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Preparation of α-Amylase Inhibitor from Seeds of White Kidney Bean Using a Novel and Scalable Process Based on Enzymatic Hydrolysis

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Abstract: This study proposed and evaluated a novel and scalable process based on enzymatic hydrolysis for the preparation of α-amylase inhibitor (α-AI) in seeds of white kidney bean (*Phaseolus vulgaris*). The process mainly involved heat treatment (70°C/30 min), enzymatic hydrolysis, isoelectric precipitation and 70% ethanol precipitation. The optimal preparation parameters for enzymatic hydrolysis and isoelectric precipitation were as follows: Flavourzyme 500MG was used for enzymatic hydrolysis and the ultimate hydrolysate was obtained at 180 min, followed by isoelectric precipitation at pH 3.6. The loss of miscellaneous proteins and purification fold in the novel process were relatively low (85.84% and 4.74, respectively), while the α-AI activity yield (67.12%) was much higher than the values obtained by chromatography. Combined with the SDS-PAGE analysis, enzymatic hydrolysis proved to have modified the pI and alcohol-solubility of the miscellaneous proteins, which has a favorable influence on isoelectric precipitation and ethanol precipitation for the preparation of the α-AI.

Keywords: α-Amylase Inhibitor (α-AI), White Kidney Bean, Enzymatic Hydrolysis, Isoelectric Precipitation

1. Introduction

White kidney bean (*Phaseolus vulgaris*) is adapted to different ecological environments and has been widely planted in the world for centuries [1]. It is popular for the excellent nutrition in its seeds as well [2]. Since Bowman reported the presence of α-amylase inhibitor (α-AI) in seeds of white kidney bean, the α-AI has been comprehensively studied and its physico-chemical properties have also been elucidated [3-4]. Nowadays, the α-AI has been regarded as a potential remedy for the treatment of diabetes and obesity because of its higher inhibition activity on the α-amylase within human intestine, as well as its physiological safety [5-6]. Besides, the α-AI makes up about 9-11% of the total seed proteins, which could provide a substantial yield of the α-AI [7]. Consequently, there is a realistic meaning to prepare α-AI from seeds of white kidney bean especially in such a world where the incidence of diabetes and obesity is on a rise [8-9].

Common strategies that have been exploited to prepare α-AI from white kidney bean comprise salting out, supercritical carbon dioxide extraction, three phase partition, ion exchange chromatography and gel filtration column chromatography [10-14]. These methods always take advantage of the physical properties of the α-AI such as solubility, polarity, partition coefficient, charge property, molecular weight and so on. However, the chemical properties of the α-AI haven't been used for the preparation of the α-AI. Although the α-AI is a kind of protein in essence, it is highly resistant to the hydrolysis of digestive enzymes in human, for example, pepsin, trypsin and even physiological amount of chymotrypsin [15-17], which indicates the α-AI could survive from enzymatic hydrolysis. On the other hand, enzymatic hydrolysis is likely to greatly modify the properties of the miscellaneous proteins, such as pI and alcohol-solubility, which might help the α-AI to separate from the miscellaneous proteins. Accordingly, a novel process for the preparation of

the α-AI based on enzymatic hydrolysis is put forward. The novel process mainly consists of heat treatment (70°C/30 min), enzymatic hydrolysis, isoelectric precipitation and 70% ethanol precipitation, so its procedure is simple and easily scalable. In this case, it is worthwhile to use the novel process to prepare the α-AI.

Although some digestive enzymes would not affect the α-AI, the pH of the hydrolysis will inevitably cause adverse influences on the α-AI activity [18]. The α-AI tends to retain its activity in the condition with pH between 3 and 7, which is suitable for some commercial proteases [16]. Consequently, the proteases used in the novel process are picked out from five commercial proteases. The objective of the present study was to propose and evaluate a novel process based on enzymatic hydrolysis for the preparation of the α-AI in seeds of white kidney bean. The novel process took advantage of the chemical properties of the α-AI and thus enriched the methods for the preparation of the α-AI in seeds of white kidney bean.

