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Fast Hydrolysis of Antler Residue (*Comu cervi pantotrichum*) by Pulsed Electric Field Assisted Pepsin

Wei Jiang[1], Yongguang Yin*, Yajun Zhou and Guidan He

College of Biological and Agricultural Engineering, Jilin University, Changchun - 130 022, PR China.

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Under optimized pulsed electric field (PEF) treatment for production of antlers calcium citrate malate (ACCM) and antlers collagen polypeptide (ACP) by one-factor test and response surface methodology (RSM) were studied. The yield of dissoluble calcium (YDC) and protein degree of hydrolysis (DH) were used as characteristic indexes, the tests were carried out using different factors, which is as follows: the molar ratio of citric acid to malic acid (2:1-1:2), mixed acid content (0.1-1.0 mol/L), E/S (1-6 %), electric field intensity (5-30 kV/cm) and pulse number (2-12) were studied. Subsequently mixed acid content, electric field intensity and pulse number were optimized by RSM for DH. The results indicated that the optimum conditions were E/S 4.0%, acid concentration 0.586 mol/L, electric field intensity 19 kV/cm, pulse number 8, the DH of 19.3±0.34 % and the YDC of 26±0.45 % were obtained. In the present study, it is feasible to utilization of antler residue in a novel nutraceutical material with a high solubility for calcium and an easy absorption for collagen peptide to people who acalcerosis.

Key words: Pulsed electric filed; Velvet antler residue; yield of dissoluble calcium; degree of hydrolysis; response surface methodology**.**

Antlers are the fastest growing tissue (protuberances) on the skull of buck[1], they are widely used as traditional Chinese medicine for over two thousand years in Asia. Up to now, many water-soluble and fat-soluble substances had been studied: immunologically activity peptide[2], anti-inflammatory peptide[3], sex hormones and phospholipids[4], etc. However, nobody pay close attention to the most collagen and calcium in the velvet antler.

At present, there are a lot of nutrition products in market, but there was not any food products used antler as the raw materials, while antler only used as medicine. Antler pharmaceutical products called pantocrine[5], whose annual output can reach more than 20 0000 bottles. Antler residue powder (ARP) was by-products of processing pantocrine, account for 75% of antler. ARP contains abundant calcium and collagen, and the composition of antler is similar to that of human bones, so one of the medicinal roles is as a source of calcium to treat osteoporsis[6,7]. Collagen peptide can be used to treat arthritis and osteoporsis[8], antihypertension[9], antioxidant[10]. Thus, this natural bioresources can be applied in food industry, health benefit products.

Pulsed electric fields (PEF) were studied on nonthermal food preservation method for 45 years[11], and PEF as a novel technique was applied in the food industry, due to its non-thermal, fast, efficient and non-negative effect. Many studies focus on sterilization of bacteria for storage of liquid food[12,13]; on inactivation of enzyme in food to prevent to be oxidized by oxidase[14,15]; on extraction effective constituent from raw materials[16,17]. Moreover, Yin reported that PEF was one of themost effective methods to quickly extract

* To whom all correspondence should be addressed.
Tel.:+86 431 85095253; Fax: +86 431 85094314;
E-mail: jlu_jw@163.com

dissoluble calcium from bone[18], and Ho found that the activity of pepsin can increase to 2.6 times under 10-15kV.cm^{-1} and 30μs [19]. But PEF assisted-hydrolysis of antler residue to extract ACCM and ACP is seldom studied.

Calcium citric malate (CCM) was recognized as a calcium source that did not increase the risk of kidney stones[20], and had high solubility. Thus, in this study mixed acid (citric acid and malic acid) will be used to provide acidic environment for pepsin to hydrolyze antler residue, and improve the degree of hydrolysis, shorten the hydrolysis time by PEF technology.

MATERIALS AND METHODS

Materials and instruments

Velvet antler residue powder were obtained from Jilin Sino-ROK institute of animal science (Changchun, China), and stored at -20°C until use. Pepsin was purchased from Sinopharm Chemical Reagent Beijing Co. (Beijing, China), citric acid and malic acid (edible grade) were purchased from Beijing Chemical Plant (Beijing, China), acrylamide and methylene diacrylamide (Chemical pure) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China), other chemicals and reagents used were of analytical grade.

