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Optimization of ultrasonification of slaughter blood for protein solubilization

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ABSTRACT

In this study, we attempted to solubilize protein in slaughter blood (SB) using ultrasonic technology. The application of ultrasonic technology can make enzymatic degradation of SB more effective, which has no comparable alternative for treatment. The SB was homogenized by grinding it for 10 minutes at 10,000 rpm as a pretreatment for preventing its clotting, and then ultrasonic treatment was attempted to solubilize protein in SB. To maximize the efficiency of ultrasonic treatment for SB, the optimum condition of ultrasonic frequency (UF) was determined to be 20 kHz. To optimize the operation conditions of ultrasonification with 20 kHz of frequency, we used response surface methodology (RSM) based on ultrasonic density (UD) and ultrasonification time (UT). The solubilization rate (SR) of protein (%) was calculated to be $101.304 - 19.4205\ X_1 + 0.0398\ X_2 + 7.9411\ X_1^2 + 0.0001\ X_2^2 + 0.0455\ X_1X_2$. From the results of the RSM study, the optimum conditions of UD and UT were determined at 0.5 W/mL and 22 minutes, respectively, and SB treated under these conditions was estimated to have a 95% SR. Also, experimentally, a 95.53% SR was observed under same conditions, accurately reflecting the theoretical prediction of 95%.

Keywords: Protein solubilization, Response surface methodology, Slaughter blood, Ultrasonification

1. Introduction

Recently, with the improvement of standards of living and increase of income in South Korea, meat consumption and numbers of cattle, pigs and chickens have increased. In 2012, 970,320 heads of cattle, 14,039,960 pigs, 787,958,258 chickens and 90,409,001 ducks were slaughtered, which is an increase of 13.8% for cattle, 29.6% for pigs, 3.7% for chickens, 5.7% for ducks against 2011 figures. With this increase in slaughter, the amount of blood produced has proportionally increased. Generally, slaughter blood (SB) makes up about 3.5% of the live weight, and the SB generated from each animal is 7-8 L for cattle, 3-4 L for pigs, and 34 mL and 51 mL for chickens and ducks, respectively [1, 2]. According to the annual amount of slaughter, blood from slaughtered pigs was calculated to be approximately 42 thousand tons in 2012. In Korea, of the SB produced, blood of cattle is consumed mostly for making food and blood of chickens and ducks are recycled with other by-products, while the blood of the pigs is mostly treated as a waste product. Annually, the amount of this wasted pig blood has been steadily increasing, except for the year 2011, when the breeding scale decreased due to an outbreak of foot-and-mouth disease.

SB is prohibited from disposal in the ocean through the enforcement of the 1996 Protocol and treaty of MARPOL, as amended, and accordingly, tremendous amounts of expenditure are required for the operation of treatment facilities for SB. Hence, it is desirable to develop technologies which can treat and utilize SB as resource rather than a waste and this study aims to produce amino acid liquefied fertilizer using the protein of SB.

In general, SB can be fractionated into liquid called plasma and a solid, which is mostly composed of red blood cells (RBCs). The plasma consists of useful proteins, such as albumin, globulin and fibrinogen, and the cells contain all of the hemoglobin [3], which is simple to convert to amino acid compared to other proteins, because of the high protein content. On the other hand, since RBCs contain hemoglobin, unlike blood plasma, it receives interruption from cellular walls. Thus solubilization of the protein through hemolysis is necessary, which involves the elution of hemoglobin and the destruction of RBCs. And several methods to convert hemoglobin to amino acid involve ultrasonic treatment, centrifugation, freeze-thaw and chemical extraction.

Ultrasound refers to frequencies above those of audible sound, and nominally refers to frequencies over 20,000 Hz. The ultrasonic effect follows the cavitation, and the ultrasonic frequencies can strongly influence the cavitation bubble. It is widely known that

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cavitation occurs more readily at low frequencies (below 40 kHz) than at high frequencies because it produces enough energy to mechanically weaken and the formation of radicals that it attacks the chemical structure during cavitation in the liquid. Also, it is widely used for the release of soluble organic matter from biological cells [4, 5]. According to Haward [6], RBCs are easily disrupted by ultrasonic treatment.