2. Materials and Methods

2.1. Materials and Reagents

Seeds of white kidney bean were produced from Lijiang, Yunnan Province, China. Porcine pancreatic α-amylase was obtained from Sigma Chemical Company (St. Louis, MO, USA). Alcalase 2.4L, Flavourzyme 500MG and Neutrase 1.5MG were purchased from Novozymes. Prote AX and Protease N "Amano" G were purchased from Amano Enzyme Inc.. Among the five proteases, except that Alcalase 2.4L was liquid protease, the others were solid proteases. All other chemicals were of analytical grade.
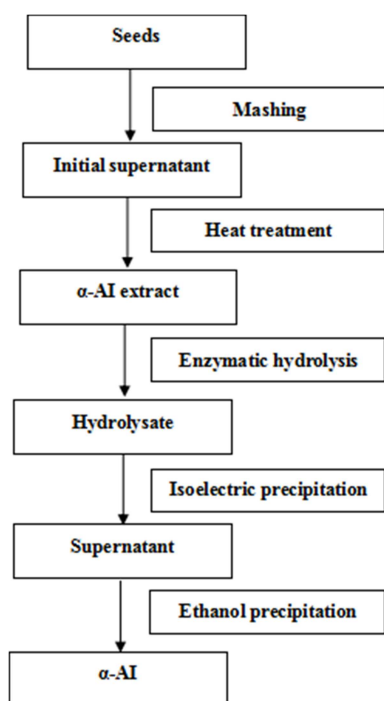
2.2. Process for the Preparation of the α-AI

Figure 1. The novel process for the preparation of the α-AI.

The process to obtain the α-AI was shown in Figure 1. The seeds were mashed with deionized water (1:4 w/v) for 10 min in a waring blender. The suspension was then passed through a 150-mesh screen to separate the fiber solids [19]. This filtered suspension was left to stand for 1 h to eliminate the starch from the initial supernatant [20]. The initial supernatant was then heated at 70°C for 30 min and then the supernatant called α-AI extract (protein concentration 25 mg/mL, pH 6.5) was collected by centrifugation (10,000 r/min, 20 min) [21]. α-AI extract was thoroughly hydrolyzed by a moderate protease. The ultimate hydrolysate was adjusted to moderate pH by using 1 mol/L HCl and stayed for 1 h. The supernatant obtained by centrifugation (10,000 r/min, 20 min) was adjusted to pH 6.9 with 1 mol/L NaOH and then to 70% ethanol at 4°C. After 2 h of stirring at 4°C, the α-AI was obtained by volatilizing the remnant ethanol in the precipitate collected by centrifugation (10,000 r/min,20 min) [21].

2.3. Enzymatic Hydrolysis

100 mL α-AI extract was hydrolyzed by proteases and hydrolysis curves were obtained using the hydrolysis parameters as follows: protein concentration 25 mg/mL; enzyme/substrate ratio 1/10 (w/w) for solid proteases and 1/300 (v/v) for liquid protease; pH 6.9; temperature 50°C. The pH was maintained by 1 mol/L NaOH. The hydrolysis was stopped by heating to 80°C for 20 min [22]. The supernants obtained by centrifugation (10,000 r/min, 20 min) were used for degree of hydrolysis measurements.

2.4. Selection of Proteases Used for Enzymatic Hydrolysis

100 mL α-AI extract was thoroughly hydrolyzed by Alcalase 2.4L, Flavourzyme 500MG, Neutrase 1.5MG, Prote AX and Protease N "Amano" G respectively. The proteases in the hydrolysates were inactivated by extremely acidic treatment, followed by adjusting the pH of the hydrolysates to 6.9 with 1 mol/L NaOH. The supernatants collected by centrifugation (10,000 r/min, 20 min) were then adjusted to 70% ethanol at 4°C. After 2 h of stirring at 4°C, the precipitates collected by centrifugation (10,000 r/min, 20 min) were dissolved in 100 mL 0.2 mol/L PBS buffer (pH 6.9) after volatilizing the remnant ethanol. α-AI extract which hadn't been hydrolyzed was served as a control. The α-AI activities of the precipitates were measured after having been diluted 8 times. All dilutions of the α-AI solutions obtained in the research were performed with 0.2 mol/L PBS (pH 6.9).