The PEF (produced in-house) was generated by apparatus described in previous study[16]; Centrifuge Biofuge Heracus (Kendro, American); Electrophoresis JY600C (Beijing, China); TU-1810 UV-vis spectrophotometer (Beijing, China).

Choice of hydrolysis methods

(A) Acid hydrolysis: 2.000g sample mixed with 25ml 0.3M citric acid, stired for 24h. (B) Pepsin hydrolysis : 2.000g sample mixed with 25ml 0.3M citric acid, and 2% pepsin (E/S), 37°C reacted for 6h. (C) PEF assisted acid hydrolysis: 2.000g sample mixed with 25ml 0.3M citric acid, then treated by PEF (field strengths 15kV/cm, pulse number 6). (D) PEF assisted pepsin hydrolysis: 2.000g sample mixed with 25ml 0.3M citric acid, and 2% pepsin (E/S), then treated by PEF (field strengths 15kV/cm, pulse number 3).

PEF treated procedure

At the beginning of each experiment, 8g antlers residue powder was weighed and mixed it with 100ml solvent (0.1-1.0mol/L mixed acid) and the mole ratio of mixed acid (citric acid: malic acid=2:1-1:2). Secondly, the mixture was boiled for 5 min, then cooled to 37°C, and then added and made the concentration of pepsin 0.5-3.0%. Thirdly, the mixture of the raw material was pumped into the PEF system with a steady velocity of flow at 20mL/min, and then the system was turned on, the electric field strength and pulse number were adjusted to 5-30kV.cm^{-1} and 2-12μs respectively. Finally, the processed mixture was boiled for 10min to inactivate enzyme and centrifuged (8500r/min) for 10min, supernatant was collected, and then the dissolvable calcium and hydrolysis degree of protein were determined.

Experimental design of Response Surface Method (RSM)

According to the principle of Box - Behnken center design, Less factors and levels will be chosen in multi-factors test[21].After determining the primary range of the hydrolysis variables through single-factor test, experiments were designed to find the interaction of three variables, i.e., acid concentration, electric field intensity and pulse number. Table 1 represents the coded values of the experimental variables. Design of experiment along with the DH is given in Table 2.

The variables were coded according to the equation:

$$x_i=(Xi - X_0)/X \quad ...(1)$$

The behavior of the system was explained according to the following quadratic polynomial model:

$$Y = \alpha_0 + \sum_{i=1}^{k} \alpha_i X_i + \sum_{i=i}^{k} \alpha_{ii} X_i^2 + \sum_{i>j}^{k} \alpha_{ij} X_I X_J \quad ...(2)$$

Where X_i and X_j are independent variables, Y is the response variable, x_i is the coded value of the variable X_i, X_0 is the value of X_i at the centre point, and X is the step change, α_0 is the regression intercept, α_i is the regression coefficients, α_{ii} is the quadratic coefficients, α_{ij} is the cross-product coefficients and k is the number of independent factors. The response surface model analysis of test data was carried out using the software package Design-Expert 7.0.0 (Stat-Ease Inc., Minneapolis, MN). Statistical analysis of the model was performed to evaluate the analysis of variance (ANVOA).

Analytical methods

Calcium content was determined by the ethylene diamine tetraacetic acid (EDTA) titration method[22], using calcium carbonate as a standard, yield of dissoluble calcium was represented according to the follow formula 4. Protein degree of hydrolysis was estimated by the ninhydrin[23].

...(3)

Statistical analysis

All data were expressed as mean ± sd. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 11.5 (SPSS Inc., Chicago, IL). A p value of ≤ 0.05 was taken as the level of statistical significance.