Response surface methodology (RSM) is one of the experiments design components. This method helps to optimize the experimental parameters with a minimum number of experiments, and to analyze the interaction between independent and dependent variables [7-11]. Optimization of experimental conditions using RSM is widely applied in many studies. Central composite design (CCD) and Box-Behnken methods are the most commonly used [12, 13].

Therefore, in this research, to recycle SB that has no alternative utilization, we attempted to solubilize protein as a product for amino acid liquid fertilizer. Clotted blood was homogenized using a grinder and the proteins were then solubilized through the disruption of RBCs in SB by ultrasonic treatment in order to increase the efficiency of conversion of protein to amino acids. To determine the optimum conditions of ultrasonic pretreatment, which can achieve a 95% solubilization rate (SR), the CCD was applied with 2 factors and 3 levels.

2. Materials and Methods

2.1. Materials

SB from slaughter pigs was used in this experiments, and was collected from a pig slaughterhouse situated in Anyang city, Korea. Pure blood from bloodletting section during the slaughtering process was used. After sampling, SB was transported to the lab in a refrigerated vehicle within 30 min, and all the experiments were conducted within a week of the sample collection date in order to prevent variation in the experimental results due to sample degeneration.

2.2. Experimental System

Fig. 1 shows ultrasonic treatment system which were used in this experiment. The blood clotting begins at the moment the blood is exposed to air. To homogenize the clotted blood, a grinder operated in the range of 3,000-15,000 rpm was used. The ultrasonic system for the solubilization of protein in SB was composed of an ultrasonic generator and reactor section. The ultrasonic generator section included a converter, control module, transducer and horn tip. The horn tip has a low-frequencies range of 20, 24, 28 kHz, which is effective for cell disruption, and the maximum powers at these frequencies are 2,000 W, 1,000 W and 300 W, respectively. The reactor section consists of a beaker, a water bath for control of temperature, a temperature sensor probe.

2.3. Method

In this study, 2L of SB was treated by a grinder which it was operated for 10 min at 10,000 rpm. The grinded SB was treated in probe type reactor for 10, 30 and 60 min at 200 rpm of agitation

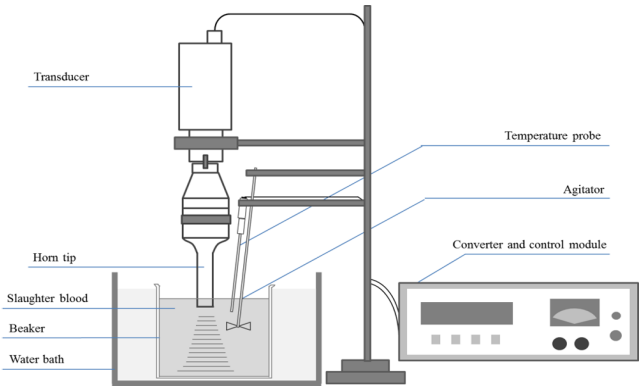

Fig. 1. Schematic diagram of ultrasonic system.

speed and room temperature by ultrasound that it was performed under condition of 20, 24 and 28 kHz of UF and 0.5, 1.0 and 1.5 W/mL of UD. All experiments were repeated three times.

2.4. Physico-chemical Analysis

To investigate the characteristics of SB, moisture, organic and inorganic content were measured by a Standard Method [14]. Total lipid was measured by a Sulfo-Phospho-Vanillin method (Ebru et al. 2007). Total protein, albumin, fibrinogen and platelets were measured using an automatic hemotology analyzer (COULTER AcT; Beckman Coulter Inc., USA), and hemoglobin was analyzed under the below method which is referred to as the Cyanmethemoglobin method [15].

The blood is mixed with a solution containing ferricyanide and cyanide. The ferrous ions (Fe^{2+}) of hemoglobin are oxidized to the ferric (Fe^{3+}) state by potassium ferricyanide($K_4[Fe(CN)_6]\cdot 3H_2O$) to form methemoglobin. Methemoglobin subsequently reacts with the cyanide ions of potassium cyanide to form cyanmethemoglobin. The amount of cyanmethemoglobin can be measured spectrophotometrically at a wavelength of 540 nm on a UV-Vis spectrophotometer (Cary 4000; Varian Australia Pty., Ltd, Australia), and can be compared to known hemoglobin standards in order to determine the hemoglobin concentration of the blood sample.

$$SR(\%) = (Ht - Hu)/(Hw - Hu) \times 100(\%) \quad (1)$$

Where, SR is solubilization rate, Ht is hemoglobin in plasma of treated SB, Hu is hemoglobin in plasma untreated SB and Hw is hemoglobin in whole blood.

