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Optimization of Hyaluronidase Inhibition Activity from *Prunus davidiana* (Carriere) Franch Fruit Extract Fermented by its Isolated *Bacillus subtilis* Strain SPF4211

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Strain SPF4211, having hyaluronidase (HAase) inhibition activity, was isolated from *P. davidiana* (Carriere) Franch fruit (PrDF) sugar extract. The phenotypic and biochemical properties based on 16S rDNA sequencing and an API 50 CHB kit suggested that the organism was *B. subtilis*. To optimize the HAase inhibition activity of PrDF extract by fermentation of strain SPF4211, a central composite design (CCD) was introduced based on three variables: concentration of PrDF extract (X_1: 1–5%), amount of starter culture (X_2: 1–5%), and fermentation time (X_3: 0–7 days). The experimental data were fitted with quadratic regression equations, and the accuracy of the equations was analyzed by ANOVA. The statistical model predicted the highest HAase inhibition activity of 37.936% under the optimal conditions of X_1 = 1%, X_2 = 2.53%, and X_3 = 7 days. The optimized conditions were validated by observation of an actual HAase inhibition activity of 38.367% from extract of PrDF fermented by SPF4211. These results agree well with the predicted model value.

Keywords: *P. davidiana* (Carriere) Franch fruit, HAase inhibition activity, optimization, response surface methodology

Introduction

P. davidiana (Carriere) Franch is a deciduous tree belonging to the *Prunus* genus and a member of the Rosaceae. Fruits of the tree are oval, smaller than peaches, with many fine hairs on the surface. In addition, the fruits are extremely hard and are therefore referred to as "*dol-bok-sung-a*" [6, 25]. *P. davidiana* (Carriere) Franch fruits (PrDF) have been used as folkloristic medicine to treat hemasthenosis, constipation, chronic rhinitis, cough, asthma, dysmenorrhea, arthritis, and diarrhea [1, 27, 28]. Various studies have shown that PrDF improves blood glucose and lipid compositions in streptozotocin-induced diabetic rats [16], reduces blood pressure level in spontaneously hypertensive rats [17], and possesses antioxidant and whitening activities [18]. Moreover, the tree extracts of this plant have been shown to have antioxidant, lipid peroxide inhibitory, anti-inflammatory [5], and anti-hyperlipidemia [7] activities.

Hyaluronidase (HAase) degrades polymeric hyaluronic acid (HA) in the extracellular matrix of connective tissue to yield HA oligosaccharides with 4 to 25 disaccharides and is known to be involved in many biological functions, including inflammation, cancer metastasis, and permeability of the vascular system [4, 9, 12, 14, 22, 29]. The modulation of HAase inhibition will be useful for maintaining normal homeostasis in the body. Therefore, evaluation of HAase inhibition could be valuable for identification of compounds with anti-inflammatory activity. Many studies have reported HAase inhibition activity as a measure of anti-inflammatory activity in compounds, including caffeic acid oligomers from *Clinopodium gracile* [3], phlorotannins of brown algae [19], pentacyclic triterpenoids from *Prismatomeris tetrandra* [24], flavonols in processed onion [10], naringenin [23], and in soybeans and sword beans fermented with *Bacillus subtilis* [13].

Here, we present the first report on the isolation of

Bacillus subtilis strain SPF4211, which is able to produce hyaluronidase inhibitory activity, from fruits of *P. davidiana* (Carriere) Franch. We also investigated the phenotypic and biochemical properties of strain SPF4211 based on 16S rDNA sequencing and an API 50 CHB kit. Furthermore, response surface methodology (RSM) was applied to analyze the effects of process parameters and to search for optimal values to produce HAase inhibition activity of PrDF extract by fermentation of strain SPF4211.

Materials and Methods

Isolation of Bacteria and Culture Condition

B. subtilis strain SPF4211, a strain that produces high HAase inhibition activity, was isolated from the sugar extract of *P. davidiana* (Carriere) Franch fruits (PrDF). Briefly, 200 µl of PrDF sugar extract was spread onto plate count agar (PCA; 2.5 g/l yeast extract, 5.0 g/l tryptone, 1.0 g/l glucose, 1.5% (w/v) agar) and incubated at 37°C for 24 h. Single colonies were then isolated and transferred to PCA plates to test their purity. The isolated strain was kept on 20% (w/v) glycerol at −80°C.

