly, freeze-dried hydrolysate (20 mg) was placed in a screw-capped vial and HCl (6 N, 5 ml) and phenol (20 μL) were added. After sealing the tube under N_2 , the tube placed into an oven (110°C , 22 h) to digest. They were then cooled and centrifuged (11000g for 20 min at 4°C). The supernatant was dried in the nitrogen-blowing instrument (Quandao, Shanghai, China), then HCl (1 ml, 0.02 N) was added. The mixture was centrifuged (11000g for 20 min at 4°C) and the supernatant (700 μL) was used for amino acid determination. Amino acid profiles of hydrolysates were quantified and expressed as g/100g (Junhao, Hu, Li, & Jiang, 2018).

2.11. Statistical analysis

Six different methods of partial purification of kiwifruit enzyme (actinidin) from a Hayward cultivar were used to determine which one can provide the highest enzyme activity for hydrolysis. In total, 16 samples were used to determine whether hard or soft gluten hydrolysates contain the highest concentration of α -glucosidase and α -amylase inhibitory activity. All the results were presented as means \pm SEM (standard error of the mean). Normal distribution was tested using SPSS statistics 18 software (IBM, New York, USA). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. P-value < 0.05 was considered as significant difference. Bar graphs were plotted using Excel software.

3. Results and discussion

3.1. Enzymatic activity of partially purified kiwifruit extract

The effects of various purification methods (crude extract, cold acetone, ethanol, ammonium sulfate precipitation, ultrafiltration, and dialysis concentration) on the enzymatic activity of the kiwifruit enzyme are presented in Table 1. The protease activity of each fraction was determined and the precipitation by ammonium sulfate showed the highest enzymatic activity. The yield, purification fold, and specific activity of the ammonium sulfate precipitation method were 95.9%, 3.6 folds, and 26 U/mg protein, respectively. Similar to these findings, the fig's protease enzyme which precipitated with ammonium sulfate has already shown the highest activity (Chaiwut, Nitsawang, Shank, & Kanasawud, 2007). Moreover, it is reported that the enzymatic activity of full-ripe kiwifruit extract was much higher than its unripe counterparts (Kaur et al., 2010). In a recent report, a specific activity around 85.6 U/mg was reported for actinidin of kiwifruit extract which was precipitated with 60% ammonium sulfate (Chalabi, Khademi, Yarani, & Mostafaei, 2014). In the present study, the kiwifruits were fully ripe and their ripening factors were 3.5 (pH), 0.7 (acidity), and 13.5° (Brix), quite similar to the ranges of brix (11.5–14.5°), pH (3.1–4.1), and acidity (<1.2) values already reported (Kaur et al., 2010). Ammonium sulfate is often used as an initial purification step and by using high concentrations of ammonium sulfate, it is possible to precipitate proteins without causing denaturation. The gradual addition of ammonium sulfate enables to achieve differential precipitation and some protein purification (Kaur et al., 2010).

Table 1 also shows that the enzyme fractions obtained from ethanol and acetone precipitation had slightly lower activity than sulfate precipitation however with a much higher specific activity and purity than those obtained from crude extracts. The low protease activity of solvent precipitation can be attributed to protein denaturation. Michail et al. (2006) reported that organic solvent fractionation should be carried out only at low temperatures, as >10 °C, and at the presence of solvents (ethanol and acetone, at concentrations of 80%), the denaturation of the enzyme is expected. The concentration of enzymes using ultrafiltration and dialysis also indicated that ultrafiltration increased the yield, specific activity, and purification fold over the dialysis membranes. This could be likely due to the long exposure to the dialysis (24 h), which can potentially reduce the activity of the extracted enzyme.

3.2. Kinetic parameters

Under the same reaction conditions, the partially-purified and crude

Table 1

Comparison of the effects of various purification methods on the yield and activities of kiwifruit enzyme.