2.5. Experimental Design and Data Analysis

RSM involving CCD was employed to obtain optimal conditions for protein SR by ultrasonic treatment systems. Experimental design used the CCD, which is the standard and the most widely used approach to RSM. It is important to determine the point and number of the experiment, to obtain optimum results with minimum experiments. The total number of required experiments was calculated according to the following equation: $N = n_f + n_a + n_0$ [16, 17]. In this study, the dependent variables were ultrasonic density (UD), (X_1) and ultrasonification time (UT), (X_2). A total of 11 different combinations of 4 factorial points coded to the (±1, ±1), 6 axial points coded to the (0, ±1) and (±1, 0) and 3 center points coded

Table 1. Central Composite Design for the Experiment

Explanatory variables	Symbol	Code levels		
		- 1	0	+ 1
UD (W/mL)	X_1	0.5	1.0	1.5
UT (min)	X_2	10	30	60

UD: ultrasonic density, UT: ultrasonification time.

to the (0, 0) were chosen in random order according to a 2^2 full factorial CCD configuration for two factors. UD (X_1, 0.5-2.0 W/mL) and UT (X_2, 10-60 min) are coded in Table 1, and we attempted to confirm optimization conditions through observing interaction between their variables.

Data were processed using Minitab 16.0 (Minitab Inc., USA) and the significance of the second-order model is shown in Eq. (2). As a scale of the effect of each variable as an objective function, unknown β in Eq. (2) clarifies the relationship between an independent variable and a dependent variable to identify optimal values of optimum design variable [18].

$$\eta = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j}^{k} \beta_{ij} x_i x_j \quad (2)$$

Where η is the predicted response, x_i and x_j are the input variables, β_0 is the intercept term, β_i is the linear effects, β_{ii} is the squared effect, and β_{ij} is the interaction tern. The effect and regression coefficients of individual linear, quadratic and interaction terms were determined through the analysis of ANOVA.

3. Results and Discussion

3.1. Physico-chemical Characteristics of Slaughter blood

The results of the SB analysis showed that moisture, organic and inorganic content were 79.14%, 19.40%, and 1.45%, respectively. Total protein was 18.22%, which was made up mostly of organic matter. The protein in SB consists of albumin, globulin, fibrinogen and hemoglobin. Hemoglobin inside RBCs was occupied mostly by 14.02%. This means that, in utilizing the protein in the SB as resource, it is necessary to destroy the cells and release hemoglobin.

Table 2. Physico-chemical Characteristics of SB

Parameters	Concentration* (wt%)
Moisture content	79.14 ± 0.15
Organic content	19.40 ± 0.23
Protein	18.22 ± 0.09
Albumin	2.08 ± 0.11
Globulin	1.99 ± 0.10
Fibrinogen	0.12 ± 0.03
Hemoglobin	14.02 ± 0.16
Lipid	0.17 ± 0.01
Inorganic content	1.46 ± 0.30

All experiments were repeated three times.
* Mean value of concentration with deviation value.

3.2. Changes in Slaughter Blood with Different Ultrasonic Frequency

In this research, SB of all experiments was homogenized using a grinder for 10 min at 10,000 rpm because SB starts to clot upon collection. The blood was treated with ultrasound for one hour in 2 W/mL of maximum UD to find out solubilization of SB by frequencies and the optimal frequency was then chosen. The solubilization of hemoglobin protein in blood cells of SB according to the pre-treatment under each ultrasonic frequency (UF) is shown in Table 3.

The results of the experiment demonstrate that the concentration of hemoglobin was highest when UF was applied at 20 kHz and SR was 96.87%. SRs at 24 kHz and 28 kHz of UF were 94.72% and 94.61%, respectively. The optimal frequency was determined to be 20 kHz.

