Bone soup: protein nutrition and enzymatic hydrolysis process optimized by response surface method

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Summary
Protein nutrition of bone soup and its enzymatic hydrolysis process were investigated. Nutrition analysis indicated that the first limiting amino acid of sheep bone soup (SBS) was cystine, and of pig bone soup (PBS) was methionine. The essential amino acid ratio, protein chemical score, and protein efficiency ratio of SBS were higher than those of PBS. These results demonstrated that the protein nutrition value of SBS was superior to that of PBS. Enzymatic hydrolysis assays indicated that papain had the best hydrolysis effect when papain, pepsin and trypsinase were compared. Quasi-target optimization results showed that the optimal process for preparing SBS was at a temperature of 82.5 °C, duration of 2.75 h, pH 3.25, and the amount of enzyme 11 500 $U \cdot g^{-1}$. Verification test confirmed the optimized process was suitable for further use in the production of SBS.

Keywords
bone soup; papain; nutrition; response surface method

Soup is generally prepared by boiling food materials such as meat or meat bone, legumes or vegetables, with salt and spices. The nutrition value and functionality of the soup largely depend on the used food materials. Chicken soup contains nutritional components such as amino acids, proteins and some microelements. It can relieve cold symptoms, remove fatigue, offer enteral nutrition and enrich the blood [1]. A mild anti-inflammatory effect of the chicken soup can result in the mitigation of symptomatic upper respiratory tract infections [2]. Soups are reportedly satiating [3], and consuming a preload of low-energy-dense soup, in a variety of forms, is one strategy for moderating energy intake in adults [4]. These features have been confirmed to be beneficial to weight control [5], providing a balanced diet and a healthy nutritional status in the overall populations [6], which highlights soups as a hot research topic in recent years [7, 8]. However, fewer investigations on protein nutrition values of bone soups have been done.

Soups made of pig and sheep bones are typical bone soups. They are widely consumed and considered as health-preserving food [9, 10]. In most cases, the pig and sheep bone soups are homemade or produced in restaurants, where the preparation process is so coarse that the nutritional components in the bone, such as amino acids and proteins, are not efficiently exploited [11]. Therefore, there is a needed to improve the process so as to enhance its nutritional value and make it suitable for industrial production.

Enzymatic hydrolysis is used to prepare chicken soup [1], but few investigations have been studied on using enzymatic hydrolysis for preparing sheep bone soup. Enzymatic hydrolysis has a potential to be developed as an effective method for protein recovery from animal bone wastes, which contains both the collagenous and non-collagenous proteins [12]. Selection of a proper enzyme can improve the soup hydrolysate properties and enhance the soup flavour [13, 14]. Therefore, the investigation our study focuses on screening of

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enzymes and developing a suitable process to prepare bone soup.

From the perspective of process optimization, advanced statistical analyses are applied to assist the determination of the optimal product formulation in terms of a matrix of desired food characteristics, including quality, sensory acceptability, shelf life, nutritional demands and physiochemical stability [15, 16]. Response surface methodology (RSM) is a statistical procedure frequently used for optimization of complex processes and evaluation of interactive effects. It has been successfully used in the optimization of process variables [17, 18]. Our previous research also demonstrated the effectiveness of using RSM for optimizing the hydrolysis process of bone extraction [19, 20].

Based on these investigations, the objectives of this research were to (1) compare the protein nutrition of the pig and sheep bone soups, and (2) establish a suitable hydrolysis process for preparing the sheep bone soup.

MATERIAL AND METHODS

Materials

Sheep backbone was supplied by Big Ears Jianyang Revival sheep (Jianyang, China). Mutton in the sheep backbone was trimmed off, washed with running water to remove blood. About 50 kilograms of sheep backbone were sampled from fifty Big Ears ewe (weight 72.2 kg ± 2.1 kg). Each sample was packed in ice in polyethylene plastic bag after slaughtering and sent to our lab within 3 h. The crude composition of the sheep backbone was water content (65.6 ± 1.2)%, fat (7.1 ± 0.4)%, protein content (11.6 ± 0.7)%, ash content (11.3 ± 0.9)%.

Pig backbone was obtained from Sichuan Gaojin Food (Suining, China) and prepared by the same sampling procedures as sheep backbone. About 50 kg of pig backbone were sampled from 50 landrace (weight 92.1 kg ± 3.4 kg). The crude composition of pig backbone was water content (26.7 ± 1.3)%, fat (27.6 ± 0.3)%, protein content (17.6 ± 0.5)%, ash content (29.1 ±0.5)%. Papain, EC 3.4.22.2 (enzymatic activity 500 000 $U \cdot g^{-1}$) was purchased from Beijing Aoboxing Biology Technology (Beijing, China); pepsin, EC 3.4.23.1 (enzymatic activity 3 800 $U \cdot g^{-1}$) was purchased from Beijing Chih-Cheng Bio-Technology (Beijing, China); trypsinase, EC 3.4.21.4 (enzymatic activity 250 000 $U \cdot g^{-1}$) was purchased from Beijing BioDee BioTech (Beijing, China). Citric acid and disodium hydrogen phosphate (analytically pure) were purchased from Chengdu Wuhuan Gaoxin Chemical Reagent factory (Chengdu, China); neutral formalin (analytically pure) was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China).

Methods

Proximate analysis

Protein analysis of bones was carried out using the Kjeldahl method [21]. Fat analysis was performed using the Soxhlet extraction method [22]. Ash analysis was carried out gravimetrically by heating the sample at 550 °C in a muffle furnace for 24 h.