Purification technique	Total activity (U)	Total protein (mg)	Yield (%)	Specific activity (U/mg protein)	Purification fold (n)
Fresh extract	366.1 ± 3.1 ^a	50.2 ± 4.6 ^a	100 ± 1 ^a	7.3 ± 0.1 ^e	1 ± 0.1 ^d
Ethanol	286.3 ± 5.5 ^d	27.3 ± 1.3 ^b	78.2 ± 1.1 ^d	10.5 ± 0.1 ^d	1.4 ± 0.1 ^c
Acetone	282.2 ± 4.5 ^d	23.3 ± 0.7 ^c	77.1 ± 2.9 ^d	12.1 ± 0.2 ^c	1.7 ± 0.1 ^c
Dialysis	106.2 ± 4.1 ^e	13.2 ± 0.2 ^e	29 ± 2.7 ^e	8.1 ± 0.3 ^e	1.1 ± 0.1 ^d
Ammonium sulfate	351.2 ± 2.1 ^b	13.5 ± 2.2 ^e	95.9 ± 1.7 ^b	26 ± 0.4 ^a	3.6 ± 0.1 ^a
Ultrafiltration	299.9 ± 3.4 ^c	16.7 ± 0.9 ^d	81.9 ± 3.7 ^c	18 ± 0.3 ^b	2.5 ± 0.2 ^b

The results represent mean ± SD for three experiments. Different small letters in each column indicate the significant difference between fractions and concentrations ($p < 0.05$).

enzyme extracts were compared to determine the effects of substrate concentration on enzyme activity. K_m , the Michaelis Menten constant, represents the substrate concentration that is required to achieve half of the maximum speed of the enzyme, and V_{max} is the maximum speed of the enzyme. When the enzyme is working at maximum efficiency, K_{cat} reflects the turnover number of the enzyme. This represents how many molecules of the substrate are converted into a product per unit of time. The K_m and V_{max} values of the purification techniques were calculated using the Lineweaver–Burk graphs (Table 2). The highest and lowest K_m values were obtained with dialysis purification and ammonium sulfate purification, respectively. This indicates the high affinity of the ammonium sulfate technique on the substrate, whereas ultrafiltration extract was the second best.

From biochemical perspective, the specificity constant (also known as kinetic efficiency or K_{cat}/K_m), is a measure of the efficiency of an enzyme to convert substrates into products. The specificity constants of enzymes can also be used to measure whether the enzyme has a preference for a particular substrate (i.e., substrate specificity). The high specificity indicates a stronger preference for the particular substrate (Voet, Pratt, & Voet, 2008). The K_{cat}/K_m values for ammonium sulfate and ultrafiltration were higher than other purification techniques. The high value of K_{cat} and a low value of K_m were expected for the most appropriate substrates and it is very clear that the ammonium sulfate purification technique, due to its lower K_m than ultrafiltration, was the best choice for hydrolysis and peptide production. These results are in line with those investigations which reported the ammonium sulfate as the most desirable purification method (Mirzapour-Kouhdasht, Moosavi-Nasab, Krishnaswamy, & Khalesi, 2020; Zhang et al., 2017). These kinetic parameters values (K_m (μM), 58.2; V_{max} ($\mu\text{M s}^{-1}$), 2.14; K_{cat} (s^{-1}), 42.8; k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$), 0.73) are already reported for the purified actinidin kiwifruit extract precipitated with 60% ammonium sulfate (Chalabi et al., 2014). Homaei and Etemadipour (2015) also noticed that K_m and k_{cat} values of free (12.5 M and 29.2 s^{-1}) and immobilized (15.92 M and 20.74 s^{-1}) actinidin were different.

3.3. SDS-PAGE analysis

Using SDS-PAGE analysis, the patterns of proteins which were resulted from various kiwifruit extract purification techniques were identified (Fig. 1). As can be seen, in all 6 methods, the Actinidin was observed around ~ 29 kDa. As expected, the ammonium sulfate purification and dialysis techniques showed the highest and lowest band intensity, respectively. Kiwellin (~20 kDa) and Thaumatin-like protein (~22 kDa) were also observed in proteins prepared by all purification methods. The ammonium sulfate purification also exhibited the highest protease activity (i.e., 26 U/mg protein) and was selected for further investigation. Zhang et al. (2017) showed that the purified enzyme from kiwifruit extract had an approximate molecular mass of 29.0 kDa. The purified kiwifruit actinidin revealed two protein bands of about 22 and 30 kDa (Grozdanovic et al., 2012). In contrast to the findings of the present study as well as the abovementioned reports, by using mass

Table 2

Comparison of the kinetic parameters of the crude enzyme extract with the extracts purified using different methods.