RESULTS AND DISCUSSION

Comparison of three hydrolysis methods

Traditionally, extraction of dissoluble calcium and collagen was hydrolyzed by acids, alkalis and enzymatic methods. Enzyme cut the protein into smaller molecules, and the calcium was dissolved to liquid. However, molecular distribution of hydrolysate is uneven and accompanied with a longer hydrolysis reaction time. Yin[18] investigated PEF assisted-hydrolysis of bone, it was found that most molecular in hydrolysate moved quickly under PEF, and electron and ions with great kinetic energy would bounce violently, so the hydrolysis reaction time could be shorten. PEF proved helpful for activity of pepsin[19].

In the present study, the hydrolysis time of PEF assist-acid and PEF assist-pepsin were ultra short (4μs) shows at Figure 1, PEF not only prompt yield of dissoluble calcium (YDC), but also prompt protein degree of hydrolysis of (DH). Treatment of the same substrate with the PEF assisted-pepsin for 4μs gave higher YDC and DH than other hydrolysis methods for 6h (pepsin hydrolysis), 4μs (PEF assisted-acid hydrolysis), 24h (acid hydrolysis), respectively. So PEF assisted pepsin hydrolysis method wound be used to hydrolyze antler residue.

The effects of mole ratio of mixed acids on YDC and DH

Fig. 2(a) shows that higher yield of dissoluble calcium 13.6±0.31% can be obtained at mole ratio of citric acid to malic acid 1:1.5, its yield

Table 1. The range of independent variables and their corresponding levels used for Box-Behnken rotatable design

Independent variables	Code value	Coded factor level (x_i) -1	0	1
Acid content(mol/L)	X_1	0.4	0.5	0.6
Field strength(kV/cm)	X_2	18	20	22
Pulse number	X_3	7	8	9

Table 2. The central composite experimental design and results for DH

Standard order	X_1 (mol/L)	X_2 (kV/cm)	X_3	DH (%)
1	0	0	0	19.5
2	0	-1	1	19.7
3	-1	-1	0	17.1
4	0	-1	-1	18.6
5	0	1	1	15.8
6	0	0	0	19.7
7	1	0	1	18.5
8	0	1	-1	15.5
9	-1	0	-1	15.2
10	-1	0	1	17.1
11	-1	1	0	14.7
12	1	0	-1	16.8
13	1	-1	0	18.2
14	1	1	0	15.3
15	0	0	0	19.6
16	0	0	0	18.9
17	0	0	0	19.4

$$YDC(\%) = \frac{Weight\ \ of\ \ dissolu\ ble\ \ calcium(g)}{Weight\ \ of\ \ antler\ \ residue(g)} \times 100\%$$

Table 3. *F*-value and *p*-value of the ANOVA analysis for the quadratic polynomial model

Factor	F-value	p-value	significant
X_1	12.35	0.0098	**
X_2	84.56	<0.0001	**
X_3	13.97	0.0073	**
X_1X_2	0.28	0.6134	
X_1X_3	0.045	0.8386	
X_2X_3	0.72	0.4256	
X_1X_1	60.83	0.0001	**
X_2X_2	31.70	0.0008	**
X_3X_3	9.83	0.0165	*
Model	24.94	0.0002	**
Lack of fit	4.05	0.1052	not significant
R^2	0.9698		
Adj-R^2	0.9309		

was 1.38 times than the yield at mole ratio of mixed acid 2:1, calcium citrate malate (CCM) had higher solubility than calcium citrate and calcium malate. Slightly influence on degree of hydrolysis by different mole ratio of mixed acid. So the mole ratio 1:1.5 will be chosen.

The effects of acid content on YDC and DH

The effects of acid concentration on YDC and DH were shown in Fig. 2(b). With the acid content increasing from 0.1mol/L to 0.9 mol/L, the yield of dissoluble calcium increased from 4.1±0.21 % to 18.3±0.21 %, and then slightly increase with the acid content increased form 0.5 mol/L to 0.9 mol/L. The DH rapidly increased with the increase of acid content before 0.5 mol/L, then decrease after 0.5 mol/L. The pepsin has the best activity in the suitable acid concentration, and the ability of hydrolysis was the strongest[25]. Under acidic condition, the helical structure of collagen was destroyed and pepsin cut collagen into smaller molecules, Ca^{2+} was combined with citric acid and malic acid, then MCC was generated[26]. So 0.5mol/L mixed acid was chosen to hydrolyze antler residue.