Preparation of soups

Sheep or pig backbone was chopped into 5 mm ± 2 mm square lumps, mixed with water at a ratio of 1 : 10 (bone weight 300 g : water 3 000 ml), and then boiled (95 °C ± 2 °C) in a temperature-controlled Media MK2102 electromagnetic furnace (Media Group, Foshan, China) for 2 h. In order to prevent water losing, the pot was covered during the boiling. After the boiling process, fat was removed from the surface of soup as it accumulated. In order not to influence the analysis of protein nutrition values and the natural flavour of soup, no spices were added during or after the boiling process. The protein contents of the sheep and pig bone soups were (1.1 ± 0.1)%, (1.9 ± 0.1)%, respectively.

Amino acid determination

After the boiling, the bone soup was naturally cooled down to 25 °C, and 100 ml of the upper layer of the soup was sampled for the analysis of amino acid composition. The total amino acid profiles of the soups were determined according to the method of Dong et al. [23] with a slight modification. The soups were hydrolysed with a 6 $mol \cdot l^{-1}$ HCl solution in a vacuum-sealed tube for 24 h at 110 °C prior to derivatization with phenyl isothiocyanate. The samples were derivatized by adding 20 μl of ethanol : water : triethylamine : phenylisothiocyanate (7 : 1 : 1 : 1) derivatizing solution, which was then allowed to react at room temperature for 10 min and then dried under vacuum for a minimum of 3 h. The samples were re-suspended in 200 μl of Picotag sample diluent (Waters, Millford, Massachusetts, USA) and 8 μl sub-sample was injected for separation by HPLC under gradient conditions. Buffer A was a sodium acetate buffer (pH 6.4) containing 5 000 $mg \cdot kg^{-1}$ EDTA, 1 : 2 000 triethylamine and 6% acetonitrile and buffer B consisted of 60% acetonitrile with 5 000 $mg \cdot kg^{-1}$ EDTA. A Waters high performance liquid

chromatography system (1525 HPLC with a binary gradient delivery, 717 auto-sampler and injector, 1500 column heater, 2487 dual-wavelength UV detector) and a Breeze data workstation (Waters) were used for the determination of amino acid composition. The contents of amino acids were calculated as grams per kilogram of protein. Three replicates were performed for each treatment.

Nutrition value analysis of amino acid

Biological values of bone soup were analysed by 5 parameters, namely, total amino acids (*TAA*) content, essential amino acid ratio (*EAAR*), protein chemical score (*PCS*) [24], essential amino acid index (*EAAI*), and protein efficiency ratio (*PER*) [24, 25]. The specific calculation formulae of the parameters are as follow:

$$TAA = \sum_{i=1}^{n} a_i \tag{1}$$

$$EAAR = \sum_{j=1}^{m} b_j \Big/ \sum_{i=1}^{n} a_i \tag{2}$$

$$PCS = Min\{(aa/AA)_1, \cdots, (aa/AA)_k\} \tag{3}$$

$$EAAI = \sqrt[k]{(aa/AA)_1 \times \cdots \times (aa/AA)_k} \tag{4}$$

$$PER = -0.468 + 0.454 \times Leu - 0.105 \times Tyr \tag{5}$$

where *a* is amino acid in meat sample; *b* – essential amino acid in meat sample; *n* – the number of amino acid; *m* – the number of essential amino acid; *AA* – the content of amino acid in reference [24] suggested pattern of protein requirement; *aa* – the content of amino acid compared to the suggested pattern of protein requirement and *k* – the number of amino acid type [24, 25], *Leu* – leucine, *Tyr* – tyrosine.

Bone hydrolysis

The chopped bone was mixed with a buffer (0.1 mol·l^{-1} citric acid and 0.2 mol·l^{-1} disodium hydrogen phosphate) at a ratio of 1:10 (20 g bone: 200 ml buffer) to perform the hydrolysis. For comparison, the producer's recommended hydrolysis condition was adopted. Bone was hydrolysed with pepsin at 37 °C, pH 7, 10 000 U·g^{-1}; trypsinase at 37 °C, pH 2.6, 10 000 U·g^{-1}; papain at 55 °C, pH 4, 10 000 U·g^{-1}. The hydrolysis optimization conditions are shown in Tab. 1. The hydrolysis reaction was done using a 1.667 Hz shaking incubator (Thermo 4520 Incubator Orbital shaker, Forma Scientific, Marietta, Ohio, USA). At the end of hydrolysis, the mixture was heated in boiling water for 15 min to inactivate the protease. The hydrolysate was stored at –20 °C until use (no longer than 90 days). All hydrolysate preparations were conducted in triplicate.

Determination of the degree of hydrolysis

The degree of hydrolysis (*DH*) was defined as the percentage of free N-terminal amino groups cleaved from proteins, which was calculated from the ratio of α–amino nitrogen to total nitrogen. According to the method of Nilsang et al. [26] and You et al. [27], the free N-terminal amino nitrogen content was determined by the formaldehyde titration method. The total protein content was determined by the Biuret method [28]. Bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, Missouri, USA) was used as the standard. The concentration of BSA was determined by using the absorbance at 280 nm (Bio-Rad Smart Spec 3000 UV/VIS spectrophotometer; Bio-Rad, Hercules, California, USA) [29], with the absorbance at 320 nm providing a background scattering correction. An extinction coefficient of 0.66 (0.1% BSA, 1 cm) was used [30]. The percentage of protein (*P*) in solutions was calculated using the following equation:

$$P = \frac{P_c \times V}{m_r} \times 100 \tag{6}$$

Tab. 1. Coded settings for the process parameters for hydrolysis, according to a central composite rotatable design.

