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Optimizing White Kidney Bean Extract for Enhanced Alpha-Amylase Inhibition: A Box-Behnken and Response Surface Methodology Approach

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Abstract

This study investigated the optimization of alpha-amylase inhibitory extract production from white kidney beans (*Phaseolus vulgaris*). A Box-Behnken design was employed to systematically evaluate the effects of three factors on yield and alpha-amylase inhibitory activity: extraction solvent concentration (0.05 M, 0.10 M, 0.15 M), extraction time (1 hour, 2 hours, 3 hours), and separation time (30 minutes, 45 minutes, 60 minutes). Response Surface Methodology (RSM) was then used to analyze complex relationships between these parameters and the desired outcomes. The strong correlation observed between the experimental data and RSM analysis validated the effectiveness of the optimization strategy. The optimal conditions, yielded an extract with the highest specific activity (0.111 units/mg) using a PBS concentration of 0.101 M, an extraction time of 1 hour, and a separation time of 30 minutes. Interestingly, a slightly modified set of parameters (0.105 M PBS, 1-hour extraction, and 30.01 minutes separation) resulted in the highest extract yield percentage (11.89%). This study demonstrates the effectiveness of a combined Box-Behnken design and RSM approach for optimizing the extraction of bioactive compounds from natural sources. This paves the way for large-scale production of a potent alpha-amylase inhibitory extract and its exploration in future health applications.

Keywords

Response Surface Methodology, Alpha-amylase inhibitory activity, Extraction time, Optimal condition, White kidney bean (*Phaseolus vulgaris*).

1. Introduction

Type 2 diabetes mellitus is a non-communicable chronic disease (NCD) in body cells that have low effective insulin action or insulin resistance that leads to hyperglycemia, also type 2 diabetes mellitus is found in the majority of people who have diabetes mellitus. Type 2 diabetes mellitus is usually found in those aged 30 or older, obese (Asian body mass index ≥ 23 kg/m^2), might not have abnormal symptoms or might have symptoms of disease, usually symptoms

not severe but gradually progress, typically appear in parents or siblings. The risk of type 2 diabetes mellitus increases by older age, higher weight, and lack of exercise, and more appear in women who used to diabetes mellitus in pregnancy. The one of reasons that lead to type 2 diabetes mellitus is consuming behavior but it can be prevented by consuming some bioactive compound from some food. One of those is *phaseolamin* or α-amylase inhibitor (αAI) which found in white bean (*Phaseolus vulgaris, P. vulgaris*) and can slower starch absorption. So that, there are many works study the effect of *P. vulgaris* on of type 2 diabetes mellitus or other NCDs in patients or animals. 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. 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 (Carai et al. 2009; Geil and Anderson, 1994). White kidney beans are known for their alpha-amylase inhibitors, first reported by Bowman (1945). Marshall and Lauda (1945) reported these inhibitors are concentrated in the embryonic axes and cotyledons, absent in other plant parts. In addition, Moreno et al. (1990) identified that they are glycoproteins that specifically inhibit alpha-amylase activity in mammals and insects, leaving plant amylases unaffected. Studies by Fantini et al. (2009), Micheli (2019), and Song (2015) have shown that *P. vulgaris* extracts can lower body weight and glycemia in animals

1.1 Objectives

This investigation leverages a randomized Box-Behnken design coupled with Response Surface Methodology (RSM) to meticulously optimize the extraction conditions for alpha-amylase inhibitors and yield percentage from white kidney beans. This approach aims to elucidate the critical factors governing the extraction process, ultimately identifying the conditions that maximize both extract yield and alpha-amylase inhibitory potency. These optimized conditions will then serve as a foundation for further exploration of their efficacy within an animal model.

2. Literature Review

White kidney beans (*Phaseolus vulgaris*) are a legume native to the Americas, particularly prevalent in the southern United States, Mexico, Central America, and northern South America (Carai et al. 2009). Introduced to Europe in the 16th century, P. vulgaris has become a crucial crop worldwide (Carai et al. 2009). These beans are a valuable source of dietary fiber and resistant starch, a fraction that remains undigested in the small intestine and ferments in the colon (Soral-Śmietana & Krupa 2005; Gordon et al.1997). Studies by Cassidy et al. (1994) suggested a negative correlation between resistant starch intake and colorectal cancer risk, potentially due to its protective effects in the colon.
Bressani (1993) reported that white kidney beans boast a significantly higher protein content compared to cereals (5-15% protein) with values ranging from 17% to 39% dry matter including primarily storage proteins which are located within membrane-bound organelles in the cotyledon and serve as a source of amino acids, ammonia, and carbon skeletons for developing seedlings during germination.