The effects of E/S on YDC and DH

Fig. 2(c) shows when the E/S increased from 1.0 % to 6.0 %, the YDC and DH increased from 16.4±0.30% to 19.1±0.25% and 2.9±0.35% to 14.3±0.20% respectively. When the E/S exceeded 4.0 %, hydrolysis reaction did not make obvious rise with the increasing of E/S, the pepsin content had reached saturation to substrate. At present, there are three methods for extraction of dissoluble calcium, and acid method was the commonly used because of its high yield and little negative effect on human body[18], but collagen can't be hydrolyzed. Collagen can be cut into peptide by acid pepsin[25], antler residue can be hydrolyzed completely by pepsin assisted acid. Considering from the economic benefits, 4% E/S should be chosen.

The effects of field strength on YDC and DH

Fig. 2(d) shows that the YDC and DH were significantly influenced by electric field strength. When the electric field strength increased from 5 kV/cm to 20 kV/cm, the yield of dissoluble calcium and degree of hydrolysis significantly increased from 10.5±0.47% to 21.4±0.25% and 2.1±0.35% to 17.3±0.45% respectively. Most molecular in hydrolysate moved quickly under PEF, and electron and ions with great kinetic energy would bounce violently. The pepsin could be activated under suitable condition of PEF, because of the structure of enzyme be changed[27]. At this suitable point, the chemical reaction was violent. Higher field strength can destroy the enzyme's active site, and the enzymatic reaction decrease[28].

The effects of pulse number on YDC and DH

Fig. 2(e) shows the effect of pulse number on YDC and DH. The yield of dissoluble calcium and degree of hydrolysis increased with the increase of pulse number before 8, and then DH obviously decrease occurred along with the pulse number increased from 8 to 14. Under the same flow rate, the reaction time is related to pulse number[18]. Thus with the increase of pulse number, the reaction time increase, and the activity of enzyme becomes unstable[28]. That might lead to

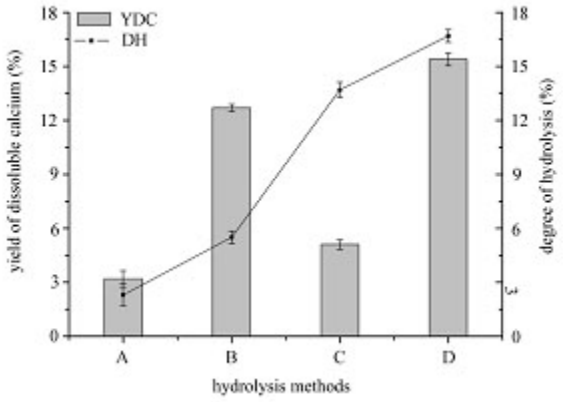

Fig. 1. Comparison of four hydrolysis methods. (A) acid hydrolysis; (B) PEF assisted-acid hydrolysis; (C) pepsin hydrolysis; (D) PEF assisted pepsin hydrolysis. Field strengths: 15kV/cm, pulse number: 6, acid content: 0.3mol/L, E/S: 2%. Results are presented as the means (n=3) ± SD. The data mean is significantly different at (P<0.05)

the DH decrease. Slightly influence on yield of dissolution calcium was indicated, so yield of dissoluble calcium with a little change after pulse number 8.

