CHAPTER III

Unveiling Optimal Extraction Conditions for Potent Alpha-Amylase Inhibitors from White Kidney Beans

3.1 Abstract

This study refines the extraction of alpha-amylase inhibitors, a valuable health-promoting compound, from white kidney beans (*Phaseolus vulgaris*). We employed a data-driven approach to identify the perfect balance between maximizing extract yield and preserving its inhibitory activity.

A Box-Behnken design strategically varied three key factors: solvent concentration, extraction time, and separation time. Response Surface Methodology (RSM) then analyzed the results, revealing a significant correlation between the chosen parameters and the extracted alpha-amylase inhibitory activity. This confirmed the effectiveness of both methods in optimizing the process.

The study analyzes the proximate analysis the composition of white kidney bean, 11.01% moisture, 3.50% ash, 0.06% sand, 0.45% fat, 5.13% crude fiber, and 19.43% protein, identified the ideal extraction conditions as 0.1 M phosphate-buffered saline (PBS), 1 hour extraction, and 30 minutes separation. This optimized approach paves the way for efficient and targeted extraction of alpha-amylase inhibitors from white kidney beans, potentially leading to the development of more potent health supplements and functional food ingredients.

Keywords: White kidney bean *(Phaseolus vulgaris)*, Alpha-amylase, inhibitory activity, Optimization

3.2 Introduction

Archaeological investigations suggest that white kidney beans (*Phaseolus vulgaris*) and other common beans originated in the Americas, specifically the southern United States, Mexico, Central America, and the northern Andes (Geil & Anderson, 1994;

Carai et al., 2009). Introduced to Europe in the 16th century, *P. vulgaris* has since become a vital crop worldwide. Its nutritional value for humans and animals stems from its high content of protein, complex carbohydrates, and dietary fibers (Geil & Anderson, 1994; Carai et al., 2009).

White kidney beans are known for their alpha-amylase inhibitors, first reported by (Bowman, 1945). These inhibitors are concentrated in the embryonic axes and cotyledons, absent in other plant parts (Marshall & Lauda, 1975). They are glycoproteins that specifically inhibit alpha-amylase activity in mammals and insects, leaving plant amylases unaffected (Marshall & Lauda, 1975; Moreno et al., 1990). Studies have shown that *P. vulgaris* extracts can lower body weight and glycemia in animals (Fantini et al., 2009; Song et al., 2016; Micheli et al., 2019).

This study employs a randomized Box-Behnken experimental design to optimize the extraction conditions for alpha-amylase inhibitors from white kidney beans. The research goal is to identify the conditions that yield the highest percentage of extract and the most potent alpha-amylase inhibitory activity.

3.3 Material and Methods

3.3.1 Sample preparation

White kidney bean (*Phaseolus vulgaris*) cultivar Pangda 2 was obtained from Royal Project Foundation, Thailand, was dried and grounded to powder, sieved by mesh size 2 mm, and kept in a vacuum package at 4 °C until used.

3.3.2 Proximate analysis

Dry white kidney beans powder was analyzed for moisture, ash, sand, fat, crude fiber and protein

3.3.2.1 Moisture (AOAC 925.10)

Empty moisture cans were putted in hot air oven at 110°C for 30 min then putted in desiccator for 30 min and weight. White kidney bean powders were placed in moisture cans around 1 gram and putted in hot air oven at 110°C for 24 hour and

then put in desiccator for 30 min and weight. The percentage moisture content was calculated by the formulation:

$$\%\text{Moisture content} = \frac{(B - C - A) \times 100}{B}$$

Where:

A = empty moisture can

B = weight of sample

C = weight of sample + moisture can after oven

3.3.2.2 Ash (AOAC 900.02A)

Empty crucibles were putted in muffle furnace at 550 ℃ for 3 hours then putted in desiccator for 30 min and weight. White kidney bean powders were placed in crucibles around 1 gram and putted in muffle furnace at 550 ℃ for 12-18 hour and then put in desiccator for 30 min and weight. The percentage ash content was calculated by the formulation:

$$\%\text{Ash content} = \frac{(B - C - A) \times 100}{B}$$

Where:

A = empty crucible

B = weight of sample

C = weight of sample + moisture can after furnace

3.3.2.3 Sand (AOAC 900.02D)

Crucibles with ash from ash analysis were added 10% hydrochloric acid and cover with watch glass, boil around 5 min, then filed through ashless filter paper and rinsed with hot water several times. Put filter paper in the same crucible, and put crucibles in muffle furnace at 550 ℃ for 12-18 hours and then put in desiccator for 30 min and weight. The percentage sand content was calculated by the formulation:

$$\%\text{Sand content} = \frac{(B - C - A) \times 100}{B}$$

Where:

A = empty crucible from ash analysis

B = weight of sample from ash analysis

C = weight of sample + moisture can after furnace

3.3.2.4 Crude fat (AOAC 945.16)

Cup and wool were putted in hot air oven at 110°C for 1 hour then putted in desiccator for 30 min, weighted cups then put in cup holder. White kidney bean powders were placed in filter paper around 1 gram wrapped up and putted in thimble with wool cover upper and lower then putted Soxtec extractor raised thimble up placed cups than add petroleum ether 80 ml in each cup. Run the Soxtec extractor around 3 hours. Draw wrapped filter paper with sample inside out of Trimble, putted its and cups in hot air oven at 110°C for 30 min then putted in desiccator for 30 min for cups but until used in crude fiber analysis for wrapped filter paper. Weight cups. The percentage sand content was calculated by the formulation:

$$\%\text{Crud fat content} = \frac{(C - A) \times 100}{B}$$

Where:

A = empty cup

B = weight of sample

C = weight of cup after extraction

3.3.2.5 Crude fiber (AOAC 978.10)

Empty crucibles were putted in muffle furnace at 500 °C for 30 hours then putted in desiccator for 30 min and weight. Weight defatted samples from crude fat analysis in crucibles then load crucibles to hot extractor poured 1.25% sulfuric acid and 1.25% sodium hydroxide to tank in hot extractor then composed each of hot extractor and run around 3 hours. After hot extraction, moved crucibles out washed samples with 95% ethanol 10 ml. Put crucibles in hot air oven at 105 °C for 3 hours then putted in desiccator for 30 min and weight then and putted in muffle furnace at 500 °C for 2 hours then putted in desiccator for 30 min and weight. The percentage crude fiber content was calculated by the formulation:

$$\%\text{Crude fiber content} = \frac{(B - C) \times 100}{A}$$

Where:

A = weight of sample

B = weight of crucible after hot air oven

C = weight of crucible after muffle furnace

3.3.2.6 Crude protein (AOAC 928.08)

0.5 N hydrochloric acid were standardized with titration with sodium tetraborate conducted by weight 5 gram of sodium tetraborate adjusted volume to 250 ml, diluted 0.5 N hydrochloric acid for 10 times, drop methyl red 2 drop and titrate with sodium tetraborate solution, the end point was changing of color from pink to no color.

White kidney bean powders were weight around 1 gram to Kjehdalh flask follow by potassium sulfate and copper sulfate 7 and 0.7 gram respectively, and did blank with same condition without sample. Add 20 ml of sulfuric acid then load in Kjedalh digestion apparatus and digested in 400 °C for 2 hours until become green color. Pour 50 ml of boric acid in Erlenmeyer flask and dropped mixed indicator 4-5 drop then placed under Kjeldahl Distillation system with tube insert in flask, Load Kjehdalh flask in to Kjeldahl Distillation system settled condition, 80 ml of distillated water and 80 ml of 32% sodium hydroxide, and run for 6 min. Brought Erlenmeyer flask to titrate with definite concentration 0.5 N hydrochloric acid, the end point was changing of color from green to pink. The percentage nitrogen content was calculated by the formulation and multiply with protein factor of white kidney bean 6.25.

$$\%N = \frac{\text{Normality of HCl}}{1000} \times \frac{V_s - V_b}{w} \times 14 \times 100$$

Where:

%N = percentage nitrogen

V_s = volume of acid used for sample

V_b = volume of acid used for blank

w = weight of sample

%crude protein = %N x 6.25

3.3.3 White kidney bean extraction

The extraction method was adapted from (Mosca et al., 2008; Manatwiyangkool, 2014). Briefly, ground white kidney beans were suspended in varying PBS (phosphate-buffered saline) solutions (0.05 M, 0.1 M, 0.15 M) at a ratio of 1.65 g beans to 10 ml PBS (10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl). The suspension was stirred for 1, 2, or 3 hours at room temperature. Subsequently, centrifugation was performed at 10,000 rpm for 30, 45, or 60 minutes at 4°C. The supernatant was collected, and the volume was adjusted to 10 ml using the corresponding PBS solution (0.05 M, 0.1 M, 0.15 M). Two milliliters of the adjusted supernatant were then aliquoted into separate tubes (5 tubes total). Finally, the aliquots were freeze-dried and stored at -20°C until further use.