2.5. Optimization for Proteases Used for the Process

Ultimate hydrolysates obtained from 100 mL α-AI extract by enzymatic hydrolysis with the selected proteases were adjusted to differnt pH (6.0, 5.2, 4.4 and 3.6) by using 1mol/L HCl and precipitated for 60 min. The supernatants obtained by centrifugation (10,000 r/min, 20 min) were adjusted to pH 6.9 with 1 mol/L NaOH and then to 70% ethanol at 4°C. After 2 h of stirring at 4°C, the precipitates (the α-AI) collected by centrifugation (10,000 r/min, 60 min) were dissolved in 100 mL 0.2 mol/L PBS buffer (pH 6.9) after volatilizing the

remnant ethanol. α-AI extract was served as a control without enzymatic hydrolysis. The protein contents of both the supernatants and the precipitates were determined. Meanwhile, the α-AI activities of the supernatants obtained in the control were measured after having been diluted 35 times.

2.6. Determination for α-AI Activity Losses

In order to determine the influence of heat treatment (70°C/30 min) and 70% ethanol precipitation on the α-AI activity, 100 mL α-AI extract obtained by heat treatment (70°C/30 min) from the initial supernatant (Method 2.2) was adjusted to pH 6.9 and then to 70% ethanol at 4°C. After 2 h of stirring at 4°C, the resulting precipitate collected by centrifugation (10,000 r/min, 20 min) were dissolved in 100 mL 0.2 mol/L PBS buffer (pH 6.9) after volatilizing the remnant ethanol. The initial supernatant, α-AI extract and the resulting precipitate were used for the α-AI activity measurement after having been diluted 35 times.

2.7. Determination for Degree of Hydrolysis

Degree of hydrolysis (DH) was calculated by determining free amino groups by reaction with TNBS [23]. For seeds of white kidney bean, the total number of peptide bonds per protein equivalent was 7.66 mmol/g of protein [19].

2.8. Assay for α-AI Activity

The α-AI activity was measured according to Yang with some modifications [21]. In this reaction system, the concentration of soluble starch solution added was 1% (w/v) and the volume of 3, 5-ditrosalicylic acid used was 1 mL. The α-AI activity was expressed as the inhibitory rate which was the ratio of the inhibited α-amylase activity and the total α-amylase activity. According to Marshall and Lauda, the α-AI activity was proportional to the amount of the α-AI when no more than approximately 50% inhibition of the α-amylase activity took place [14]. In order to ensure the inhibitory rates below 50%, samples had been moderately diluted before measurements. In the present study, the dilution times of the α-AI solutions in a consistent procedure were kept same, so the changes of the inhibitory rates were equal to those of the α-AI contents. Protein content was measured by the Lowry method, using bovine serum albumin as the standard [24].

2.9. Extremely Acidic Treatment on Proteases

50 mg solid proteases and 0.25 mL liquid protease were respectively dissolved in 50 mL 0.2 mol/L PBS buffer (pH 6.9), followed by adjusting the pH of the enzyme solutions to 2.0 with 1 mol/L HCl at room temperature. Keep this treatment for 30 min with stirring and then adjust the pH back to 6.9 with 1 mol/L NaOH. Determinate the protease activities before and after the treatment according to Maria [25].

2.10. Polyacrylamide Gel Electrophoresis and Statistical Analysis

SDS–PAGE of solutions with 12% (w/v) gel strength was performed at a constant voltage (80 V) in a MiniProtean Tetra system with PowerPac Basic Power Supply (Bio-Rad Laboratories, Inc., Richmond, CA) [26]. Protein markers, α-lactalbumin (14.4 kDa); trypsin inhibitor (20.1 kDa); carbonic anhydrase (31.0 kDa); ovalbumin (43.0 kDa); bovine serum albumin (66.2 kDa); phosphorylase B (97.4 kDa) were used as standard molecular weight markers. Following electrophoresis, the gels were stained by the Coomassie blue G-250 method.

Values were given as mean of three determinations ± standard deviation (SD). Statistical calculation employed a one-way analysis of variance (ANOVA) test by using SPSS version 17 and means were separated using a least significant difference (LSD) test at $p < 0.05$.