Phaseolus vulgaris contains three isoforms of alpha-amylase inhibitors (αAI): αAI-1, αAI-2, and αAI-3 (or αAI-L) (Lee et al. 2002). Despite sharing a high degree of amino acid sequence similarity, αAI-1 and αAI-2 exhibit distinct specificities towards α-amylases (Guzman-Partida et al. 2007; Obiro et al. 2008). Notably, αAI-1, found in cultivated beans, inhibits porcine pancreatic α-amylase (PPA) (Lee et al. 2002). αAI-2 is present in some wild bean accessions, while αAI-L lacks activity against all tested α-amylases and is likely an evolutionary intermediate between plant defense proteins (Lee et al. 2002; Guzman-Partida et al. 2007).

The αAI-1, a typical bean lectin, undergoes synthesis in the rough endoplasmic reticulum (ER). It is then modified in the Golgi apparatus through the removal of a signal peptide and N-glycosylation before being transported to protein storage vacuoles. Analysis using SDS-PAGE reveals that fractions with a molecular weight of 30,000-35,000 are associated with the ER, while those at 14 and 19 kDa are linked to the Golgi apparatus and storage vacuoles. The αAI-1 becomes detectable in cotyledons and the seed axis 17 days after pollination, with its amount steadily increasing until reaching a maximum at 28 days. Although the total αAI-1 content decreases slightly during drying on a dry weight basis, it remains consistently high throughout maturation (Obiro et al. 2008).

The αAI-1 inhibitor functions by completely blocking the substrate-binding end of the enzyme's cavity, hindering access to the other end through a steric hindrance process. This inhibition involves "mimetic" interactions with the binding subsites on the enzyme, effectively targeting all its catalytically competent components (Payan 2004).
In recent years, researchers have made significant strides in optimizing the extraction of valuable compounds from legumes. Cubas et al. (2008) developed a method using N, N-dimethylformamide and response surface methodology

to extract and analyze chlorophylls a and b from green beans (Phaseolus vulgaris L.). This method identified the number of extractions as the key factor, with five repetitions yielding the optimal results (Cubas et al. 2008). This approach offers advantages like faster data collection and a clearer understanding of the impact of extraction parameters on chlorophyll content. Furthermore, it proved applicable across different green bean cultivars. Similarly, Gomes et al. (2019) focused on Carioca beans, a staple food rich in health-promoting phenolics. Recognizing the lack of a standardized extraction method, they employed response surface methodology to identify the optimal conditions for phenolic compound extraction (Gomes et al. 2019).

Their research established 70% acetone at 25°C with a 1:15 sample-to-solvent ratio as the superior method compared to existing techniques. This not only provides a valuable tool for future studies on Carioca bean health benefits but also underscores the importance of optimizing extraction methods to fully understand the potential of various food components. Beyond traditional methods, Yang et al. (2019) explored the effectiveness of ultrasonic treatment for extracting antioxidants from common beans (Yang et al.2019). This innovative approach utilizes ultrasound waves to disrupt the cellular structure of the beans, significantly increasing the surface area and exposure of antioxidant compounds. Consequently, the study observed a seven-fold increase in extraction efficiency compared to conventional methods. Additionally, they identified ten specific phenolic compounds in the extracts, providing valuable insights into the health benefits of common beans. Overall, these studies highlight the ongoing advancements in extraction techniques, paving the way for maximizing the utilization of valuable compounds present in legumes.

3. Methods

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.

3.2 White kidney bean extraction

Building upon established protocols by Fantini et al. (2009) and Micheli (2019), the extraction procedure was optimized for alpha-amylase inhibitors from white kidney beans. Ground beans (1.65 g) were suspended in varying concentrations of phosphate-buffered saline (PBS) (0.05 M, 0.1 M, 0.15 M) at a 1:6.06 (w/v) ratio. The suspension buffer consisted of 10 mM phosphate buffer (pH 7.2) supplemented with 150 mM NaCl. This mixture was stirred for different durations (1, 2, or 3 hours) at room temperature to facilitate inhibitor release. Subsequently, centrifugation was performed at 10,000 rpm for varying time points (30, 45, or 60 minutes) at 4°C to separate the extract from the cellular debris. The collected supernatant was then adjusted to a final volume of 10 ml using the corresponding PBS solution. Aliquots (2 ml) of the adjusted supernatant were dispensed into separate tubes for further analysis. Finally, all aliquots were freeze-dried and stored at -20°C until subsequent use.