Purification technique	K_m (μM)	V_{max} (U)	K_{cat} (1/s)	K_{cat}/K_m
Fresh extract	0.5 ± 0.1 ^c	345.0 ± 2.9 ^f	2.1 ± 0.1 ^d	15.6 ± 1.2 ^b
Ethanol	0.3 ± 0.1 ^b	1000.0 ± 5.1 ^d	2.6 ± 0.1 ^c	13.1 ± 0.3 ^d
Acetone	0.3 ± 0.1 ^b	526.0 ± 5.6 ^b	2.3 ± 0.1 ^d	14.5 ± 0.7 ^c
Dialysis	0.4 ± 0.1 ^b	5000.0 ± 5.5 ^c	3.8 ± 0.1 ^a	10.3 ± 1.7 ^e
Ammonium sulfate	0.2 ± 0.1 ^a	6667.0 ± 2.8 ^a	0.7 ± 0.1 ^e	17.2 ± 0.5 ^a
Ultrafiltration	0.3 ± 0.1 ^b	492.0 ± 2.3 ^c	3.1 ± 0.1 ^b	17.1 ± 1.1 ^a

The results represent mean ± SD for three experiments. Different small letters in each column indicate the significant difference between fractions and concentrations ($p < 0.05$).

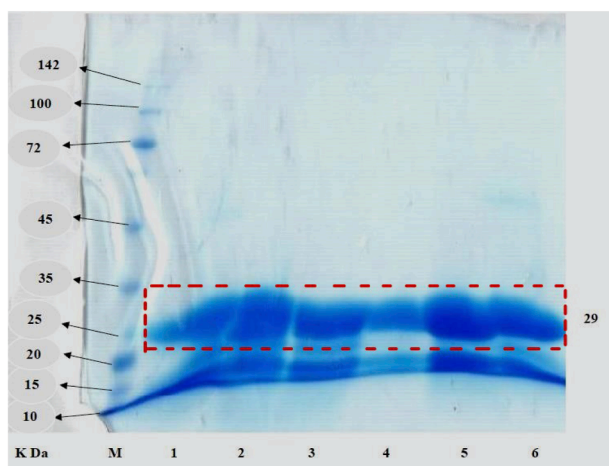


Fig. 1. Illustration and comparison of the SDS-PAGE chromatograms of kiwi-fruit extract purified by using different methods (M, low molecular weight protein markers; 1, Fresh extract; 2, Ethanol precipitation; 3, Acetone precipitation; 4, Dialysis purification; 5, Ammonium sulfate; 6, Ultrafiltration).

spectrometry, under reducing conditions, the molecular weight of actinidin was 23.5 kDa (Yuk, Hwang, & Lee, 2017). The inconsistency regarding the molecular weight of actinidin can be likely attributed to the various experimental conditions of the existing reports as well as the partial purification of the fractions.

3.4. Inhibitory assays

The small intestine brush border membrane contains complex enzymes (alpha-glucosidases) which break down oligosaccharides into monosaccharides (Ibrahim, Bester, Neitz, & Gaspar, 2018). To inhibit the degradation of disaccharides, one strategy is to inhibit the secretion of α -glucosidase, as a treatment for diabetic patients. The active side of α -glucosidase attaches to the inhibitor through hydrogen bonds and electrostatic interactions. The peptides can potentially act as α -glucosidase inhibitors, therefore can modify its activity (Roskar et al., 2015). Tetra-peptide is already reported as an effective α -glucosidase inhibitor, which contains amino acids with hydroxyl side groups (González-Montoya, Hernández-Ledesma, Mora-Escobedo, & Martínez-Villalunga, 2018). In this study, the conversion of PNPG to p-nitrophenol catalyzed by α -glucosidase in the presence of hydrolysis products of the wheat gluten subunit was measured (Table 3). The highest inhibition ($18.4 \pm 0.7\%$) was attained by the very fine fractions of WGLYH (<1 kDa). This could happen due to the presence of hydroxyl-containing amino acids (i.e., tyrosine, threonine, and serine) at their N-terminals, a proline residue closer to the C-terminal, as well as sulfur-containing amino acids that significantly promote α -glucosidase inhibitory activity peptides (González-Montoya et al., 2018; Ibrahim et al., 2018). Wang et al. (2019) reported the production of bio-active peptides, with α -glucosidase inhibitory activity, from soybean protein. According to this study, the papain-based fermented soybean (Tempe) hydrolysates (<5 kDa) showed the highest α -glucosidase inhibitory activity. Pino (2020) mentioned that trypsin can cleave the Tenebrio Molitor protein to the smaller peptides (0.8–2 kDa) with α -glucosidase inhibitory capability. Ren et al. (2020) also stated that alcalase can release the hemp protein hydrolysates (≤ 1 kDa) with the highest degree of α -glucosidase inhibition compared to the other enzymes (i.e., trypsin, fluorescein, neutralase, and papain). OsegueraToledo, GonzalezMejia, Sivaguru, and Amaya-Llano (2016), who isolated the peptides from peeled beans, also suggested that the fine fractions (<1 kDa) expressed a good inhibitory ($76.4 \pm 0.5\%$) effect on α -glucosidase.