Parameter	X1	X2	X3	X4
	Temperature [°C]	Time [h]	pH	Enzyme activity [U·g^{-1}]
–2	32.5	2.00	3.25	3500
–1	45.0	2.75	4.50	5500
0	57.5	3.50	5.75	7500
1	70.0	4.25	7.00	9500
2	82.5	5.00	8.25	11500

Tab. 2. Standard score sheet and corresponding concentration for taste analysis.

Score	Bitterness [g·l^{-1}]	Umami [g·l^{-1}]
5	0.005	0.03
4	0.0025	0.015
3	0.0012	0.0075
2	0.0006	0.0038
1	0.0003	0.0019

Bitterness is expressed as grams of quinine hydrochloride. Umami is expressed as grams of monosodium glutamate.

where P is expressed in percent, P_c is protein concentration in milligrams per millilitre, V is volume of solution in millilitres and m_r is weight of the raw material in grams. All the tests were conducted in triplicate.

Response surface experiment design

On the basis of the single-factor test, we designed a series of experiments by response surface methodology (RSM) to optimize the hydrolysis conditions. The coded and non-coded values of four independent variables by Central Composite (Uniform Precision) Rotatable design are described in Tab. 1. Four variables were used to determine the response pattern and then to establish a model. The four variables used in this study were hydrolysis temperature ($X1$), hydrolysis time ($X2$), hydrolysis pH ($X3$), and enzyme activity ($X4$), with 5 levels of each variable, while the dependent variables were degree of hydrolysis ($Y1$), bitterness ($Y2$), and umami taste ($Y3$), respectively. The symbols and levels are shown in Tab. 1. Seven replicates at the centre of the design were used to allow for estimation of a pure error sum of squares. Experiments were randomized to maximize the effects of unexplained variability in the observed responses because of extraneous factors. A full quadratic equation or the diminished form of this equation, shown as follows, was used for this model

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j \quad (7)$$

where Y is the estimated response and β_0, β_j, β_{jj}, and β_{ij} are the regression coefficients for intercept, linearity, square and interaction terms, respectively.

Sensory analysis

The tastes (bitterness and umami) of the hydrolysate were analysed as the inactivated hydrolysate was cooled down to (25 ± 2) °C. A panel comprising 10 females and 10 males (all panellists 21–31 years old) was recruited from the Department of Food Science and Engineering at Chengdu University, Chengdu, China.

The final 20 panellists had been trained to the standard level of proficiency for sensory evaluation. Briefly, prospective panel members were first tested for their ability to distinguish between the standard solution for the primary taste (bitterness, umami) and water by the threshold test [31]. Quinine hydrochloride (0.0003 g·l^{-1}) and monosodium glutamate (0.0019 g·l^{-1}) solutions were taken as the threshold. Then the qualified panel was asked to assess the difference in the strength of taste using the respective standard solution for the primary taste. Forty-five individuals participated in the screening session and 10 females and 10 males were selected. The evaluation panellists were trained by the strength of bitterness and umami of standard solution (Tab. 2), then let to score the taste of hydrolysate. The panellists were permitted to score between the standard scores, such as 0~1, 1~2, etc. Each sample (10 ml) was served in a 50 ml plastic cup at room temperature (25 °C ± 2 °C) in individual booths to each panel member. The score sheet is shown as Tab. 2.

Statistical analysis

The results were analysed by ANOVA at a significance level of 5% (H0: $p < 0.05$). The comparison of means was analysed by Fisher's LSD tests using the SAS statistical package (SAS Institute, Cary, North Carolina, USA).

RESULTS AND DISCUSSION

Comparison of protein nutrition values

Protein is one of the main nutrients of the bone soup, but its nutrition value was little investigated. Tab. 3 presents data on the amino acids of the sheep and pig bone soups. These data show that the first limiting amino acid of the sheep bone soup was cystine, and the first limiting amino acid of the pig bone soup was methionine. Glycine, glutamic acid and proline were three amino acids with the highest contents in the sheep or pig bone soup. Except for glycine, proline, alanine, phenylalanine and methionine, all other amino acids were contained at higher levels in the sheep bone soup than in the pig bone soup, indicating that the amino acids in the sheep bone soup were richer than those in the pig bone soup.

The ranked contents of the amino acids in the sheep or pig bone soup (Tab. 3) showed that

glycine, glutamic acid and proline were the three major amino acids both in the sheep and pig bone soups. These results are similar to those of An et al. [32] and Dickson [33], who determined the amino acid contents of sheep bone and pig bone. Comparing other amino acids in the sheep bone and pig bone with their corresponding soups, it was found that the ranked orders of the contents of the amino acids in the bones were similar to those in the corresponding soups, but the contents of amino acids in the sheep bone were higher than those in its soup, in particular for the three main amino acids glycine, glutamic acid and proline. Furthermore, according to results of protein determination, the protein content of the sheep bone and the sheep bone soup were 11.6% ± 0.7% (w/w) and 1.1% ± 0.0% (w/w), respectively. The dissolved protein in the sheep bone soup accounted only for 9.4% of the sheep bone protein, while almost 90% protein in the sheep bone was not dissolved. Similarly, the dissolved protein in the pig bone soup was 11.0%. These results indicated that large amounts of bone protein were not dissolved in the soup, in particular in case of the sheep bone.