Optimization of hydrolysis conditions

Statistical analysis and model fitting

The experimental data for degree of hydrolysis under different treatment conditions

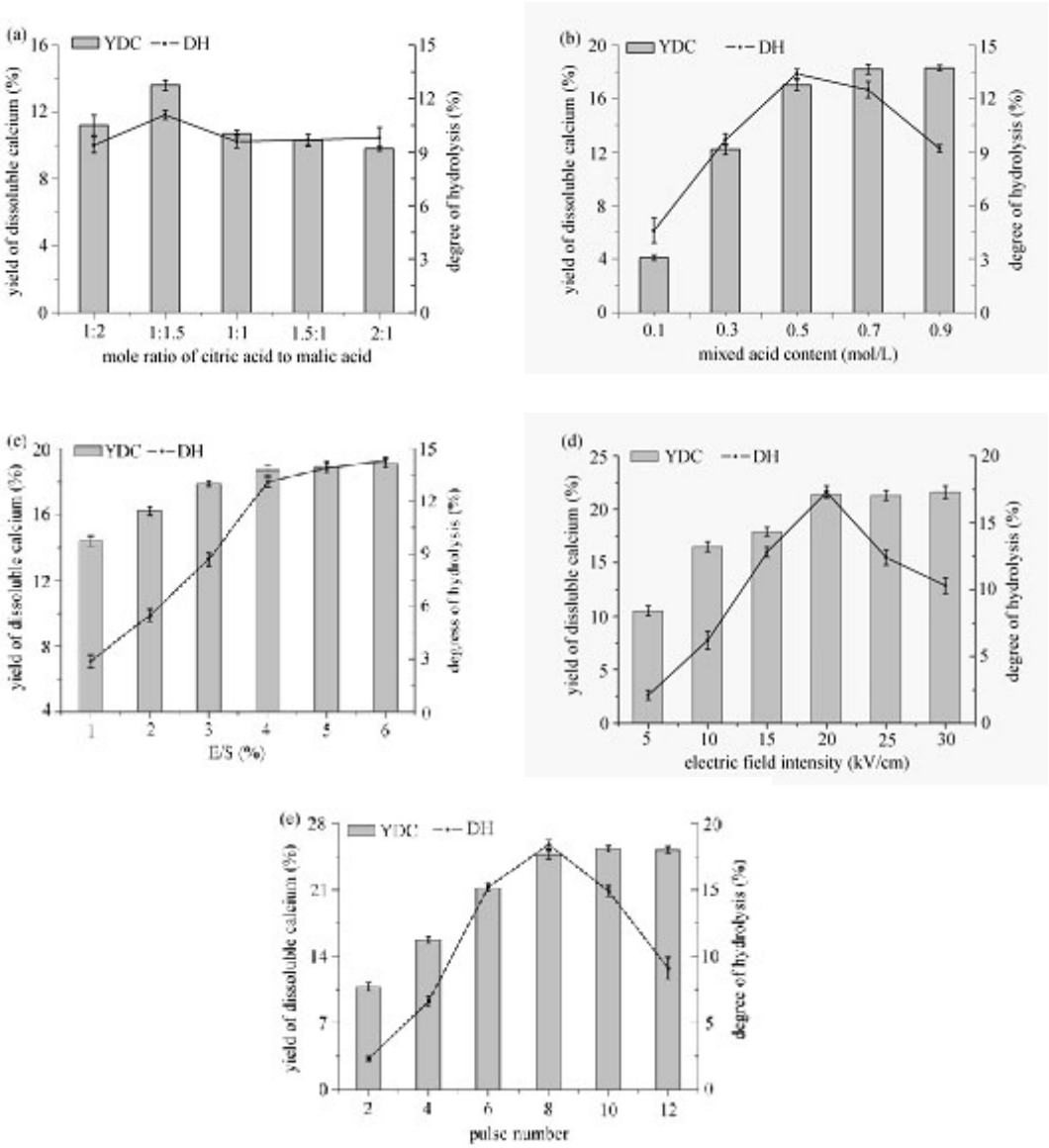

Fig. 2. The effects of molar ratio of mixed acid, mixed acid content, E/S, electric field intensity and pulse number on the YDC and DH. (a) Effects of molar ratio of mixed acid on the YDC and DH. Mixed acid content: 0.3mol/L, E/S: 2.0%, electric field intensity: 10kV/cm, pulse number: 6. (b) Effects of mixed acid content on the YDC and DH. Molar ratio of mixed acid: 1:1.5, E/S: 2.0%, electric field intensity: 10kV/cm, pulse number: 6. (c) Effect of E/S on the YDC and DH. Molar ratio of mixed acid: 1:1.5, acid concentration: 0.5mol/L, electric field intensity: 10kV/cm, pulse number: 6. (d) Effect of electric field intensity on the YDC and DH. Molar ratio of mixed acid: 1:1.5, acid concentration: 0.5mol/L, E/S: 2.0%, pulse number: 6. (e) Effect of pulse number on the YDC and DH. Molar ratio of mixed acid: 1:1.5, acid concentration: 0.5mol/L, E/S: 2.0%, electric field intensity: 20kV/cm. Results are presented as the means (n=3) ± SD. The data mean is significantly different at (P<0.05)