3. Results and Discussion

3.1. Selection of Proteases Used for Enzymatic Hydrolysis

3.1.1. Duration for Enzymatic Hydrolysis

Figure 2 showed the hydrolysis curves of the five proteases. For Flavourzyme 500MG, the rate of hydrolysis was nearly constant and its degree of hydrolysis (6.46%) was the lowest of all. For the others, the rates of hydrolysis were much higher at the beginning and tended to decrease with the duration of time. Prote AX had the highest initial rate of hydrolysis and the highest degree of hydrolysis (25.55%). What's in common was that the degree of hydrolysis reached a maximum at 180 min, which meant the proteases had interacted thoroughly with the proteins including the α-AI and the ultimate hydrolysates had also been obtained at this time.
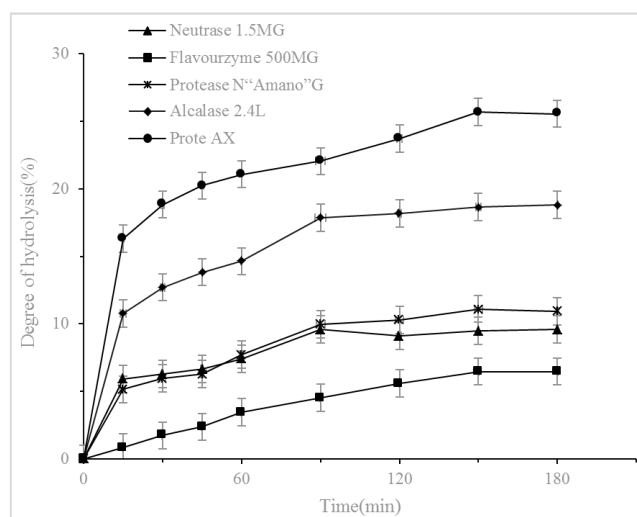

Figure 2. Hydrolysis curves of the five proteases.

3.1.2. Inactivation Effect of Extremely Acidic Treatment on Proteases

It was well known to all, proteases would interfere in the determination of the α-AI activity, so it was necessary to inactivate proteases in the hydrolysates. Although heat treatment at 80°C caused a subtle activity loss of the α-AI in cranberry bean, it nearly destroyed the α-AI activity in the

present research, which might attribute to the differences of the varieties [6]. Thus, an extremely acidic treatment was exerted to inactivate the proteases instead of heat treatment. Table 1 showed the losses of protease activities caused by such a treatment. Except that Prote AX remained subtle activity (0.76%), the other four proteases were all irreversibly inactivated. Although Prote AX wouldn't be thoroughly inactivated, the residual activity was so low and the preincubation period (10 min) was so short that Prote AX could hardly perform its function and might have no influence on the determination of the α-AI activity [21]. Consequently, the extremely acidic treatment was applicable to inactivate the proteases. Because the α-AI concentration in α-AI extract was pretty high, the extremely acidic treatment wouldn't lead a low α-AI activity which was difficult to determine exactly. It was the reason why only high concentration of the α-AI was effective in vivo [27]. Besides, the α-AI would get into gastric environment, so it was not necessary to inactivate the proteases during the process.

Table 1. *Activity changes of proteases when treated by extremely acidic treatment.*

Protease	Activity unit	Activity before treatment	Activity after treatment	Residual activity (%)
Prote AX	U/g	1927.62±0.00	14.58±1.30 a	0.76
Alcalase 2.4L	U/mL	1286.11±19.67	-1.61±0.34 b	0
Neutrase 1.5MG	U/g	631.15±1.04	-5.03±0.25 b	0
Flavourzyme 500MG	U/g	208.40±2.07	-0.76±1.01 b	0
Protease N "Amano" G	U/g	4411.90±122.03	0.27±1.63 b	0

Values with same letters within one column are not significantly different at $p < 0.05$.

3.1.3. Suitable Proteases for Enzymatic Hydrolysis

During enzymatic hydrolysis, the short peptides produced might possess α-AI activity and should be separated from the α-AI in case of interference [28]. Because of the excellent solubility of short peptides in high concentration of ethanol, the ultimate hydrolysates with the proteases inactivated were precipitated with 70% ethanol to separate the short peptides from the α-AI. Table 2 showed the loss of the α-AI activity caused by enzymatic hydrolysis with the five proteases. Compared with the control, Flavourzyme 500MG and Neutrase 1.5MG had no significant effect on the α-AI activity ($p<0.05$) and the loss of the α-AI activity caused by them was 3.54% and 2.67% respectively. The other proteases significantly reduced the α-AI activity ($p > 0.05$) and the influence of Alcalase 2.4L on the α-AI activity was the greatest of all (88.53%). Consequently, Flavourzyme 500MG and Neutrase 1.5MG were suitable for enzymatic hydrolysis.