The amino acid compositions of the P1–P3 sub-fractions are shown in Table 4. Depending on their amino acid composition, the inhibitory

Table 3

Comparison of the antidiabetic activity of four MWCOS of hard and soft wheat gluten subunit hydrolysates.

	Peptides with Membrane MWCOS	Inhibitory activity (%)
	α -glucosidase	α -amylase
≥ 100 kDa		
SGUTH	N.D.	6.1 ± 1.5^g
WGUTH	N.D.	7.3 ± 0.1^g
SGLYH	4.7 ± 0.7^e	6.6 ± 0.2^g
WGLYH	2.8 ± 0.1^f	6.5 ± 0.3^g
10–100 kDa		
SGUTH	3.6 ± 0.9^f	13.1 ± 0.4^f
WGUTH	2.1 ± 0.1^f	11.1 ± 0.8^f
SGLYH	5.2 ± 0.3^e	12.2 ± 0.1^f
WGLYH	5.2 ± 0.1^e	12.1 ± 0.3^f
1–10 kDa		
SGUTH	7.3 ± 2.4^d	26.8 ± 0.6^e
WGUTH	8.2 ± 3.1^d	28.3 ± 0.7^e
SGLYH	8.6 ± 0.2^d	32.1 ± 0.9^d
WGLYH	10.2 ± 0.9^c	27.2 ± 0.5^e
≤ 1 kDa		
SGUTH	15.3 ± 3.9^b	42.5 ± 0.7^c
WGUTH	14.3 ± 0.7^b	42.4 ± 1.1^c
SGLYH	15.6 ± 1.2^b	48.2 ± 0.9^b
WGLYH	18.4 ± 0.7^a	53.7 ± 1.2^a

The results represent mean \pm SD for three experiments. N.D. represents for not detected.

Different letters in each column indicate the significant difference between fractions and concentrations ($p < 0.05$).

Table 4

Comparison of the amino acid compositions of P1, P2, and P3 sub-fractions.

Amino acid	3-letter code	P1	P2	P3
Alanine**	Ala	2.5 ± 0.7^b	0.3 ± 0.1^c	3.4 ± 0.6^a
Cysteine**	Cys	1.2 ± 0.7^b	2.2 ± 0.4^a	1.9 ± 0.1^a
Aspartic acid**	Asp	9.9 ± 0.1^b	10.2 ± 0.3^b	15.4 ± 0.1^a
Glutamic acid**	Glu	3.5 ± 0.2^b	4.3 ± 0.1^a	4.0 ± 0.5^a
Phenylalanine*	Phe	0.0 ± 0.0	0.0 ± 0.0	1.9 ± 0.1
Glycine**	Gly	0.8 ± 0.1^c	1.1 ± 0.1^b	3.2 ± 0.7^a
Histidine*	His	2.3 ± 0.2^a	0.9 ± 0.1^b	0.6 ± 0.1^b
Isoleucine*	Ile	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 0.1
Lysine*	Lys	0.9 ± 0.1^a	0.2 ± 0.1^b	1.4 ± 0.1^a
Leucine*	Leu	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 0.2
Methionine*	Met	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1
Asparagine**	Asn	4.6 ± 0.1^b	3.3 ± 0.1^b	12.9 ± 0.2^a
Proline**	Pro	1.5 ± 0.9^c	7.9 ± 2.2^b	29.1 ± 0.6^a
Glutamine**	Gln	24.2 ± 0.1^a	25.1 ± 0.7^a	22.3 ± 0.3^b
Arginine**	Arg	2.9 ± 0.5^b	3.7 ± 0.6^a	3.2 ± 0.1^b
Serine**	Ser	7.5 ± 0.6^b	2.9 ± 0.1^c	30.5 ± 0.8^a
Threonine*	Thr	2.3 ± 0.9^b	4.8 ± 0.2^a	2.6 ± 0.5^b
Valine*	Val	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.1
Tryptophan*	Trp	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.1
Tyrosine**	Tyr	2.3 ± 0.2^b	2.2 ± 0.4^b	3.2 ± 0.5^a
% Amino acid distribution				
Essential AA*/ Non-essential AA**		0.1	0.1	0.1
Essential AA*/ Total AA (%)		8.5	8.7	10.5