The inoculum was prepared in 14 ml polypropylene round-bottomed tubes (BD Biosciences, San Jose, CA, USA) containing plate count broth medium (2.5 g/l yeast extract, 5.0 g/l tryptone, 1.0 g/l glucose). The seed cultures were grown in a shaking incubator (VS-8480; Vision Scientific, Bucheon, Korea) to a final cell density of approximately $10^{7.76}$ CFU/ml (OD_{600} = 0.4) at 35°C and 200 rpm for 12 h.

16S rRNA Analysis

Genomic DNA was isolated from pure cultures using a DNeasy tissue kit (Qiagen, Germany). The 16S rRNA gene was then amplified using HiPi PCR Premix (ElpisBio, Korea) with primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTG TTACGACTT) by polymerase chain reaction (PCR) using a Swift MiniPro Thermal Cycler (Esco Micro Pte. Ltd., Singapore). DNA sequencing of the resultant PCR products was carried out at Cosmo Genetech Institute (Cosmo Genetech Co., Ltd., Korea), after which Basic Local Alignment Search Tool (BLAST) analysis was performed to determine the identity of the bacterial isolate at the National Center for Biotechnology Information Web site [2].

Hyaluronidase Inhibition Assay

Samples were prepared by centrifugation at 12,000 ×*g* for 5 min after fermentation of PrDF hot water extract with strain SPF4211. HAase inhibition was investigated by the Morgan-Elson method [8, 15, 20]. Briefly, 12 µl of 1% (w/v) HAase solution in 0.1 M acetate buffer (pH 3.5) was mixed with 12 µl of sample, and then pre-incubated at 37°C for 20 min. The resulting mixture was added to 12 µl of 12.5 mM $CaCl_2$ as the HAase activator and incubated for an additional 20 min. For the HAase reaction, 24 µl of 0.6% (w/v) hyaluronate solution in 0.1 M acetate buffer (pH 3.5) was added and incubated in a water bath at 37°C for 40 min. Following incubation, 12 µl of 0.4 N NaOH and 12 µl of 0.4 M potassium tetraborate were added separately, and then incubated in boiling water for 3 min to terminate the HAase reaction. After cooling to room temperature, 360 µl of *p*-dimethylaminobenzaldehyde (DMAB) reagent (4 g of DMAB in 350 ml of glacial acetic acid and 50 ml of 10 N HCl) was added to the reaction mixture and incubated at 37°C for 20 min. The absorbance was then measured at 540 nm using a microplate reader (Tecan Sunrise, Tecan, Switzerland), after which the percentage inhibition activity was calculated by the following equation:

$$\text{HAase inhibition activity (\%)} = (1 - [\text{Sample}_{Abs}/\text{Control}_{Abs}]) \times 100 \quad (1)$$

Experimental Design and Statistical Analysis

Statistical analysis of the HAase inhibition activity during fermentation by the isolated *Bacillus subtilis* strain SPF4211 was conducted using the Design Expert 8 program (State-Easy Co., USA). The central composite design (CCD) was introduced to study the interaction of process variables and predict the optimal fermentation conditions for the HAase inhibition activity of the PrDF extract by applying RSM. To evaluate the effects of factors on the response surface in the region of the investigation, the ranges and coded level of fermentation process variables, such as the concentration of PrDF extract (X_1), amount of starter culture (X_2), and fermentation time (X_3), listed in Table 1 were used. For the regression model, variables were transformed to coded variables according to the following equation based on a three-factor-three-level CCD:

$$x_i = (X_i - X_i^*)/\Delta X_i, \quad (2)$$

Table 1. Experimental ranges and levels of three independent variables in terms of actual and coded factors based on response surface methodology.

Level	Variables		
	Concentration of PrDF extract (X_1) (%)	Amount of starter culture (X_2) (%)	Fermentation time (X_3) (days)
-1	1.0	1.0	0.0
0	3.0	3.0	3.5
1	5.0	5.0	7.0

Table 2. Central composite design arrangement, and the observed and predicted values for RSM.