Table 2. *The loss of the α-AI activity caused by enzymatic hydrolysis with the five proteases.*

Protease	Inhibitory rate (%)	Activity loss (%)
Flavourzyme 500MG	41.12±0.91 a	3.54
Protease N "Amano" G	31.52±0.12 b	26.06
Alcalase 2.4L	4.89±0.66 d	88.53
Neutrase 1.5MG	41.49±0.36 a	2.67
Prote AX	28.45±0.36 c	33.26
Control	42.63±0.36 a	0

Values with same letters within one column are not significantly different at $p < 0.05$.

3.2. Optimization of Proteases Used for the Novel Process

3.2.1. Effects of Isoelectric Precipitation on the α-AI

Table 3 showed the α-AI activities in the supernatants obtained from the α-AI extract by isoelectric precipitation at pH between 3.6 and 6.0. Compared with α-AI extract, the α-AI activities in the supernatants reduced with the decrease of the pH while the differences were not significant ($p > 0.05$). The loss of the α-AI activity at pH 6.0 and 3.6 was 0.64% and 5.34% respectively (Table 3). This result demonstrated few of the α-AI precipitated in acidic solutions. The stability of the α-AI activity in acidic solutions was accordance with the conclusion reported by Golparvar & Naseri [16]. Although the pI of the α-AI ranged from 4.0 to 6.0, the α-AI near pI did't sediment, which might attribute to its carbohydrate contents which ranged from 8.6% to 15% and accordingly improved its solubility [4, 15]. Consequently, during isoelctric precipitation, the α-AI nearly had no loss and even distributed in one phase, which made it easy to recover all of the α-AI. Thus, isoelectric precipitation was an ideal method for the preparation of the α-AI.

Table 3. *The α-AI activities in the supernatants obtained by isoelectric precipitation.*

Isoelectric precipitation	Inhibitory rate (%)	Activity loss (%)
α-AI extract	24.92±0.72 a	
Supernatant		
pH 6.0	24.76±1.37 a	0.64
pH 5.2	24.30±0.80 a	2.49
pH 4.4	23.79±0.72 a	4.53
pH 3.6	23.59±0.64 a	5.34

Values with same letters within one column are not significantly different at $p < 0.05$.

3.2.2. Optimal Operation Parameters for the Process

Two ultimate hydrolysates (hydrolysate F and hydrolysate N) obtained by hydrolysis with Flavourzyme 500MG and Neutrase 1.5MG respectively were precipitated at pH between 3.6 and 6.0. Table 4 showed the protein contents in the supernatants. The pH had a significant influence on protein contents in the supernatants and the protein contents significantly decreased with the decrease of the pH ($p<0.05$). At the same pH used for isoelectric precipitation, the protein contents in the supernatants deriving from hydrolysate F were significantly lower than those deriving from hydrolysate N. In order to collect the α-AI, the supernatants were precipitated with 70% ethanol and the protein contents of the precipitates (the α-AI) were presented in Table 4. The protein contents of

the α-AI were always less than those of the corresponding supernatants. The pH used for electric precipitation had a significant influence on protein contents in the α-AI and the protein contents significantly reduced with the decrease of the pH ($p<0.05$). Consequently, isoelectric precipitation at pH 3.6 eliminated the most of miscellaneous proteins. At the same pH, the protein contents of the α-AI deriving from hydrolysate F were significantly lower than the ones deriving from hydrolysate N ($p<0.05$), so Flavourzyme 500MG was more suitable for the preparation of the α-AI considering the purity of the α-AI.

So far, the optimal operation parameters of the novel process described in 2.2 had been obtained as follows: the protease used for enzymatic hydrolysis was Flavourzyme 500MG; the hydrolysis time was 180 min; the pH used for isoelectric precipitation was 3.6.