Different letters in each column indicate the significant difference between fractions and concentrations ($p < 0.05$).

activity of sub-fractional peptides (P1–P3) may differ (Ibrahim et al., 2018). Hydrogen bonds and electrostatic interactions are proposed to be the main mechanisms by which inhibitory peptides interact with the active sites of α -glucosidase. It follows that the presence of amino acids with hydroxyl groups (i.e., tyrosine, threonine, and serine) or basic amino acids (i.e., arginine and lysine) at the N-terminal of the peptides might be critical to α -glucosidase inhibition (Table 4). The results of this study demonstrated that serine and lysine were the major amino acids

that inhibited enzymatic reactions in peptide fractions (Table 4). The P3 sub-fraction contained Gly (3.2 g/100 g) and Pro (29.1 g/100 g). The high inhibitory activity of this peptide could be attributed to its high Pro content. This is in line with previous reports where amino acids acted as α -glucosidase inhibitor. Pro, Gly, and Tyr derived from sardine muscle hydrolysate were reported as the most active compounds against diabetes (Elnur et al., 2021).

Food products that contain glycosidic bonds, such as carbohydrates and starches, are hydrolyzed more rapidly by α -amylase (1, 4, D-glucan glucanohydrolase). α -amylase inhibitors usually slow down the hydrolysis of (α -1,4) glycosidic bonds, delay the digestion of carbohydrates, and lower the blood glucose level after a meal, by slowing down the digestion of carbohydrates (Ren et al., 2020). In this study, the inhibitory activity of α -amylase was evaluated as a potential indicator of the antidiabetic response. The inhibitory activity was observed in the hydrolysates of the gluten subunit, which was fractionated with 4 different molecular weight cut-offs (Table 3). All hydrolysates showed inhibitory effect but the highest ($53.3 \pm 1.9\%$) α -amylase inhibitory activity was observed on the fine fractions of WGLYH while the coarse fractions (≥ 100 kDa) showed the lowest inhibitory activity ($6.1 \pm 1.5\%$). No significant difference was observed between these peptides with those over the range of 10–100 kDa. These results confirm that the size of the molecular peptide could affect the α -amylase inhibitory activity. Aludatt et al. (2016) found that the highest level of α -amylase inhibitory activity (66%) was obtained from prolamine peptides of barley protein fractions and the lowest values exhibited in glutenin fraction. Ngoh and Gan (2016) also confirmed that pinto bean peptides (< 3 kDa) had a very good α -amylase inhibitory activity ($62.1 \pm 3.5\%$) but larger polypeptides ($M_w > 100$ kDa) exhibited the lowest inhibition. The tartaric buckwheat protein hydrolysates, hydrolyzed by catalase, also significantly inhibited the activities of α -amylase (79%) and α -glucosidase (90%) (Tao et al., 2019). Although the specific relations between the structure and activity of these peptides for inhibiting α -glucosidase and α -amylase at the molecular level are not fully understood. However, it can be concluded that WGLYH peptide has potential for anti-diabetic applications and might contain anti-diabetic peptides.