Run	Coded variable levels			HAase inhibition (%)	
	X_1	X_2	X_3	Observed values[a]	Predicted values
1	-1	-1	-1	3.147	3.220
2	+1	-1	-1	4.720	3.980
3	-1	+1	-1	4.196	2.400
4	+1	+1	-1	4.021	5.960
5	-1	-1	+1	35.490	33.780
6	+1	-1	+1	13.811	15.820
7	-1	+1	+1	26.224	27.200
8	+1	+1	+1	11.888	12.040
9	-1	0	0	14.336	16.790
10	+1	0	0	12.937	9.590
11	0	-1	0	0.699	1.060
12	0	+1	0	0.000	-1.240
13	0	0	-1	4.196	4.730
14	0	0	+1	24.476	23.050
15	0	0	0	6.993	6.970
16	0	0	0	6.818	6.970
17	0	0	0	6.818	6.970
18	0	0	0	6.469	6.970
19	0	0	0	6.294	6.970
20	0	0	0	6.644	6.970

[a]Data are the means of three replications.

where x_i, X_i, X_i^*, and ΔX_i are the coded value, uncoded value, uncoded value of X_i at the selected center value, and step size for the *i*th independent variable, respectively [30]. The total number of experiments with three factors was 20 ($2^k + 2k + 6$, when k = 3, where k is the number of factors), with six replications to evaluate error. The design matrix with three variables and three levels is presented in Table 2. During optimization, the response can be related to chosen factors by the full quadratic model, which is as follows:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2, \quad (3)$$

where Y is the predicted response; β_0 is the intercept; β_1, β_2, and β_3 are linear coefficients; β_{12}, β_{13}, and β_{23} are interaction coefficients; and β_{11}, β_{22}, and β_{33} are squared coefficients. Analysis of variance (ANOVA) was employed to evaluate the empirical mathematical model at the 5% significance level and measure the interactive effects between process variables and the response. The quality of fit of the polynomial model was expressed by the coefficient of determination R^2, and its statistical significance was checked by an *F* test in the same program.

Results and Discussion

Identification of Isolated Bacteria from Sugared Extract of PrDF

In this study, strain SPF4211, which is able to produce a high HAase inhibition activity, was collected from the sugared extract of PrDF, and its partial 16S rRNA nucleotide sequence was determined for a 1,423 base region. BLAST analysis of the SPF4211 sequences showed that it had the highest similarity with *Bacillus subtilis* strains (99.9%) sequences presented in the database. Upon biochemical identification by API 50 CHB, strain SPF4211 showed 99.9% identity with the predicted carbohydrate fermentation profiles for the *B. subtilis*/*B. amyloliquefaciens* clade [21]. Based on the results of the above analyses, strain SPF4211 is believed to be *B. subtilis*. This is the first report showing isolation of a microorganism from *P. davidiana*.

Model Development and Optimization of HAase Inhibition Activity

To optimize the HAase inhibition activity of the PrDF hot water extract fermented by *B. subtilis* strain SPF4211, a three-variable-three-level matrix CCD was employed in which the concentration of PrDF extract, amount of starter culture, and fermentation time were investigated. The RSM experimental values of HAase inhibition at points based on the CCD experimental design are summarized in Table 2. Using the data presented in Table 2, an empirical relationship between HAase inhibition activities and test variables that resulted in the following regression equation was developed:

$$Y = 6.97 - 3.60X_1 - 1.15X_2 + 9.16X_3 + 0.70X_1X_2 - 4.68X_1X_3 - 1.44X_2X_3 + 6.22X_1^2 - 7.06X_2^2 + 6.92X_3^2, \quad (4)$$

where Y is the HAase inhibition activity of fermented PrDF extract as a function of concentration of PrDF extract (X_1), amount of starter culture (X_2), and fermentation time (X_3). Based on the experimental response, HAase inhibition ranged from 0 to 35.49%. The highest HAase inhibition activity was attained when the concentration of PrDF extract, amount of starter culture, and fermentation time were 1.0%, 1.0%, and 7 days, respectively (Run 5 in Table 2).