3.3.4 Protein concentration

Method of Bradford (1976) using BSA as a standard. Prepare a series of protein standards using BSA diluted with 0.15 M NaCl to final concentrations of 0 (blank = NaCl only), 0.1, 0.2, 0.3, 0.5, 0.7 and 1 mg BSA/mL. Also prepare serial dilutions of the unknown sample to be measured. Add 100 µL of each of the above to a separate test tube. Add 5.0 mL of Coomassie Blue to each tube and mix by vortex, or inversion. Adjust the spectrophotometer to a wavelength of 595 nm, and blank using the tube which contains 0 BSA. Wait 5 minutes and read each of the standards and each of the samples at 595 nm wavelength. Plot the absorbance of the standards vs. their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

3.3.5 Specific α-amylase inhibitory activity measurement

Specific α-amylase inhibitory activity was measured using the method described by Bernfeld (1955) with modifications, by measuring the residual α-amylase

activity. The αAI and 40 unit/ml porcine pancreas α-amylase solution in succinate buffer (15 mM, 20 mM $CaCl_2$, and 0.5 M NaCl, pH 5.6) was preincubated at 37 ˚C for 30 min, after that 2% (w/v) soluble starch solution was added and incubated at 37 ˚C for 1 min. The reaction was stopped by addition of 0.8 ml of dinitrosalicylic acid reagent (DNS) solution and boiled for 10 min then cooled and diluted with 6 ml of water then the absorbance was read at 540 nm. Appropriate blank was prepared without α-amylase also the residual activity was measured. A control without enzyme inhibitor addition was done for the specific inhibition activity calculation.

$$\text{Inhibition} = \frac{\text{liberated maltose in control} - \text{liberated maltose in sample}}{\text{sample}}$$

$$\text{Specific inhibitory activity} = \frac{\text{Inhibition}}{\text{mg protein}}$$

3.3.6 Experimental design

Response surface methodology (RSM) was chosen to identify the optimal settings for three critical variables influencing both extract yield and alpha-amylase inhibitory activity. These variables, PBS concentration (denoted as X_1), extraction time (X_2), and separation time (X_3) were investigated using a randomized Box-Behnken design. This specific design ensures all experiments are conducted in a fully randomized order, minimizing bias and strengthening the statistical analysis (Table 3.1).

Table 3.1 Encoded and coded levels of independent variables used in the experimental design.

Symbols	Independent variables	Coded levels		
		-1	0	1
X_1	PBS concentration (M)	0.05	0.1	0.15
X_2	Extraction time (hour)	1	2	3
X_3	Separation time (min)	30	45	60

3.4 Results and Discussion

3.4.1 Proximate analysis results

Proximate analysis results showed in Table 4.4. These results had each attribute, except fat, and crude fiber, near Manastwiyangkool (2014) who reported composition of whit kidney bean, 11.07% moisture, 4.10% ash, 0.01% sand, 1.8%fat, 31.73% crud fiber, and 20.28% protein.

Table. 3.2 Proximate analysis

Nutrient	% Composition
Moisture	11.01 ± 0.05
Ash	3.50 ± 0.12
Sand	0.06 ± 0.01
Fat	0.45 ± 0.04
Crude fiber	5.13 ± 0.17
Protein	19.43 ± 0.07

3.4.2 Optimization of bean extraction conditions

To identify the ideal extraction conditions for maximizing alpha-amylase inhibitory activity in white kidney beans, a response surface methodology (RSM) analysis was conducted on the data presented in Table 3.2 This analysis focused on the influence of three independent variables – PBS concentration, extraction time, and separation time – on the two key responses: percentage yield (Y_1) and alpha-amylase inhibitory activity (Y_2). The RSM analysis revealed a strong correlation between these variables and the desired outcomes. Notably, the analysis identified the following conditions as optimal for achieving the highest alpha-amylase inhibitory activity: PBS concentration: 0.1 M, extraction time at 1 h, and separation time for 30 min. These optimized extraction conditions are expected to yield white kidney bean extracts with both a desirable yield and potent alpha-amylase inhibitory activity, paving the way for further research on their potential health benefits.