Type 2 diabetes can be successfully controlled by blocking or limiting the activity of α -amylase (Arise, Yekken, & Emmanuel, 2016). It was suggested that bioactive peptides may interact or bind to the active site of the enzyme and prevent the binding of α -amylase to the substrate. Consequently, a less extensive interaction surface creates between the substrate and the enzyme, and a lesser number of interactions arise between them (Ngoh & Gan, 2016). An alternative mechanism for biopeptide inhibition involves the binding of biopeptides to the enzyme allosteric sites (such as calcium ions and chloride ions) to create an unstable conformation. These conformational changes can restrict enzyme displacement from substrates (Ibrahim et al., 2018). Some α -amylases can be inactive if calcium ions are removed because many of the functions, structures, and stability of organisms depend on calcium ions (Karimi et al., 2020). According to a recent report (Karimi et al., 2020), metal ion chelating and enzyme inhibition are strongly correlated. A new theory confirms that the interaction between chelator peptides and calcium ions in the enzyme structure is one of the reasons why the enzyme activity further decreases by the WGLYH fraction (Mousavi et al., 2022). The amino acids such as tyrosine, glycine, arginine, proline, lysine, glutamic acid, glutamine, aspartic acid, asparagine, and serine which were found in peptides in the present study (P1–P3, Table 4) have the potential to bind to active domains and block the enzyme (Ngoh & Gan, 2016). In the present study, the stronger enzymatic inhibition of the P3 fraction was likely due to the higher levels of some amino acids such as glycine, glutamic acid, glutamine, proline, aspartic acid, asparagine, leucine, and lysine. The current results supported the hypothesis of this study.

3.5. Fractionation via RP-HPLC

Small molecules and bioactive substances, especially those with $M_w < 1$ kDa, can be purified using RP-HPLC, a highly sensitive and rapid technique (Garcia, Far, Diesis, & Melnyk, 2006). The WGLYH obtained by ultrafiltration (< 1 kDa) was further purified by RP-HPLC (Fig. 2A). Three fractions were isolated, and their α -glucosidase and α -amylase inhibitory activities were measured. The results (Fig. 2B) revealed their different inhibition activities. The α -glucosidase inhibitory activities of P1, P2, and P3 sub-fractions were 9.4, 33.2, and 47.2%, respectively. According to these results, α -glucosidase inhibitory activities of P3 and P2 were more than P1 and the former (P3) was significantly ($p < 0.05$) higher than the others. Similarly, the α -amylase inhibition activity of P3 ($71.2 \pm 0.3\%$) was significantly higher than others (Fig. 2B). Though, that of P2 ($66.2 \pm 0.7\%$) was very close to P3.

An RP-HPLC system usually separates peptides and proteins based on their hydrophobicity. It is possible to separate different proteins rapidly under different media conditions with a high recovery rate, high efficiency, and other unique features. In the present study, the large retention time of fraction P3 indicates that it had low polarity and likely contained peptides with numerous nonpolar residues. To elucidate the relationship between the inhibition activity and amino acid composition of peptides, their (P1, P2, and P3) amino acid profiles were carefully determined. The hydrolysates, which were performed by kiwifruit actinidin, were further purified by RP-HPLC and 9 fractions were obtained. The F1 fraction (retention time 5.05 min) had the highest intensity and bioactivity (97.68% and 82.98% anticancer and antioxidant activity, respectively) (Mirzapour-Kouhdasht et al., 2020). The results of a study worked on antidiabetic peptides from Chinese giant salamander (*Andrias davidianus*) protein hydrolysate showed that among 14 fractions obtained from RP-HPLC assayed by α -amylase and α -glucosidase, only four peaks responded to inhibition and the activities were above 50%. The inhibitory activity, at IC_{50} of the fractions, indicated that the F8 (2.862×10^3 , 42.93 $\mu\text{g/mL}$) had the highest activity of α -amylase and α -glucosidase, respectively (Ramadhan et al., 2017). The hydrolysis of salmon skin collagen in a study showed that dipeptidyl peptidase IV (DPP-IV) inhibitory activities of RP-HPLC fractions such as A9–12 (retention time of 25–35 min) were significantly ($p < 0.05$) higher than those of A3–A8 fractions (retention time of 9–24 min). This may indicate the effective inhibition of DPP-IV activity by hydrophobic peptides. The concentration of ACN increased with elution time (within 35 min), which resulted in the elution of the hydrophobic peptide (Jin, Teng, Shang, Wang, & Liu, 2020).