The amino acid nutrition values of the sheep and pig bone soups are shown in Tab. 4. The data indicate that the total amino acids (*TAA*), essential amino acid index (*EAAI*) based on amino acid requirements of school children (10–12 years) or adult [24] of the sheep bone soup, were lower than those of the pig bone soup. However, the essential amino acid ratio (*EAAR*), protein chemical score (*PCS*) based on amino acid requirements of school children (10–12 years) or adults, protein efficiency ratio (*PER*) of the sheep bone soup were higher than those of the pig bone soup. *PCS, EAAR* and *PER* of the sheep bone soup were higher than those of the pig bone soup. These results suggest that the sheep bone soup provided higher protein nutrition values than the pig bone soup. Therefore, it is worth to make a good use of the sheep bone protein.

Tab. 3. Amino acid composition of soups prepared from pork bone and sheep bone.

Amino acids	Sheep bone	Pig bone
	Content [g·kg^{-1}]	
Glycine	1.1013 ± 0.0006	1.2887 ± 0.0035
Glutamic acid	1.0087 ± 0.0006	0.8767 ± 0.0006
Proline	0.7337 ± 0.0015	0.7740 ± 0.0010
Alanine	0.6060 ± 0.0020	0.6183 ± 0.0012
Aspartic acid	0.5693 ± 0.0016	0.5147 ± 0.0015
Arginine	0.5323 ± 0.0015	0.5147 ± 0.0006
Leucine	0.4130 ± 0.0010	0.3607 ± 0.0006
Lysine	0.3943 ± 0.0015	0.3507 ± 0.0012
Serine	0.2943 ± 0.0006	0.2780 ± 0.0010
Histidine	0.2657 ± 0.0014	0.2423 ± 0.0013
Valine	0.2477 ± 0.0013	0.2320 ± 0.0010
Threonine	0.2293 ± 0.0015	0.1963 ± 0.0011
Cystine	0.0500 ± 0.0001	0.4333 ± 0.0015
Phenylalanine	0.1740 ± 0.0010	0.2270 ± 0.0372
Tyrosine	0.1190 ± 0.0010	0.1130 ± 0.0000
Isoleucine	0.1467 ± 0.0012	0.1193 ± 0.0014
Methionine	0.0666 ± 0.0006	0.0670 ± 0.0001

Values are expressed as milligrams of amino acid per kilogram of proteins.

Effect of proteases on hydrolysis of sheep bone

In order to investigate action of enzymes at hydrolysis of sheep bone, papain, pepsin and trypsinase were chosen to hydrolyse the sheep bone. Fig. 1 shows that use of papain resulted in higher *DH* compared to pepsin and trypsinase, when the sheep bone was hydrolysed for 1.5 h and 3 h, even though there were no significant ($p > 0.05$) differ-

Tab. 4. Comparison of nutrition values of soups prepared from pork bone and sheep bone.

Nutrition of amino acids	Sheep bone soup	Pig bone soup
Total amino acids (*TAA*) [g·kg^{-1}]	6.9520 ± 0.0116	7.2067 ± 0.0456
Essential amino acids (*EAAR*) [%]	24.0 ± 0.0	21.5 ± 0.4
Protein chemical score (*PCS*)	0.0113 ± 0.0001 *	0.0092 ± 0.0001 *
	0.0052 ± 0.0001 **	0.0043 ± 0.0001 **
Essential amino acid index (*EAAI*)	0.0077 ± 0.0000 *	0.0086 ± 0.0001 *
	0.0193 ± 0.0000 **	0.0217 ± 0.0003 **
Protein efficiency ratio (*PER*)	0.1282 ± 0.0003	0.1051 ± 0.0003

Means ($n = 3$) without a common letter differ significantly ($p < 0.05$).
* – based on amino acid requirements of school children (10–12 years); ** – based on amino acid requirements of adults.

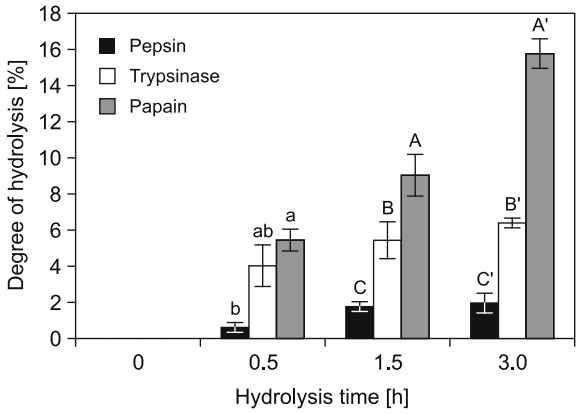

Fig. 1. Effect of enzyme categories on hydrolysis degree of the sheep bone soup.
Different letters indicate significant differences ($p < 0.05$)

ences when the sheep bone was hydrolysed by papain and trypsinase for 0.5 h. Similar results were found by Pena-Ramos et al. [34] and Akeson et al. [35], reporting that papain yielded higher *DH* when it was used to hydrolyse whey and egg protein compared with pepsin and trypsinase.

Pepsin and trypsinase were commonly adopted to mimic gastrointestinal digestion of protein in vitro [36–38]. Lower *DH* values of pepsin or trypsinase (Fig. 1) mean that the sheep bone protein was difficult to digest by human stomach directly. The higher *DH* of papain means that papain hydrolysis is more helpful to the exploitation of sheep bone protein and beneficial to the protein digestion. Therefore, papain was used to hydrolyse the sheep bone to prepare sheep bone soup.