3.3. Evaluation for the Novel Process

The novel process was evaluated by the α-AI activity yield and the loss of miscellaneous proteins. As Flavourzyme 500MG was introduced into the process, it was hard to directly determine the changes of the α-AI activity during the process because of the interference. Thus, the α-AI activity yield was determined indirectly by calculating the losses of the α-AI activity caused by influential factors which included heat treatment (70°C/30 min), enzymatic hydrolysis, isoelectric precipitation and 70% ethanol precipitation. The α-AI activities of the initial supernatant, α-AI extract and the resultant precipitate were 31.64%, 24.92% and 23.26% respectively, which indicated the loss of the α-AI activity caused by heat treatment (70°C/30 min) and ethanol precipitation was 21.24% and 6.66% respectively. Besides, enzymatic hydrolysis and isoelectric precipitation caused 3.54% and 5.34% loss of the α-AI activity respectively as mentioned in 3.1.3 and 3.2.1. Consequently, the α-AI activity yield was 67.12% in total (Table 5). The value was much higher than the result (37%) presented by Marshall and Lauda who had used CM-cellulose chromatography [14]. The protein contents in different steps of the process were presented in Table 5. Every procedure could significantly reduce the protein contents ($p<0.05$) except for enzymatic hydrolysis. 85.84% of the miscellaneous proteins were eliminated by the novel process. The loss of miscellaneous proteins was much lower than the result (94.96%) reported by Mridu et al. who had used ion exchange chromatography, which lead to a relatively low purification fold (4.74) [13]. However, according to Jay et al., the α-AI was only effective at high doses in vivo, thus the α-AI activity yield might be the most important indicator considering the practical application of the α-AI [29]. Consequently, the novel process could better ensure the efficacy of the α-AI.

Table 5. The loss of miscellaneous proteins, the α-AI activity yield and purification fold during the novel process.

Step	Protein content (mg)	Protein loss (%)	Activity yield (%)	Purification fold
Initial supernatant	2064.30±55.76 a	0	100	1
α-AI extract	1255.87±22.94 b	39.16	78.76	1.29
Hydrolysate	1227.23±4.63 b	40.55	75.97	1.28
Supernatant	481.20±24.55 c	76.69	71.91	3.09
α-AI	292.39±2.31 d	85.84	67.12	4.74

Values with same letters within one column are not significantly different at $p < 0.05$.

3.4. SDS-PAGE Analysis

SDS-PAGE analyses of the proteins in different steps of the novel process were shown in Figure 3 (A). The α-AI obtained by the process contained two peptide fractions with the molecular weight between 14.4 and 20.1 kDa, together with another two peptide fractions around 28 to 32 kDa (Figure 3 (A), Lane e). This profile was similar to the α-AI profile for a common bean mutant obtained by three phase partition [12]. The α-AI purified from seeds of white kidney bean should have ranged between 36 and 56 kDa, which might be because the α-AI was dissolved into relatively smaller peptides during electrophoresis [30]. Although heat treatment (70°C/30 min) reduced 39.16% of the protein contents in the initial supernatant, the protein profile of the initial supernatant and α-AI extract were the same (Figure 3 (A), Lane a and Lane b), which might be because the heat-sensitive proteins were in a relatively high amount and couldn't be effectively eliminated by the heat treatment (70°C/30 min). Compared with α-AI extract (Figure 3 (A) Lane b), the bands of peptide fractions with molecular weight above 97.2 kDa and near 29 kDa (Figure 3 (A) Lane b) slightly moved downward and two peptide fractions with lower molecular weight above 97.2 kDa

and near 27 kDa appeared in the hydrolysate (Figure 3 (A) Lane c), which indicated some new proteins with smaller molecular weight might be produced by enzymatic hydrolysis.
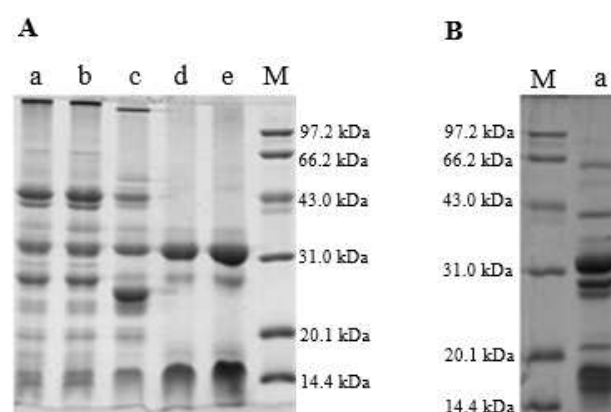

Figure 3. SDS–PAGE analysis of (A): Lane a: initial supernatant; Lane b: α-AI extract; Lane c: hydrolysate; Lane d: supernatant; Lane e: precipitate; Lane M: standard molar mass markers; and (B): Lane M: standard molar mass markers; Lane a: the supernatant obtained from α-AI extract without enzymatic hydrolysis by acidic precipitation at pH 3.6.