Eq. (4) is described in Table 3 as the ANOVA results for the quadratic regression model for the HAase inhibition activity of fermented PrDF extract. The *F* value of 46.22 indicates that the model is significant ($p < 0.001$). The determination coefficient (R^2) describing the goodness of the model fit [32] was 0.9765, indicating that approximately

Table 3. Analysis of variance of the experimental results for the quadratic model.[a]

Source	Sum of squares	Degrees of freedom	Mean square	*F* value	Prob>*F*
Model	1,561.67	9	173.52	46.22	<0.0001
Residual	37.55	10	3.75		
Lack of fit	37.21	5	7.44	112.39	<0.0001
Pure error	0.33	5	0.07		
Total	1,599.21	19			

[a]Coefficient of determination (R^2) = 0.9765; Adjusted R^2 = 0.9554; Coefficient of variation (CV) = 19.36%; Adeq precision = 25.568.

97% of the variations in HAase inhibition activity could be explained by this model [33]. The lack of fit *F* value of 112.39 implies that lack of fit was significant relative to the pure error, but the noise value was below 0.01% probability [31]. The adequate precision, which measures the signal-to-noise ratio and should not be less than 4, was 25.568 [11, 34]. Therefore, this model could be considered reasonable to navigate the design space.

The regression coefficients for the surface quadratic model of HAase inhibition activity in the fermented PrDF extract were estimated by the *F* test and the corresponding *p* values (Table 4). A smaller *p* value indicates a greater effect on the response variable, Y [26]. The estimated coefficient and the corresponding *p* values suggest that among the independent variables, the regression coefficients of the linear terms (β_1 and β_3), interaction term (β_{13}), and quadratic terms (β_{11}, β_{22}, and β_{33}) had significant effects on the HAase inhibition activity of fermented PrDF extract at the 0.1% level ($p < 0.001$). Thus, the variables with the largest effect on HAase inhibition activity were linear terms of β_3, followed by the quadratic terms (β_{11}, β_{22}, and β_{33}), interaction term (β_{13}), and another linear term (β_1).

The response surface and contour plots of the quadratic model were obtained to study the interaction among the variables, and to determine the optimal conditions of each factor for the maximum HAase inhibition activity. As shown in Fig. 1A, the effects of X_1 (concentration of PrDF extract) and X_2 (amount of starter culture) on the appearance of HAase inhibition activity were determined when the other variable (X_3, fermentation time) was at its center point. When X_2 was at a medium level, the HAase inhibition activity was high, but further increasing X_2 did not improve the HAase inhibition activity. The interaction of X_1 and X_3 on the HAase inhibition activity when X_2 was at its center point was statistically significant (Fig. 1B). When X_3 was at a high level and X_1 was at a low level, the fermented PrDF extract had more HAase inhibition activity. In the case of the effects of the interaction between X_2 and X_3 on the HAase inhibition activity, as shown in Fig. 1C, more HAase inhibition activity appeared when X_2 was at a medium level and X_3 was at a high level. The good correlation between the model (predicted) and experimental values yielded the goodness of fit of the model, as shown in Fig. 1D.

Validation of Model

The optimum values of the selected variables for the HAase inhibition activity of fermented PrDF extract, obtained by

Table 4. Significance test of the regression coefficient model.

Model term	Estimated coefficient	Standard error	*F* value	*p* value
β_0	6.97	0.67		
β_1	-3.60	0.61	34.55	0.0002***
β_2	-1.15	0.61	3.55	0.0891
β_3	9.16	0.61	223.52	<0.0001***
β_{12}	0.70	0.69	1.04	0.3314
β_{13}	-4.68	0.69	46.60	<0.0001***
β_{23}	-1.44	0.69	4.43	0.0615
β_{11}	6.22	1.17	28.36	0.0003***
β_{22}	-7.06	1.17	36.56	0.0001***
β_{33}	6.92	1.17	35.09	0.0001***