Table 3. Changes in SR with Different UF

	Untreated SB	Ultrasonic treated SB*		
		20 kHz	24 kHz	28 kHz
Hemoglobin in plasma (%)	0.00	15.34 ± 0.05	14.99 ± 0.01	14.98 ± 0.01
SR (%)	-	96.87 ± 0.89	94.67 ± 0.61	94.61 ± 0.57

SB: slaughter blood, SR: solubilization rate.
All experiments were repeated three times.
The experiments were tested under ultrasonification for 1 h under agitation speed at 200 rpm after grinding for 10 min.
* Mean value of concentration with deviation value.

3.3. Response Surface Modeling by CCD

In this experiment, two factors (UD and UT) of the frequency of 20 kHz were optimized, and were coded at three levels between -1 and +1. The whole design consisted of 11 experimental points, as listed in Table 4.

It was confirmed that the protein SR was the highest with 97.72% in 0.5 W/mL of UD at 60 min, and the lowest with 91% in 1.0 W/mL at 10 min. In general it is widely known that when the UD increases, ultrasonic efficiency is improved. But, in this experiment, ultrasonic efficiency by UD is not significantly different because SR has little the change according 0.1-2.0 W/mL of UD under condition of equivalent UT and the 95% of SR set to an objective value, and as UT was extended, the SR was increased. It is considered that the blood was not affected by UD because it underwent hemolysis with minimal physical force which it such as sheer stress to active cavitation bubble has lower occurrence.

The regression coefficients of the second order equation were calculated using the designed experimental data, leading to the following model of SR as a function of UD (X_1) and UT (X_2) : Solubilization rate = $101.304 - 19.4205\ X_1 + 0.0398\ X_2 + 7.9411\ X_1^2 + 0.0001\ X_2^2 + 0.0455\ X_1X_2$.

The analysis of ANOVA of regression parameters of the predicted response surface quadratic model for protein solubilization efficiency is shown in Table 5. *p* values less than 0.05 indicate that model terms are significant, while *p* values greater than 0.1 indicate that model terms are not significant. In this model, F value and

Table 4. Response Values for Different Experimental Conditions by the Central Composite Design

Run order	Coded factor		Explanatory variables		SR (%)
	UD	UT	UD (W/mL)	UT (min)	
1	-1	-1	0.5	10	93.94
2	1	-1	1.5	10	91.00
3	-1	1	0.5	60	97.72
4	1	1	1.5	60	97.01
5	-1	0	0.5	30	96.01
6	1	0	1.5	30	93.60
7	0	-1	1.0	10	90.91
8	0	1	1.0	60	95.66
9	0	0	1.0	30	92.34
10	0	0	1.0	30	92.42
11	0	0	1.0	30	92.19

UD: ultrasonic density, UT: ultrasonification time, SR: solubilization rate.
The experiments were treated with an ultrasonic frequency of 20 kHz under agitation speed at 200 rpm after homogenization at 10,000 rpm for 10 min.

Table 5. Analysis of Variance for Response Surface Quadratic Model

Source	SS	DF	MS	*F* value	*p* value
Model	54.9600	5	10.9920	79.99	< 0.001*
Linear	42.6427	2	6.8159	49.60	0.001*
X_1	6.1206	1	13.6235	99.14	< 0.001*
X_2	36.5221	1	0.2086	1.52	0.277
Square	11.0032	2	5.5016	40.04	0.001*
X_1^2	10.9842	1	9.9846	72.66	< 0.001*
X_2^2	0.0190	1	0.0190	0.14	0.725
Interaction	1.3142	1	1.3142	9.56	0.027*
X_1X_2	1.3142	1	1.3142	9.56	0.027*
Residual	0.6871	5	0.1374	-	-
Lack of Fit	0.6598	3	0.2199	16.13	0.059
Pure Error	0.0273	2	0.0136	-	-
Cor Total	55.6471	10	-	-	-

SS: sum of squares, DF: degree of freedom, MS: mean square, *F* value: F statistics test to determine significance, *p* value: probability value, X_1: ultrasonic density, X_2: ultrasonification time.
* significant at 0.05 level.