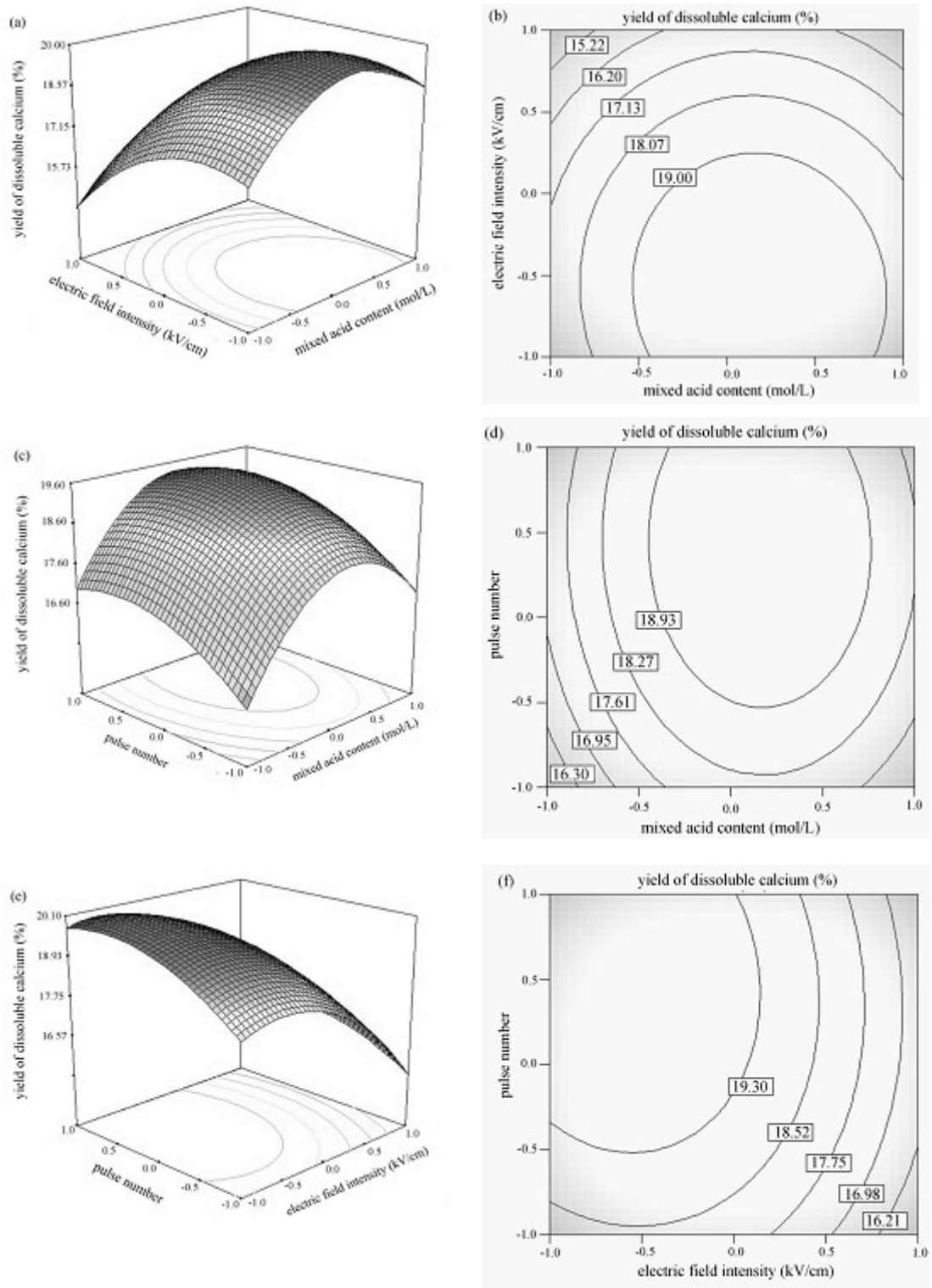

Fig. 3. Response surface plots and contour plots of enzymatic hydrolysis reaction. The degree of hydrolysis is shown as a function of the interactions between acid concentration and electric field intensity (a, b), acid concentration and pulse number (c, d), pulse number and field intensity (e, f)

were presented in Table 2. By analyzing the plots, the predicted values (19.28 %) of the degree of hydrolysis lay in the following condition: 0.518 mol/L mixed acid, electric field intensity 19 kV/cm , pulse number 9. In these optimal conditions, the validated experimental DH was 19.3±0.34% and the yield of dissoluble calcium was 26±0.45%. By applying multiple regression analysis on the experimental data, the response variable and the test variables are related by the following second-order polynomial equation:

$$Y=19.70+1.09X1-0.58X2+0.61X3-0.27X1X2 -0.1X1X3-0.27X_2X_3-1.73X_1^2-1.9X_2^2-0.98X_3^2 \quad (4)$$

Statistical testing of the model was performed at Table 3 in the form of ANOVA, which is required to test the significance and adequacy of the model. *F*-value for the lack of fit (0.1052) was no significant (P<0.05), confirming the validity of the models. The value of R^2 (0.9698) for the model is reasonably close to 1, and indicated a high degree of correlation between the observed and predicted values. The *P*-values were used as methods to check the significance of each coefficient, the smaller *P*-value, the more significant influence on *Y* by the corresponding coefficient. It can be seen from Table 3, the variables with the largest influence were X_1, X_2, X_3, X_1X_1, X_2X_2 (p<0.01), then X_3X_3 (p<0.05) indicated that the experimental factors were not simple linear relationship on degree of hydrolysis, the quadratic of each variable also had very close relationship with degree of hydrolysis, the interactive effect of variables (X_1X_2, X_1X_3, X_2X_3) were no significant (Pÿ0.05).

Response surface plot and contour plot