Although two chief peptide fractions with molecular weight near 32 and 16 kDa were nearly close to each other in the supernatant deriving from the hydrolysate and α-AI extract (Figure 3 (A), Lane d; Figure 3 (B), Lane a), the peptide fractions in the supernatant deriving from the hydrolysate were less than those in the supernatant deriving from α-AI extract, which was accordance with the significant differences ($p<0.05$) in protein contents of the two supernatants (Table 4). On the other hand, in the supernatant deriving from the hydrolysate, the third chief peptide fraction near 29 kDa in the supernatant deriving from α-AI extract turned much weaker and the newly produced peptide fraction near 27 kDa in the hydrolysate even disappeared. This result demonstrated that enzymatic hydrolysis was in favor of isoelectric precipitation by altering the pI of more miscellaneous proteins to 3.6.

During the novel process, although the peptide fractions of the supernatant and the α-AI were the same (Figure 3 (A), Lane d and Lane e), 39.24% of the miscellaneous proteins in the supernatant were eliminated by ethanol precipitation. As to the supernatants obtained in the control, the highest miscellaneous protein loss caused by ethanol precipitation was 21.67% (Table 4). This might attribute to the existence of the newly produced proteins with smaller molecular weight. Because of the relatively low molecular weight, these proteins weren't presented in the SDS-PAGE and were able to disperse in higher concentration of ethanol. Consequently, enzymatic hydrolysis was in favor of ethanol precipitation to eliminate the miscellaneous proteins by elevating the amount of alcohol-soluble proteins.

Table 4. *Protein contents in the supernatants and the precipitates (the α-AI) when the ultimate hydrolysates obtained from the α-AI extract were subjected to isoelectric precipitation, followed by ethanol precipitation. Meanwhile, the α-AI extract was served as a control without enzymatic hydrolysis.*

Treatment conditions	Protein contents (mg)	
	Supernatants obtained by acidic precipitation	Precipitates obtained by ethanol precipitation
α-AI extract	1255.87±22.94* a	
Control		
pH 6.0	783.69±30.10 de	628.44±14.46 c
pH 5.2	582.14±19.63 fg	461.23±9.57 de
pH 4.4	570.47±16.58 fg	446.85±13.90 de
pH 3.6	511.73±22.85 fg	462.60±17.64 de
Flavourzyme 500MG		
pH 6.0	1086.44±37.04 b	851.47±3.47 b
pH 5.2	739.35±16.11 de	559.77±45.14 c
pH 4.4	601.39±6.93 f	393.09±28.94 e
pH 3.6	481.20±24.55 g	292.39±2.31 f
Neutrase 1.5MG		
pH 6.0	1212.40±51.15 a	1021.05±38.85 a
pH 5.2	943.98±51.15 c	839.10±30.08 b
pH 4.4	827.40±67.65 d	609.59±18.56 c
pH 3.6	708.52±2.90 e	478.26±20.05 d

* This value presented here referred to the protein content of the α-AI extract.
Values with same letters within columns are not significantly different at $p<0.05$.

4. Conclusions

Flavourzyme 500MG and Neutrase 1.5MG had no significant influence on the α-AI activity ($p<0.05$), which indicated the α-AI was highly resistant to the hydrolysis of the two proteases. Besides, the α-AI had a quite good solubility in acidic solutions with pH between 3.6 and 6.0, where the α-AI activity had no significant decrease ($p<0.05$). On the other hand, the novel process based on enzymatic hydrolysis proved feasible for the preparation of the α-AI in seeds of white kidney bean. Although the loss of miscellaneous proteins and purification fold in the novel process were relatively low, the α-AI activity yield was higher than the methods which used chromatography, which was crucial for the practical application of the α-AI. Combined with the SDS-PAGE analysis, enzymatic hydrolysis proved to have modified the pI and alcohol-solubility of the miscellaneous proteins, which had a favorable influence on isoelectric precipitation and ethanol precipitation.

Acknowledgements

Author Contributions

Wei Zhao designed the study and interpreted the results. Yifeng Rang further adjusted the study design, collected test data and drafted the manuscript.

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