4. Data Collection

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 1).

Table 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

The experiment adopted a Box-Behnken design, where each treatment combination was evaluated. A total of 15 unique treatment conditions were established based on the design. To ensure data strength, each treatment was replicated three times, resulting in a total of 45 experimental runs (15 treatments x 3 replicates). Following data collection, the standard deviation was calculated for the dependent variable associated with each treatment. This

calculation provides a measure of the variability within each treatment group and aids in assessing the overall precision of the experimental results.

5. Results and Discussion

To pinpoint the ideal extraction conditions that maximize alpha-amylase inhibitory activity in white kidney beans, a response surface methodology (RSM) analysis was undertaken on the data presented in Table 2. This analysis centered on elucidating the influence of three independent variables; PBS concentration, extraction time, and separation time; on the two crucial responses: percentage yield (Y_1) and alpha-amylase inhibitory activity (Y_2). The contour plot of RSM are shown in Figure 1. The RSM analysis revealed a robust correlation between these variables and the desired outcomes (Figure 2). Notably, the optimization strategy, the optimal conditions yielded an extract with the highest specific activity (0.111 units/mg) using a PBS concentration of 0.101 M, an extraction time of 1 hour, and a separation time of 30 minutes. Interestingly, a slightly modified set of parameters (0.105 M PBS, 1 hour extraction, and 30.01 minutes separation) resulted in the highest extract yield percentage (11.89%) (Table 2). This paves the way for further exploration of their potential health benefits in future studies.

The regression models are presented in the equation:

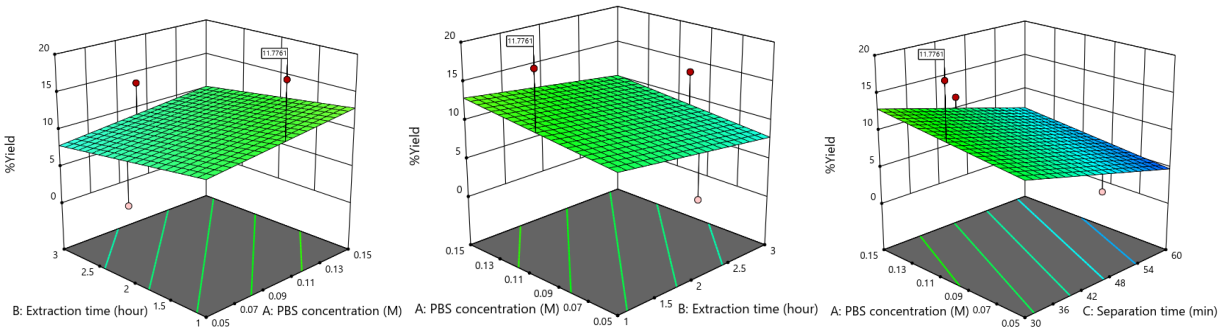
$$Y1\ (\%Yield) = 16.50108 + 23.725\ X1 - 1.34X2 - 0.191917X3 \quad (1)$$

$$Y2\ (\text{specific inhibitory activity}) = 0.0633 + 0.0087X1 + 0.0012\ X2 - 0.0075\ X3 - 0.0175\ X1\ X2 - 0.02\ X1\ X3 + 0.015\ X2\ X3 - 0.0129\ X1^2 + 0.0121\ X2^2 + 0.0146\ X3^2 \quad (2)$$

Table 2. Experimental design and responses of the dependent variables to the extract parameter

Exp. No.	Independent variables					Predicted	
	PBS concentration (M) (X_1)	Extraction time (hour) (X_2)	Separation time (min) (X_3)	%yield (Y_1)	specific inhibitory activity (unit/mg) (Y_2)	%yield	specific inhibitory activity (unit/mg)
1	0.1	2	45	9.32 ± 0.96	0.07 ± 0.00	7.56	0.0633
2	0.15	3	45	5.94 ± 0.85	0.06 ± 0.00	7.40	0.0550
3	0.1	3	30	13.53 ± 2.13	0.09 ± 0.00	9.10	0.0838
4	0.05	1	45	5.4 ± 1.25	0.03 ± 0.00	7.71	0.0350
5	0.1	1	60	4.67 ± 1.72	0.06 ± 0.00	6.02	0.0663
6	0.15	2	60	4.4 ± 0.66	0.06 ± 0.00	5.86	0.0463
7	0.1	2	45	5.12 ± 1.40	0.06 ± 0.00	7.56	0.0633
8	0.05	2	30	3.47 ± 2.03	0.03 ± 0.00	9.25	0.0438
9	0.15	1	45	11.65 ± 0.92	0.08 ± 0.00	10.08	0.0875
10	0.1	1	30	19.45 ± 0.88	0.13 ± 0.00	11.78	0.1113
11	0.15	2	30	8.43 ± 1.38	0.09 ± 0.00	11.62	0.1013
12	0.1	2	45	4.07 ± 1.32	0.06 ± 0.00	7.56	0.0633
13	0.1	3	60	5.85 ± 0.89	0.08 ± 0.00	3.34	0.0988
14	0.05	3	45	5.13 ± 2.21	0.08 ± 0.00	5.03	0.0725
15	0.05	2	60	6.93 ± 1.08	0.08 ± 0.00	3.49	0.0688