Processing optimization

Effect of papain on hydrolysis of sheep bone

Orthogonal test and response surface experiment design are two major experiment design methods, they were frequently adopted to optimize the processing of food or other products [39–44]. Results of our recent research showed that the response surface method was superior to the orthogonal test when it was adopted to optimize the processing of bone extraction [45]. Based on these facts and our previous single factorial experiments [20], the response surface method was used to design the experiment. Hydrolysis temperature ($X1$ = TEM), hydrolysis time ($X2$ = TIM), hydrolysis pH ($X3$ = PH) and the amount of enzyme ($X4$ = ENZYME) were taken as independent factors, degree of hydrolysis ($Y1$ = DH), bitterness ($Y2$ = BITTER) and umami taste ($Y3$ = UMAMI) were as response or dependent factors. The designed experiments and experimental results are shown in Tab. 5.

Analysis of model fitting

Model fitting is the first step to analyse experimental results and is a key step to obtain the optimal processing parameters. A good fitting model is based on a rational experiment design and the corresponding results. If a significant model is established, there will be a quantitative relationship between the independent factors and the dependent factors. The model can be used to obtain the optimal process parameters or to perform the target optimization. The fitting model obtained from the response surface experiment results (Tab. 5) was shown as the following equation:

$$\begin{aligned} DH = & -5.5537013 + 0.4084345 \cdot X1 + \\ & + 0.1831944 \cdot X2 + 3.3848454 \cdot X3 - \\ & - 0.0009459 \cdot X4 - 0.0001343 \cdot X12 - \\ & - 0.0073333 \cdot X1 \cdot X2 - 0.0676 \cdot X1 \cdot X3 + \\ & + 0.000007.25 \cdot X1 \cdot X4 - 0.1484127 \cdot X22 + \\ & + 0.3133333 \cdot X2 \cdot X3 + 0.0000375 \cdot X2 \cdot X4 + \\ & + 0.0745714 \cdot X32 - 0.0001425 \cdot X3 \cdot X4 + \\ & + 0.0000001 \cdot X42 \end{aligned} \quad (8)$$

where *DH* is the degree of hydrolysis, $X1$ is the hydrolysis temperature, $X2$ is the hydrolysis time, $X3$ is the hydrolysis pH, $X4$ is the amount of enzyme.

The ANOVA results for the independent factors and the fitting model are summarized in Tab. 6. *DH* of the sheep bone was significantly ($p < 0.05$) influenced by the hydrolysis temperature ($p = 0.012609 < 0.05$), hydrolysis time ($p = 0.000812 < 0.05$), hydrolysis pH ($p = 0.004888 < 0.05$) and the amount of enzyme ($p = 0.000915 < 0.05$), the influenced extent was hydrolysis time > the amount of enzyme > hydrolysis pH > hydrolysis temperature. ANOVA for the quadratic terms showed that *DH* of the sheep bone was significantly ($p < 0.05$) influenced by the interaction effects of hydrolysis temperature and pH ($p = 0.0001 < 0.05$), and the amount of enzyme ($p = 0.008427 < 0.05$). The established model ANOVA (Tab. 6) indicated that the founded model was significant ($p = 0.000135 < 0.05$) when it was used to reflect the quantitative relationship between the independent factors and *DH*. In addition, "lack of fit" in Tab. 6 was not significant with p value of $0.101633 > 0.05$. All these significance analyses combined with the regression parameter $R^2 = 0.8675$ suggested that the developed model for *DH* was suitable to be used to cal-

culate the optimal processing parameters and to predict *DH* of the sheep bone hydrolysis [46].

The independent factors' ANOVA (Tab. 6) for bitterness of the hydrolysate indicated that it was significantly ($p < 0.05$) influenced by the hydrolysis temperature ($p = 0.000436 < 0.05$) and hydrolysis time ($p = 0.044912 < 0.05$). Therefore, temperature and time are apparently the key parameters and should be controlled strictly when the sheep bone is hydrolysed by papain. The model ANOVA (Tab. 6) for bitterness showed that the fitting model for bitterness was significant ($p = 0.000436 < 0.05$), and combining this significance with $R^2 = 0.7186$ reflected that the obtained model was feasible to predict bitterness of the hydrolysate. Similarly, the model ANOVA (Tab. 6) for the umami taste ($p = 0.018446 < 0.05$) and $R^2 = 8.21$ suggested that the developed model was effective to predict the umami taste of the hydrolysate.

Effectiveness test of the fitting model

In order to further confirm the effectiveness of the obtained model, the confirmatory test was done and its results are shown in Tab. 7. The determined *DH* values were compared with those calculated, the relative errors ranging from –2.9% to 8.0% (Tab. 7). These results of the confirmatory tests were consistent with the significance analysis (Tab. 6), which meant that the obtained model for *DH* was effective to be used to optimize and predict the hydrolysis parameters.

Process optimization of the papain hydrolysis

Based on the founded fitting model for *DH*, the optimal process of the sheep bone hydrolysis

Tab. 5. Response surface design for hydrolysis of sheep bone soup.