***$p < 0.001$.

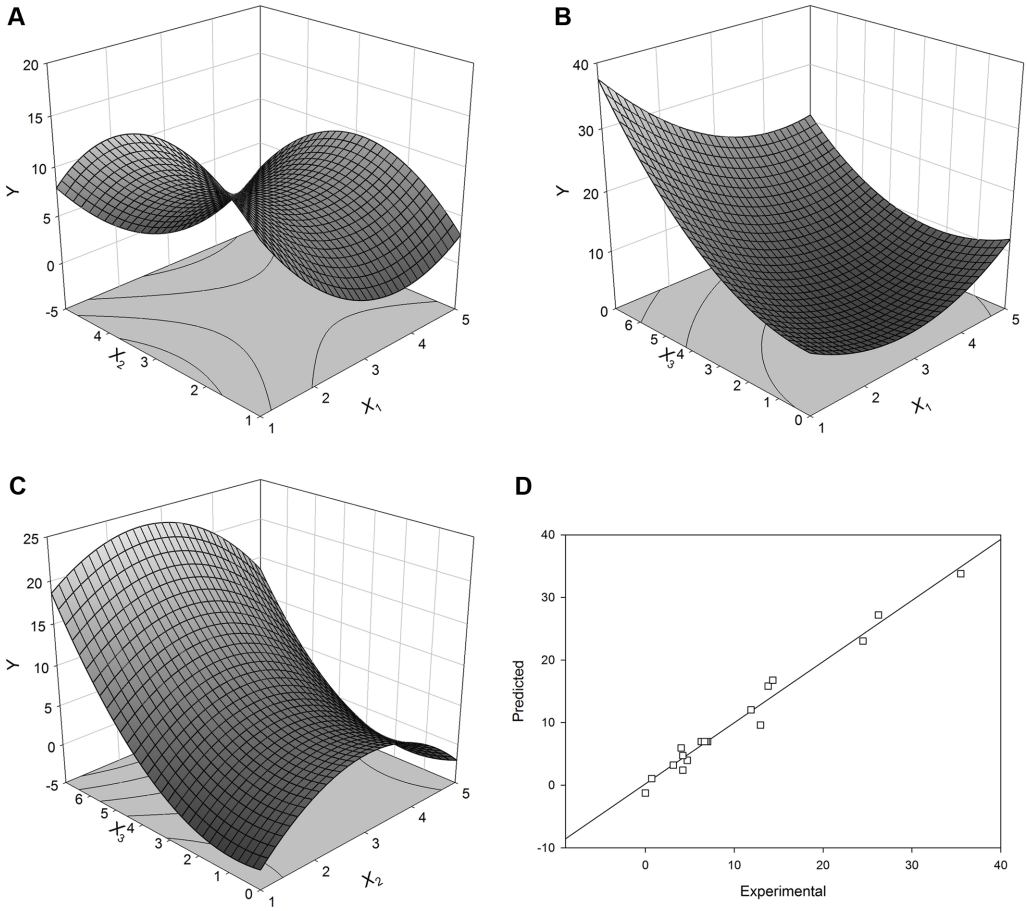

Fig. 1. Response surface curve of the effect of different factors on the production of HAase inhibition activity from the PrDF hot water extract.

(**A**) Response surface as a function of concentration of PrDF extract (X_1) and amount of starter culture (X_2); (**B**) response surface as a function of concentration of PrDF extract (X_1) and fermentation time (X_3); (**C**) response surface as a function of amount of starter culture (X_2) and fermentation time (X_3); (**D**) model (predicted) versus experimental values.

solving the quadratic regression equation (Eq. (4)) using the Design Expert program, were X_1 = 1%, X_2 = 2.53%, and X_3 = 7 days. The predicted response (HAase inhibition activity) at the optimum condition was 37.936%. To verify the prediction of this model, fermentation was conducted by *B. subtilis* strain SPF4211 under the optimum conditions. The generated product showed 38.367% of HAase inhibition activity, which was in good agreement with the predicted value. Based on these results, the above model is adequate to predict the HAase inhibition activity of fermented PrDF extract within the range of variables tested.

B. subtilis strain SPF4211 is the first microorganism isolated from *P. davidiana*. The results of this study showed that the fermentation of PrDF extract by this organism under the optimum conditions elevated the HAase inhibition activity to 38.4% from <5% of the unfermented PrDF extract (X_3 = 0 day). Based on these results, further studies on the analysis of the active compounds corresponding to HAase inhibition activity through the bioconversion by *B. subtilis* strain SPF4211 are warranted.

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