The 3-D response surface and 2-D contour plots were showed at Fig. 3(a-f), which were graphical representations of regression function, the interactions of variables can be intuitively seen from the shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables. A circular contour plot indicated that the interactions between the corresponding variables were no significant, while elliptical contour plot indicated interactions were significant [24]. DH maximum can be seen from the highest point in 3-D response surface and the center point of minimum ellipse in the 2-D contour plots. The interaction of variables X_1, X_2, X_3 were displayed quadratic effect on the response yield. When the variable was kept at a low level, the degree of hydrolysis increase at first and then decreased with the increase of variable. Under suitable conditions, the pepsin have higher activity, but its activity will be decreased under higher acid concentration, field strength and pulse number, the influence of X_2 (field strength) to Y was the most obvious, similar to the results of ANOVA.

CONCLUSIONS

In this investigation, The PEF technique was performed for hydrolysis of antler residue in order to increase yield of dissoluble calcium and protein degree of hydrolysis. Based on the results of single-factor experiments, , RSM was used to optimize the hydrolysis parameters in terms of mixed acid content, electric field intensity and pulse number. A desirable quadratic mathematical model was built with the following optimal conditions for hydrolysis of protein: acid concentration 0.586 mol/L, electric field intensity 19 kV/cm, pulse number 8 (the mole ratio of citric acid to malic acid 1:1.5, E/S 4.0%), under these conditions, the DH of 19.3±0.34% and the YDC of 26±0.45% were obtained at 4 µs. The optimization of hydrolysis condition resulted in a increase yield of dissoluble calcium and degree of hydrolysis, the chosen method of hydrolysis was efficient, relatively simple and time saving.

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