Run	*X*1	*X*2	*X*3	*X*4	*Y*1	*Y*2	*Y*3
	TEM [°C]	TIM [h]	PH	ENZYME [U·g^{-1}]	DH [%]	BITTER	UMAMI
1	–1.00	–1.00	–1.00	–1.00	15.3 ± 0.3	1.39 ± 0.08	2.52 ± 0.26
2	–1.00	–1.00	–1.00	1.00	15.7 ± 0.2	1.33 ± 0.17	2.06 ± 0.18
3	–1.00	–1.00	1.00	–1.00	18.0 ± 0.3	1.49 ± 0.15	1.52 ± 0.21
4	–1.00	–1.00	1.00	1.00	18.3 ± 0.1	1.28 ± 0.17	2.17 ± 0.12
5	–1.00	1.00	–1.00	–1.00	15.1 ± 0.1	1.53 ± 0.05	2.48 ± 0.28
6	–1.00	1.00	–1.00	1.00	16.7 ± 0.2	1.29 ± 0.11	2.51 ± 0.25
7	–1.00	1.00	1.00	–1.00	20.2 ± 0.6	1.47 ± 0.10	3.08 ± 0.21
8	–1.00	1.00	1.00	1.00	20.5 ± 0.5	1.25 ± 0.16	1.26 ± 0.16
9	1.00	–1.00	–1.00	–1.00	16.9 ± 0.3	1.33 ± 0.17	2.16 ± 0.12
10	1.00	–1.00	–1.00	1.00	19.7 ± 0.2	1.27 ± 0.10	3.17 ± 0.25
11	1.00	–1.00	1.00	–1.00	17.3 ± 0.4	1.93 ± 0.08	1.37 ± 0.21
12	1.00	–1.00	1.00	1.00	17.4 ± 0.3	1.93 ± 0.09	2.37 ± 0.24
13	1.00	1.00	–1.00	–1.00	18.0 ± 0.2	2.09 ± 0.14	1.42 ± 0.10
14	1.00	1.00	–1.00	1.00	20.1 ± 0.2	2.17 ± 0.12	2.68 ± 0.27
15	1.00	1.00	1.00	–1.00	18.4 ± 0.4	1.53 ± 0.15	2.23 ± 0.12
16	1.00	1.00	1.00	1.00	18.9 ± 0.2	1.81 ± 0.18	2.32 ± 0.23
17	–2.00	0.00	0.00	0.00	16.0 ± 0.1	1.49 ± 0.19	2.37 ± 0.14
18	2.00	0.00	0.00	0.00	17.5 ± 0.2	1.94 ± 0.29	1.52 ± 0.15
19	0.00	–2.00	0.00	0.00	15.2 ± 0.3	1.62 ± 0.16	2.06 ± 0.21
20	0.00	2.00	0.00	0.00	17.8 ± 0.1	1.99 ± 0.10	2.38 ± 0.14
21	0.00	0.00	–2.00	0.00	17.3 ± 0.1	1.98 ± 0.18	1.76 ± 0.11
22	0.00	0.00	2.00	0.00	17.3 ± 0.2	1.90 ± 0.12	1.43 ± 0.21
23	0.00	0.00	0.00	–2.00	16.9 ± 0.2	1.48 ± 0.08	1.87 ± 0.21
24	0.00	0.00	0.00	2.00	20.0 ± 0.3	1.53 ± 0.15	2.38 ± 0.14
25	0.00	0.00	0.00	0.00	16.9 ± 0.3	1.42 ± 0.14	1.58 ± 0.21
26	0.00	0.00	0.00	0.00	17.2 ± 0.5	1.58 ± 0.16	1.53 ± 0.16
27	0.00	0.00	0.00	0.00	16.9 ± 0.2	1.47 ± 0.15	1.68 ± 0.21
28	0.00	0.00	0.00	0.00	17.8 ± 0.5	1.54 ± 0.15	1.58 ± 0.17
29	0.00	0.00	0.00	0.00	18.1 ± 0.1	1.48 ± 0.15	1.37 ± 0.13
30	0.00	0.00	0.00	0.00	16.9 ± 0.3	1.51 ± 0.14	1.52 ± 0.17
31	0.00	0.00	0.00	0.00	17.1 ± 0.3	1.48 ± 0.17	1.54 ± 0.14

TEM – hydrolysis temperature, TIM – hydrolysis time, PH – hydrolysis pH, ENZYME – amount of enzyme, DH – degree of hydrolysis, BITTER – bitterness, UMAMI – umami taste.

Tab. 6. Significance analysis for factors and master model.

Independent factors' ANOVA							
		Degree of hydrolysis [%]		Bitterness		Umami taste	
Source	*DF*	*MS*	*p* values	*MS*	*p* values	*MS*	*p* values
TEM	1	4.08375	0.012609	0.643538	0.000436	0.103359	0.404249
TIM	1	8.760417	0.000812	0.156494	0.044912	0.067947	0.497256
PH	1	5.510417	0.004888	0.00057	0.897137	0.4704	0.086309
ENZYME	1	8.520417	0.000915	0.004293	0.723285	0.319243	0.151643
TEM*TEM	1	0.012589	0.878019	0.018551	0.464678	0.565588	0.062293
TEM*TIM	1	0.075625	0.707316	0.07412	0.153777	0.134506	0.342961
TEM*PH	1	17.85063	0.0001	0.009025	0.608495	0.009264	0.800844
TEM*ENZYME	1	0.525625	0.328591	0.068513	0.169267	1.540081	0.004453
TIM*TIM	1	0.199291	0.543666	0.067877	0.171145	1.246246	0.008936
TIM*PH	1	1.380625	0.121951	0.337561	0.005632	0.325756	0.147792
TIM*ENZYME	1	0.050625	0.758519	0.003393	0.752839	0.433622	0.098426
PH*PH	1	0.388228	0.399265	0.189801	0.029147	0.081312	0.458382
PH*ENZYME	1	2.030625	0.065088	0.001225	0.849778	0.228962	0.220478
ENZYME*ENZYME	1	4.667696	0.008427	0.021183	0.435167	0.978815	0.01796
Model ANOVA							
	DF	*MS*	*p* values	*MS*	*p* values	*MS*	*p* values
Model	14	3.874066	0.000135	0.113498	0.010246	0.425397	0.018446
linear	4	6.71875	0.0001	0.201224	0.003565	0.240237	0.197827
quadratic	4	1.362043	0.073154	0.072561	0.115865	0.580606	0.017497
cross product	6	3.652292	0.000825	0.082306	0.067836	0.445365	0.030566
Error	16	0.517612		0.03306		0.140823	
lack of fit	10	0.68675	0.101633	0.051244	0.000971	0.22005	0.000416
pure error	6	0.235714		0.002754		0.008777	
Regression coef. R^2	0.8675			0.7186		8.208726	