(a)



(b)

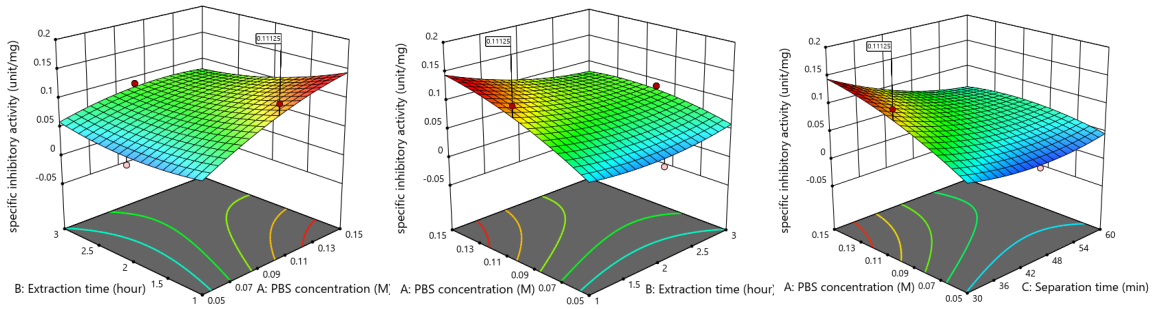


Figure 1. Response surface plots indicate the interaction effect of PBS concentration and extraction time, the interaction effect of PBS concentration and separation time, and the interaction effect of extraction time separation time on percentage yield (a) and alpha-amylase inhibitory activity (b).

5.1. Validation of the model

The improved extraction method was selected by grouping of extraction variables at the highest percentage yield (11.89%) for white bean extract. Precisely, this agreed to the 0.105 M PBS concentration, 1 hour extraction, and 30.01 minutes separation for white bean extract; three extractions were performed under those conditions to confirm the model's prediction (Table 2). No alterations were made between the predicted and experimental values of the percentage yield and alpha-amylase inhibitory activity which established the model's accuracy (Figure 2). In addition, data was investigated for correlation among independent variable and percentage yield, statically analysis showed significant at *p value* less than 0.05. Table 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.

Table 3. Analysis of variance of (ANOVA) independent variables for the extraction of bioactive compound from *P. vulgaris*.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	91.92	3	30.64	1.92	0.1846	not significant
A-X1	11.26	1	11.26	0.7060	0.4187	
B-X2	14.36	1	14.36	0.9009	0.3629	
C-X3	66.30	1	66.30	4.16	0.0662	
Residual	175.39	11	15.94			
Lack of Fit	159.96	9	17.77	2.30	0.3394	not significant
Pure Error	15.44	2	7.72			
Cor Total	267.31	14				
		Std.Dev = 3.99 R-Squared = 0.3439 Mean = 7.56 R-Squared = 0.1649 C.V. % = 52.84 Adeq Precision = 4.0919				

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0069	6	0.0008	2.32	0.1837	not significant
A-X1	0.0006	1	0.0006	1.87	0.2302	
B-X2	0.0000	1	0.0000	0.0381	0.8530	
C-X3	0.0005	1	0.0005	1.37	0.2945	
AB	0.0012	1	0.0012	3.73	0.1113	
AC	0.0016	1	0.0016	4.87	0.0783	
BC	0.0009	1	0.0009	2.74	0.1587	
A^2	0.0006	1	0.0006	1.88	0.2291	
B^2	0.0005	1	0.0005	1.64	0.2563	
C^2	0.0008	1	0.0008	2.39	0.1827	
Residual	0.0016	5	0.0003			
Lack of Fit	0.0016	3	0.0005	15.75	0.0624	not significant
Pure Error	0.0001	2	0.0000			
Cor Total	0.0085	14				
	Std.Dev = 0.0181 R-Squared = 0.8067 Mean = 0.0707 R-Squared = 0.4588 C.V. % = 25.64 Adeq Precision = 5.1538					

Note: Degree of freedom (DF).