3.6. Amino acid composition

Table 4 represents the amino acid composition of the most active antidiabetic fractions (P1, P2, and P3) derived from prep-RPH-HPLC of WGLYH (≤ 1 kDa). The essential/non-essential amino acid (EAA/NEAA) and essential /total amino acid (EAA/TAA) ratios of P1 (0.1, 0.1), P2 (0.1 and 8.5) and P3 (8.7 and 10.5) sub-fractions were calculated. According to FAO/WHO joint committee recommendations, in adults, the EAA/NEAA and EAA/TAA ratios should not be smaller than 0.6 and 40%, respectively. However, the results of the present study indicate that none of the fractions fell within the recommended ranges therefore enrichment of these peptides with essential amino acids or the utilization of appropriate pretreatment to release essential amino acids seems necessary. P3 also contained high content of aspartic acid (9.9–15.4 g/100 g), proline (1.5–29.1 g/100 g), and serine (7.5–30.5 g/100 g) which are vital for many bodily functions. Glutamic acid and glycine play critical roles in memory, learning, and neurotransmittance. A key role for arginine is in blood circulation, wound healing, erectile dysfunction treatment, and immune function (Lacroix & Li-Chan, 2012). The amino acids associated with biological activity in the P3 sub-fraction were 81 g/100 g of hydrophobic, 5.2 g/100 g of amino acids with a positive charge, 6.6 g/100 g of aromatic amino acids that were significantly ($p \leq$

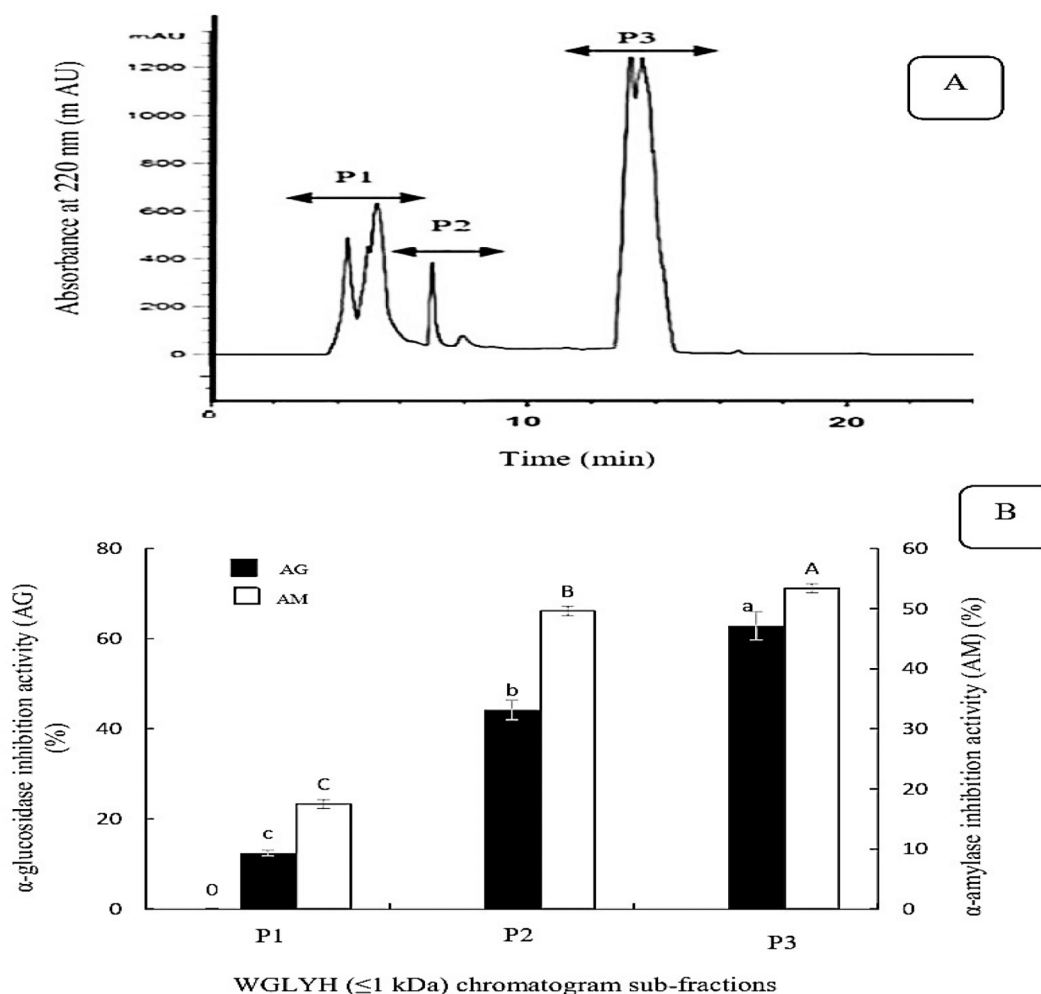


Fig. 2. RP-HPLC profile (A) and inhibitory activities (B) of different fractions of antidiabetic peptides obtained from chromatogram.