Table 3.3 Experimental design and responses of the dependent variables to the extract parameter

	Independent variables				
Exp. No.	PBS concentration (M) (X_1)	Extraction time (hour) (X_2)	Separation time (min) (X_3)	%yield (Y_1)	alpha-amylase inhibitory activity (unit/g) (Y_2)
1	0.1 (0)	1 (-1)	30 (-1)	56.57 ± 0.37	2.58 ± 0.02
2	0.1 (0)	3 (1)	30 (-1)	41.29 ± 0.23	1.88 ± 0.01
3	0.1 (0)	1 (-1)	60 (1)	35.64 ± 0.21	1.62 ± 0.02
4	0.1 (0)	3 (1)	60 (1)	38.88 ± 0.57	1.77 ± 0.02
5	0.05 (-1)	1 (-1)	45 (0)	23.91 ± 0.52	1.09 ± 0.15
6	0.05 (-1)	3 (1)	45 (0)	38.24 ± 0.52	1.74 ± 0.03
7	0.15 (1)	1 (-1)	45 (0)	43.69 ± 0.21	2.00 ± 0.01
8	0.15 (1)	3 (1)	45 (0)	36.18 ± 0.29	1.67 ± 0.01
9	0.05 (-1)	2 (0)	30 (-1)	24.31 ± 0.30	1.11 ± 0.29
10	0.05 (-1)	2 (0)	60 (1)	54.51 ± 0.38	2.49 ± 0.03
11	0.15 (1)	2 (0)	30 (-1)	49.69 ± 0.14	2.26 ± 0.02
12	0.15 (1)	2 (0)	60 (1)	38.39 ± 0.17	1.76 ± 0.03
13	0.1 (0)	2 (0)	45 (0)	37.86 ± 0.57	1.72 ± 0.02
14	0.1 (0)	2 (0)	45 (0)	30.85 ± 0.21	1.41 ± 0.02
15	0.1 (0)	2 (0)	45 (0)	29.91 ± 0.17	1.37 ± 0.01

Significant at $p < 0.05$ expressed by letters

1 = High level, 0 = Medium level, -1 Low level

Table 3.4 ANOVA of independent variables for percentage yield and alpha-amylase inhibitory activity respond

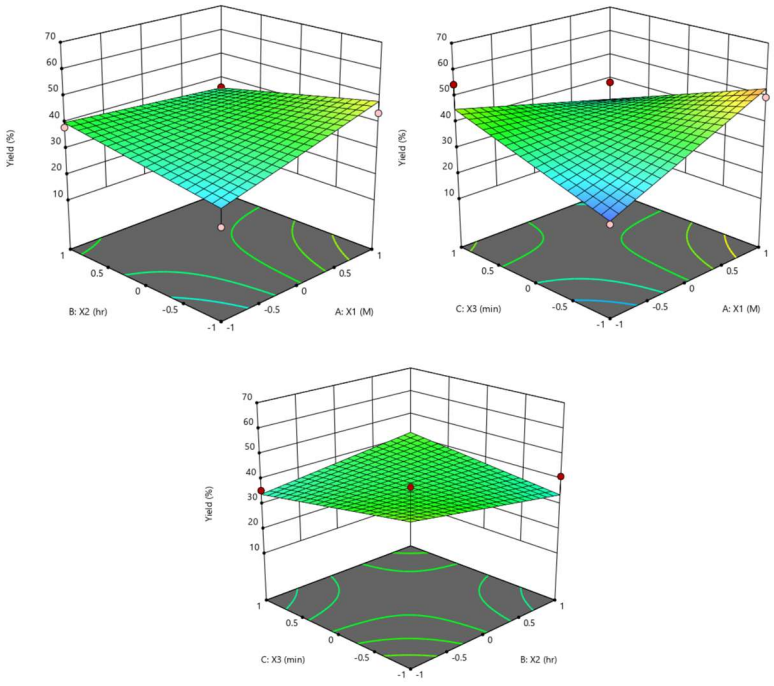
Source	Sum of Squares	df	Mean Square	F-value	p-value		Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	732.42	6	122.07	1.71	0.2357	not significant	Model	1.51	6	0.2523	1.7	0.2388	not significant
A-X1	90.99	1	90.99	1.28	0.2915		A-X1	0.1984	1	0.1984	1.34	0.2812	
B-X2	3.41	1	3.41	0.0477	0.8325		B-X2	0.0066	1	0.0066	0.0445	0.8382	
C-X3	2.46	1	2.46	0.0345	0.8572		C-X3	0.0045	1	0.0045	0.0304	0.866	
AB	119.25	1	119.25	1.67	0.2321		AB	0.2401	1	0.2401	1.62	0.2394	
AC	430.56	1	430.56	6.04	0.0395		AC	0.8836	1	0.8836	5.95	0.0407	
BC	85.75	1	85.75	1.2	0.3048		BC	0.1806	1	0.1806	1.22	0.3023	
Residual	570.7	8	71.34				Residual	1.19	8	0.1486			
Lack of Fit	532.96	6	88.83	4.71	0.1856	not significant	Lack of Fit	1.12	6	0.1859	5.07	0.174	not significant
Pure Error	37.74	2	18.87				Pure Error	0.0734	2	0.0367			
Cor Total	1303.12	14					Cor Total	2.7	14				
		Std.Dev = 8.45							Std.Dev = 0.3855				
		R-Squared = 0.562							R-Squared = 0.5601				
		Mean = 38.66							Mean = 1.76				
		R-Squared = 0.2336							R-Squared = 0.2302				
		C.V. % = 21.85							C.V. % = 21.85				
		Adeq Precision = 4.7653							Adeq Precision = 4.7656				