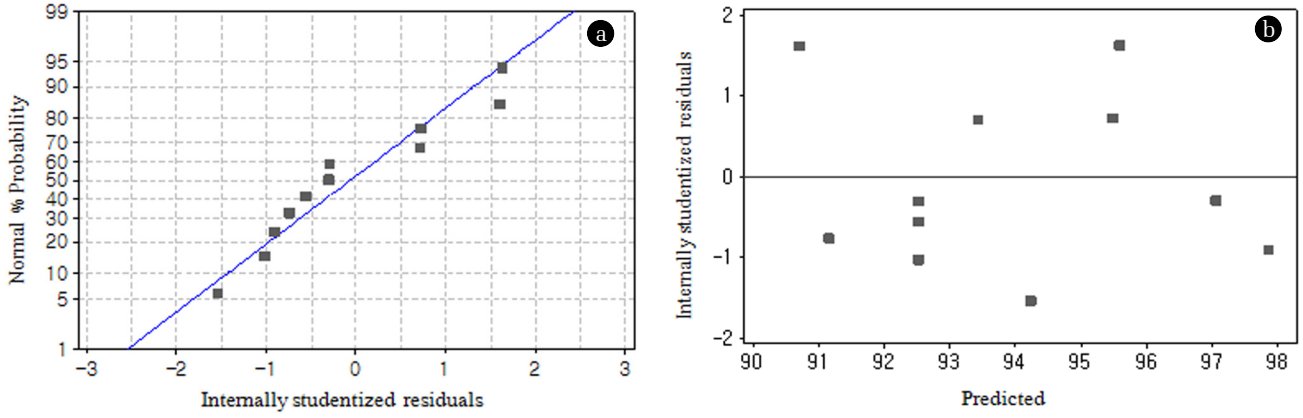

Fig. 2. Residual plot of model for error values: (a) normal probability plot of the residuals, (b) scatter plot of the residuals and predicted.

p values were indicated to be 79.99 and < 0.001. In addition, the linear value was 0.001 (< 0.05), with a very high validity, and the p value of the square and interaction were 0.001 (< 0.05) and 0.027 (< 0.05) respectively, illustrating a significant effect. In the verification of lack of fit, if the value of p is smaller than 0.05, it is assumed to be inappropriate for a prediction model [19]. Yet, in this study, the calculated value of p was 0.059 which indicates that it is appropriate for use as a prediction model. As a result of variance analysis with complete second-order formula for the verification of the validity of the model, the coefficient of determination (R^2) of the SR model formula was 98.77%, and the modified coefficient of determination was 97.53%, displaying the appropriateness of the experimental design [18, 20].

The distribution of residuals was analyzed to evaluate the adequacy of the model obtained from the response surface analysis if the residuals followed a normal distribution. Residuals are the deviation between predicted and actual values, and are expected to follow a normal distribution if the experimental errors are random [21, 22]. As shown in (a) of Fig. 2, which indicates the regular probability of standardized residuals, all data follow the normal distribution, being distributed in a straight line, and the analysis result of residual-fitted value in (b) showed that it was randomly distributed around 0, and thus, the model used in the experiment was appropriate.

3.4. Optimization of Ultrasonification of Slaughter Blood

The main effect plot is appropriate for analyzing data in a designed experiment, with respect to important factors. The main effect plot of two variables (UD and UT) of SR is shown in Fig. 3. Note the slope of the effect is larger, the larger the effect, the experiment of UT results in increased protein SR was found to have a big influence. On the other hand, if the density of ultrasound in low-density, high SR of research seems to not be significantly different according to the density. In this study, SR was highest in 0.5 W/mL of UD or 60 min of UT.

Fig. 4 is the response surface plot for SR prepared by using the presumed response surface model to determine the optimal response condition. Looking at the response surface plot by response variable, high SR is observed at 0.5 W/mL of the investigated density and SR increases with as the experiment is continued. In this study, an optimal response condition which minimizes the variables by using the presumed response surface model and response surface plot on the basis of the set objective of 95% SR was determined considering economic factor because it was noneconomic that the increase of UT consumes too much the power consumption, and to quantify it further, it was calculated using a desirability function.