0.05) higher than the others. The major amino acids found in sub-fractions were glutamic acid (Glu), glutamine (Gln), aspartic acid (Asp), asparagine (Asn), proline (Pro), tyrosine (Tyr), threonine (Thr), arginine (Arg), and Cysteine (Cys). All sub-fractions were found to be rich in serine (2.5–30.5 g/100 g), proline (1.5–29.1 g/100 g), and glutamine (22.3–25.1 g/100 g). There were differences ($p < 0.05$) in the amino acid composition between the sub-fractions, which could be the reason why the antidiabetic activity of the P3 sub-fraction was slightly better as described in Section 3.4.

While most of these methods are effective in a laboratory setting, they cannot be usually used in large-scale applications due to high manufacturing costs. The unavailability of large-scale technologies and the high costs of purification techniques are major limiting factors for the marketing of peptide-based products. A majority (70%) of the capital and operational costs of industrial biotechnology processes are incurred in separation and purification (Brady, Woonton, Gee, & O'Connor, 2008). In this study, the hydrolysates of the gluten subunits were prepared, isolated, and identified. Recent advances in food science and technology have raised new hope for preventing, treating, and curing serious illnesses such as diabetes. Peptide therapeutics does offer a promising approach to address this concern. The physiological properties of proteins potentially candidate them for the development of therapeutic agents targeted at a biological activity in a living cell. Under optimal conditions, 10 g of wheat gluten was able to yield 1.4 g (14% w/w) of the purified peptides with α -glucosidase (18.4%) and α -amylase (53.3%) inhibitory activities. According to the present study, gluten subunit hydrolysate inhibitory efficiency was insufficient and low,

therefore further investigations are required to identify any efficient sources. The main causes of this decline are related to the type of protein source, low content of hydrophobic amino acids in peptides, and poor and costly purification techniques. Moreover, prior to the commercialization of these peptides as dietary supplements, their sensory properties should be further studied as bitterness is an inherent characteristic of peptides.

4. Conclusions

In this study, the kinetics of kiwifruit actinidin, which was extracted using different precipitation concentration procedures, were investigated. The ammonium sulfate precipitation method was selected as the most effective method and its recovery, purification fold, and specific activity were 95.9%, 3.6 folds, and 26 per mg of protein, respectively. The high value of K_{cat} and a low value of K_m were expected for the most appropriate substrates and it is very clear that the ammonium sulfate purification technique, due to its lower K_m than ultrafiltration, was the best choice for hydrolysis and peptide production. The SDS-PAGE analysis also confirmed the presence of actinidin (~29 kDa) in fractions prepared by various purification methods. The gluten hydrolysate subunits were digested using enzyme prepared with ammonium sulfate precipitation method and a significant difference between the peptide fractions was observed where WGLYH fractions (≤ 1 kDa) showed the highest α -amylase ($53.3 \pm 1.9\%$) and α -glucosidase ($18.4 \pm 0.7\%$) inhibitory activities. The peptides with the highest inhibitory activities were then identified by RP-HPLC and their amino acid compositions

were determined. The presence of high levels of amino acids with hydroxyl groups (tyrosine, threonine, and serine) or basic amino acid (arginine) and proline could be the reason why the antidiabetic activity of P3 sub-fraction was slightly better than the others. These were *in vitro* findings, therefore further *in vivo* investigations are needed to determine whether α -amylase and α -glucosidase inhibitory peptides could be used as agents for the prevention or treatment of type 2 diabetes.

CRedit authorship contribution statement

Babak Mousavi: Conceptualization, Investigation, Writing – original draft. **Mohammad-Hossein Azizi:** Supervision, Funding acquisition. **Soleiman Abbasi:** Supervision, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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