TEM – hydrolysis temperature, TIM – hydrolysis time, PH – hydrolysis pH, ENZYME – amount of enzyme, *DF* – degree of freedom, *MS* – mean square.

Tab. 7. Effectiveness of relativity model in predicting the possible processing of the sheep bone soup.

RUN	*X*1	*X*2	*X*3	*X*4	Degree of hydrolysis		Relative error [%]
	TEM [°C]	TIM [h]	PH	ENZYME [U·g^{-1}]	Determined [%]	Calculated [%]	
1	45.00	2.75	7.00	9500	18.3	17.8	–2.9
2	45.00	4.25	4.50	5500	15.1	15.3	0.9
3	45.00	4.25	7.00	5500	20.2	19.6	–2.9
4	57.50	3.50	8.25	7500	17.3	18.6	8.0
5	70.00	2.75	4.50	9500	19.7	19.4	–1.3
6	70.00	2.75	7.00	9500	17.4	17.0	–2.4
7	70.00	4.25	4.50	9500	20.1	20.0	–0.2
8	70.00	4.25	7.00	9500	18.9	18.7	–0.6
9	82.50	3.50	5.75	7500	17.5	18.0	2.5
10	57.50	3.50	5.75	11500	20.0	19.9	–0.4
11	57.50	3.50	5.75	7500	16.9	17.2	1.7

TEM – hydrolysis temperature, TIM – hydrolysis time, PH – hydrolysis pH, ENZYME – amtount of enzyme.

was optimized. Taking the *DH* value as the maximum to optimize the process, results showed that the optimal hydrolysis process was hydrolysis temperature 82.5 °C, hydrolysis time 3.5 h, pH 3.25, the amount of enzyme 11500 U·g^{-1}. Under these conditions, the corresponding *DH* was 26.7%, sensory value for the bitterness was 2.24 and sensory value for the umami taste was 5.05.

According to the standard sensory score sheet (Tab. 2), the bitterness score over 2 suggests that the panellists can feel the bitter taste obviously. Enzymatic hydrolysis of proteins often causes

a bitter taste [47, 48]. Numerous bitter peptides have been isolated from enzymatic hydrolysate of casein [49, 50], soybean protein [51, 52], cheese [53, 54] and some other foods. Bitterness greatly affects the flavour of the hydrolysate [19], mainly due to the presence of strongly hydrophobic bitter peptides formed during the hydrolysis degradation [48]. In order to decrease the influence of bitterness on the flavour of sheep bone hydrolysate, quasi-target optimization method was adopted to optimize the hydrolysis process of the sheep bone. Quasi-target optimization means that part of the optimizing targets were limited. To optimize the hydrolysis process, the maximum *DH* was taken as the target, the value of the bitterness was limited to 2, and the process temperature was limited to 50 °C. Results showed that the quasi-target optimized process was temperature 82.5 °C, hydrolysis time 2.75 h, pH 3.25, the enzyme amount 11 500 $U \cdot g^{-1}$. Under these conditions, the corresponding *DH* was 26.6%, sensory value for the bitterness was 1.75, sensory value for the umami