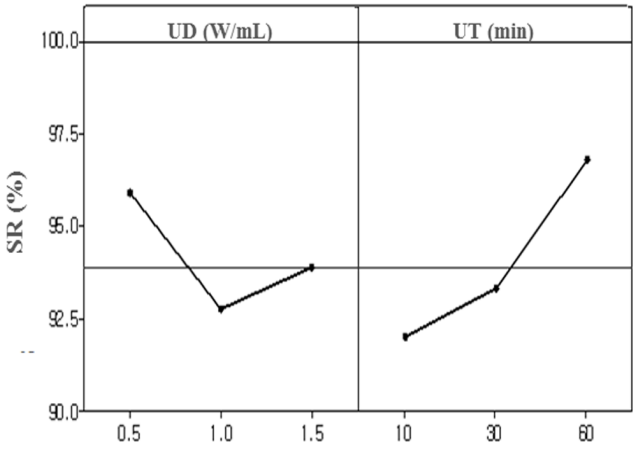

Fig. 3. Results of ANOVA test for solubilization rate (SR) (%) at main effect.

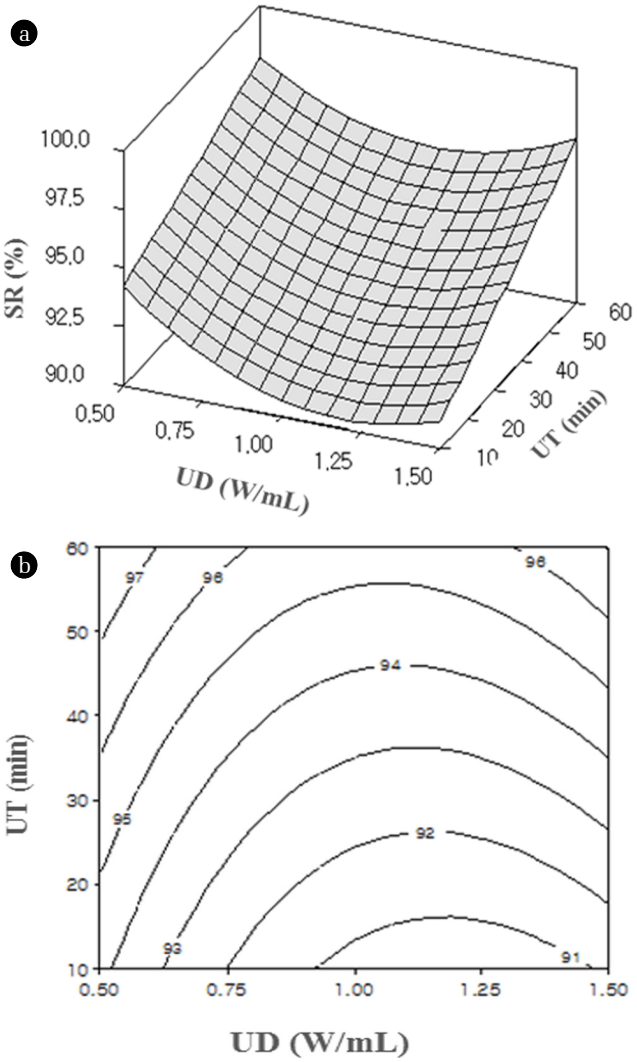

Fig. 4. 3D response surface (a) and contour plot (b) of ultrasonic density (UD) and ultrasonification time (UT) effect on solubilization rate (SR) (%).

Table 6. Predicted and Experimental Values of Response Variables for Optimal Conditions

Response variables	UD (W/mL)	UT (min)	Predicted values (%)	Experimental values (%)
Y	0.5	21.62	95.00	95.53

UD: ultrasonic density, UT: ultrasonification time.

Table 7. Experimental Results of SR with Different UD and UT

UT (min)	UD (W/mL)							
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**1.0**	**1.5**	**2.0**
0*	87.68	87.68	87.68	87.68	87.68	87.68	87.68	87.68
10	88.34	91.82	90.88	91.84	93.64	91.73	91.00	87.68
20	89.10	91.19	91.51	92.87	94.62	92.73	92.88	91.76
30	89.73	92.49	92.03	93.43	96.01	92.34	93.60	93.36
40	89.60	93.79	92.66	93.50	96.12	93.43	93.49	94.32
50	90.99	93.74	92.45	94.21	97.72	93.60	94.08	96.57
60	91.21	93.80	92.88	93.83	97.72	95.66	97.01	96.05

UD: ultrasonic density, UT: ultrasonification time.
The experiments were treated at an ultrasonic frequency of 20 kHz under an agitation speed of 200 rpm after homogenization at 10,000 rpm for 10 min.
* only grinded.