Significant at $p < 0.05$

Table 3.3 shows the ANOVA of independent variables for percentage yield and alpha-amylase inhibitory activity which is not significantly different at a p-value lower than 0.05 in both percentage yield and alpha-amylase inhibitory.

Figure 3.1 showed response surface plots of the interactive effect of PBS concentration, extraction time, and separation time on percentage yield and alpha-amylase inhibitory activity respectively which had the same trend.

(a)



(b)

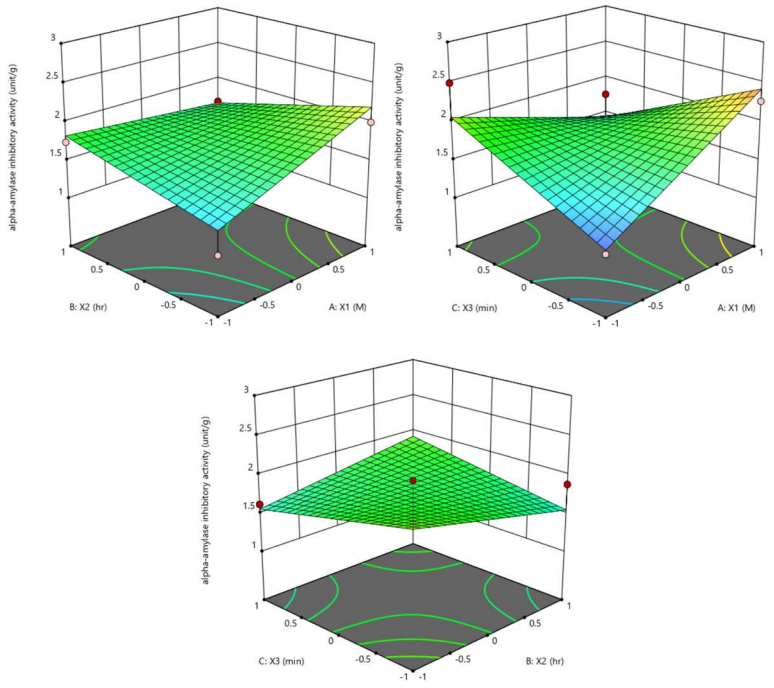


Figure 3.1 Response surface plots indicate the interaction effect of PBS concentration and extraction time, the interaction effect of PBS concentration and separation time, and the interaction effect of extraction time separation time on percentage yield (a) and alpha-amylase inhibitory activity (b).

The regression models are presented in the equation

$Y_1 = 38.66133 + 3.3725\ X_1 - 0.6525\ X_2 - 0.555\ X_3 - 5.46\ X_1\ X_2 - 10.375\ X_1\ X_3 + 4.63\ X_2\ X_3$ (1)

$Y_2 = 1.76467 + 0.1575\ X_1 - 0.02875\ X_2 - 0.02375\ X_3 - 0.245\ X_1\ X_2 - 0.47\ X_1\ X_3 + 0.2125\ X_2\ X_3$ (2)

3.4 Conclusion

This study successfully gained proximate analysis showed the composition of white kidney bean, 11.01% moisture, 3.50% ash, 0.06% sand, 0.45% fat, 5.13% crude fiber, and 19.43% protein, employed a randomized Box-Behnken design to identify the optimal conditions for extracting white kidney bean extract with maximized yield and alpha-amylase inhibitory activity. Under these parameters, the extract achieved a promising yield of 56.57% and a notable inhibitory activity of 2.85 units/g.

However, future research could research deeper into the specific components responsible for the inhibitory activity. Employing high-performance liquid chromatography (HPLC) would enable the quantification of total alpha-amylase inhibitors and active glycoproteins within the white kidney beans. This approach holds the potential to refine extraction conditions even further, potentially leading to even higher yields of extracts boasting even more potent inhibitory activity. By optimizing the extraction process based on specific bioactive components, this research paves the way for the development of more effective functional foods and nutraceuticals derived from white kidney beans.

3.5 References

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