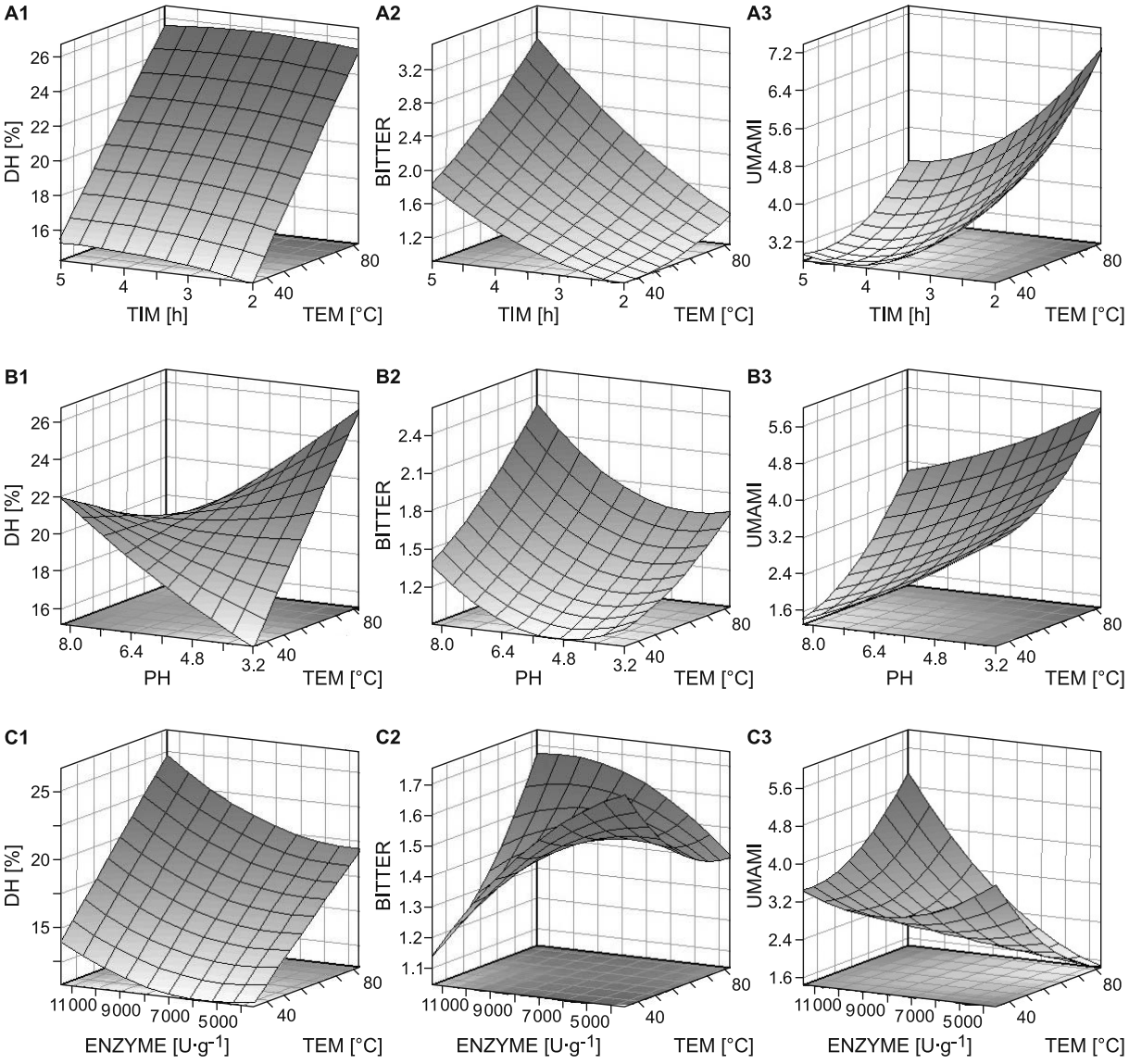

Fig. 2. Response surface of the optimized processing for preparing sheep bone soup.

A – response surfaces of degree of hydrolysis, bitterness and umami taste with temperature and time (fixed levels: pH 3.25, amount of enzyme 11 500 $U \cdot g^{-1}$); B – response surfaces of degree of hydrolysis, bitterness and umami taste with temperature and pH (fixed levels: hydrolysis time 2.75 h, amount of enzyme 11 500 $U \cdot g^{-1}$); C – response surfaces of degree of hydrolysis, bitterness and umami taste with temperature and the amount of enzyme (fixed levels: hydrolysis time 2.75 h, pH 3.25).
DH – degree of hydrolysis, BITTER – bitterness, UMAMI – umami taste, TIM – hydrolysis time, TEM – hydrolysis temperature, PH – hydrolysis pH, ENZYME – amount of enzyme.

taste was 6.01. Comparing the two optimized processes, the hydrolysis time was curtailed to 0.5 h. This result is consistent with NILSANG et al. [26] and ZHU et al.[55], who found that shorter hydrolysis time resulted in lower bitterness of the hydrolysate. *DH* was little influenced (decreased by 0.4%), but the bitterness score was decreased obviously (decreased by 21.9%), and the umami taste score, which is popular to soup products [56, 57], increased by 19.0%. This result showed that the second optimized process was acceptable and beneficial to the hydrolysis. Therefore, hydrolysis temperature 82.5 °C, hydrolysis time 2.75 h, pH 3.25 and the enzyme amount 11 500 $U \cdot g^{-1}$ were taken as the optimal process conditions to prepare the sheep bone soup.

The changes of *DH*, bitterness and umami taste scores are visualized in the response surfaces (Fig. 2). In Fig. 2A are the response surfaces of *DH*, bitterness and the umami score with the hydrolysis temperature and time. Fig. 2B shows the response surfaces of *DH*, bitterness and the umami score with the hydrolysis temperature and pH. Fig. 2C shows the response surfaces of *DH*, bitterness and the umami score with the hydrolysis temperature and the amount of enzyme. As shown in Fig. 2A1, 2B1 and 2C1, higher temperatures correspond to higher *DH* values. Fig. 2A2, 2B2 and 2C2 show that shorter hydrolysis time, optimal pH and larger amounts of enzyme resulted in a weaker bitterness. Fig. 2A3, 2B3 and 2C3 show that a higher temperature and shorter time or pH, higher temperature and higher amount of enzyme led to stronger umami taste.

CONCLUSIONS

The first limiting amino acid of the sheep bone soup was cystine, and of the pig bone soup was methionine. Glycine, glutamic acid and proline were the three amino acids with the highest contents in both the sheep and pig bone soups. The protein nutrition value of the sheep bone soup was higher than that of the pig bone soup. Application of papain provided a higher degree of hydrolysis compared to pepsin or trypsinase. The quasi-target optimized hydrolysis process for preparing the protein enriched sheep bone soup was determined by temperature 82.5 °C, hydrolysis time 2.75 h, pH 3.25, and the amount of enzyme 11 500 $U \cdot g^{-1}$. Under these conditions, *DH*, bitterness value and the umami taste score were 26.5%, 1.75 and 6.01, respectively. These results allow further application of the developed process in the production of the sheep bone soup.

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