95% of SR was determined to result due to 0.5 W/mL of UD being applied for approximately 22 min. The experimental result using optimization conditions was confirmed to be 95.53%, which corresponds extremely well to the target value of 95%.

Additionally, the experiments were performed for SR at 10 min intervals over the following 60 min in the investigation range of 0.1-2.0 W/mL, in order to determine the correlation between the experimental values and the actual predicted value using the RSM, as shown in Fig. 5 and Table 7. In the experimental results of SR, the highest value was observed as 0.5 W/mL of UD among the various densities because efficiency of ultrasonic treatment is increased in the smaller UD but the effect of UD in SB is lower. And there was no remarkable differences between progress of UT and increase of SR.

Predicted and experimental values resulted in a similar curve shape of the response surface plot, and the RSM result suggested that the predicted value of optimization conditions was similar with the experimental data. Therefore, the model and experimental data were similar, and it is possible that the SR was calculated using estimation regression equation without experiment.

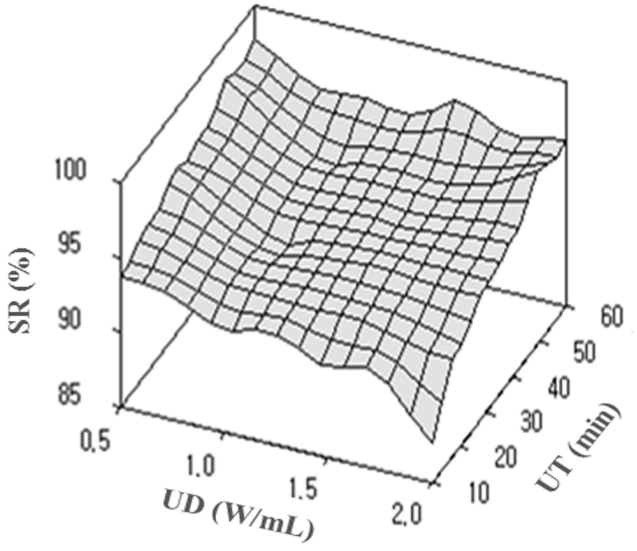

Fig. 5. Experimental results of solubilization rate (SR) with different ultrasonic density (UD) and ultrasonification time (UT).

4. Conclusions

In this study, to treat SB which has no alternative usage, we tested the solubilization of protein in SB using ultrasonic technology. The result of analysis from SB showed that moisture, organic matter and inorganic matter were 79.14%, 19.40% and 1.45%, respectively. Most organic matter was composed of total protein (18.22%), and hemoglobin was 14.02% protein. Hemoglobin protein in SB was solubilized through UT after homogenization as a pretreatment method. The protein SR when treated at an UF of 20 kHz for one hour was confirmed to be at its highest at 96.82%, and was therefore determined as the optimum frequency. The result of RSM in UF of 20 kHz, SR (%) was calculated $101.304 - 19.4205\ X_1 + 0.0398\ X_2 + 7.9411\ X_1^2 + 0.0001\ X_2^2 + 0.0455\ X_1X_2$. The value of correlation coefficient (R^2) in SR model was 98.77%, and the adjusted correlation coefficient (R^2) was 97.53%. The result of regression and variance analysis, p value of linear, square, and interaction effects were 0.001, 0.001, and 0.02, respectively. And the p value of lack of fit was 0.059. Thus, the statistical analysis indicated that the proposed model was adequate, possessing no significant lack of fit and very satisfactory values of R^2 for all responses. The best combination of each significant factor was determined by RSM and optimum pretreatment conditions for 95% of SR were established when blood was treated with 0.5 W/mL of UD for 22 min. Under these conditions, 95.53% of SR was observed experimentally, similar to the theoretical prediction of 95%. As a result, it is expected that enzymatic degradation of SB is most effective when the optimum conditions determined by this research are applied.

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