The previous studies (Cubas et al. 2008; Gomes et al. 2019; Yang et al.2019) explored optimizing extraction methods for various components from different legumes.

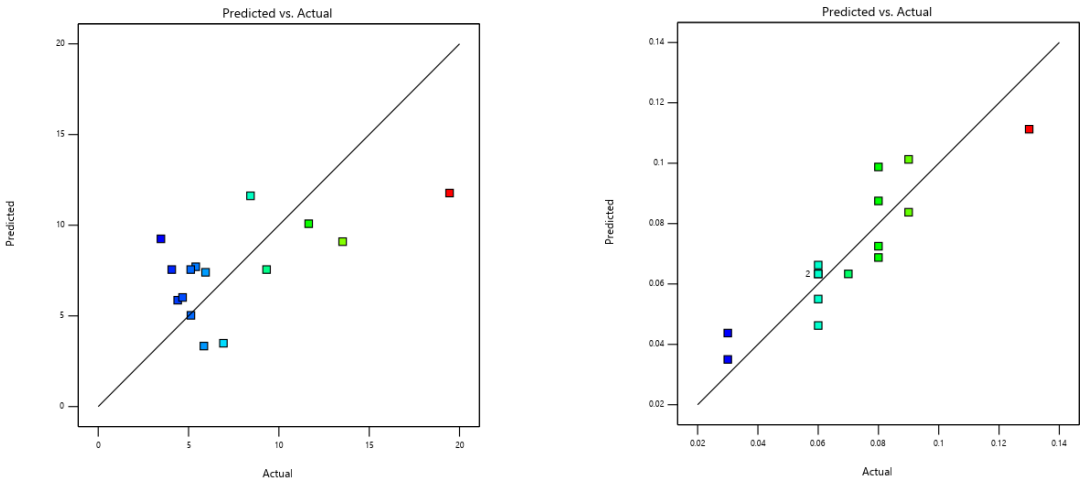


Figure 2. A plot of predicted and experimental value for the percentage yield (a) and alpha-amylase inhibitory activity (b) using RSM.

They focused on improving efficiency, identifying optimal conditions for specific targets (chlorophyll, phenolics, antioxidants), and exploring novel techniques (ultrasonic treatment) while as this study aimed to optimize the extraction process for alpha-amylase inhibitory activity in white kidney beans. It focused on three independent variables (PBS concentration, extraction time, and separation time) and their impact on two responses (percentage yield and alpha-amylase inhibitory activity).

In addition to the outcome of research, this study successfully identified the optimal extraction conditions to maximize alpha-amylase inhibitory activity, potentially leading to potent white kidney bean extracts for further health benefits research, while as combined legume extraction studies which each study presented different outcomes; Cubas et al. (2008) showed optimized chlorophyll extraction in green beans with five repetitions as the key factor; Gomes et al. (2019) established the best conditions for phenolic compound extraction in Carioca beans (70% acetone, 25°C, 1:15 ratio), Yang et al. (2019) demonstrated the effectiveness of ultrasonic treatment for extracting antioxidants from common beans, achieving a seven-fold increase in efficiency. In similarities, both sets of research highlight the importance of optimizing extraction methods for legumes. They all utilize Response Surface Methodology (RSM) as a valuable tool for identifying optimal conditions. For future research directions, the application of the optimized white kidney bean extraction conditions to larger-scale production and investigating its impact on alpha-amylase inhibition in vivo (within a living organism) including exploring the potential health benefits of the extracted white kidney bean compounds. Furthermore, the utilizing RSM to optimize ultrasonic treatment for alpha-amylase inhibitory extract in white kidney beans (combining findings from both sets of research) and investigating the applicability of the optimized conditions for extracting similar compounds from other legumes.

In conclusion, the presented results showcase diverse applications of RSM optimization in legume extraction. While the white kidney bean study focuses on a specific target compound and its activity, the combined studies offer broader insights into optimizing extraction methods for various legume components. Both sets of research pave the way for further investigation into the health benefits of legumes and the development of improved extraction techniques.

6. Conclusion

This study successfully 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 11.89 % and a notable inhibitory activity of 0.111 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.

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