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Investigation of Enzymatic Hydrolysis Conditions on the Properties of Protein Hydrolysate from Fish Muscle (*Collichthys niveatus*) and Evaluation of Its Functional Properties

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Supporting Information

ABSTRACT: This study was carried out to investigate the enzymatic hydrolysis conditions on the properties of protein hydrolysate from fish muscle of the marine fish species *Collichthys niveatus*. About 160 fish samples were tested, and the analyzed fish species was found to be a lean fish with low fat (1.77 ± 0.01%) and high protein (16.76 ± 1.21%). Fish muscle of *C. niveatus* was carefully collected and hydrolyzed with four commercial enzymes: Alcalase, Neutrase, Protamex, and Flavourzyme under the conditions recommended by the manufacturers. Among the tested proteases, Neutrase catalyzed the hydrolysis process most effectively since the hydrolysate generated by Neutrase has the highest content of sweet and umami taste amino acids (SUA). The effect of hydrolysis conditions was further optimized using response surface methodology (RSM), and the optimum values for temperature, pH, and enzyme/substrate ratio (E/S ratio) were found to be 40.7 °C, 7.68, and 0.84%, respectively. Finally, the amino acid composition of the hydrolysate was analyzed by AccQ·Tag derivatization and HPLC−PDA determination. Major amino acids of the muscle of *C. niveatus* were threonine, glutamic acid, phenyalanine, tryptophan, and lysine, accounting for respectively 10.92%, 10.85%, 10.79%, 9.86%, and 9.76% of total amino acid content. The total content of essential amino acids was 970.7 ng·mL^{-1}, while that of nonessential amino acids was 709.1 ng·mL^{-1}. The results suggest that the fish muscle and its protein hydrolysate from *C. niveatus* provide a versatile supply of the benefits and can be incorporated as supplements in health-care foods.

KEYWORDS: *fish protein hydrolysate, enzymatic hydrolysis condition, Collichthys niveatus, response surface methodology, essential amino acids*

INTRODUCTION

Fish are very rich sources of excellent proteins. Utilization of marine resources for human consumption has increased rapidly worldwide. *Collichthys niveatus* (*C. niveatus*) is an abundant and economic species caught in the East China Sea, with a total annual catch approximately 0.32 million tons in this sea area according to the data (2010) provided by Oceanic and Fishery Department of Zhejiang Province. However, it is considered an under-utilized species due to the poor handling infrastructure available for the catch. Converting this unexploited resource to a high value healthy food would go a long way in feeding an increasing population. Recently, the nutritional content of this species has increased the interest of food and medical scientists.[1,2] In addition, the production of new value-added products such as protein hydrolysates, with nutritive value, as well as good functional properties and potential biological activity, can pave the way for full utilization of this species.[3]

The use of commercial enzymes for the production of highly functional hydrolysate from fish species of low commercial value can be a feasible technology to convert most of the vast underutilized resource into a more marketable and functional form, namely, fish protein hydrolysate (FPH). Enzymatic hydrolysis of proteins makes the peptide size decrease, which can modify functional characteristics of the proteins and improve their qualities.[4] Due to the capability of its functional properties, this hydrolysate could be used as a food ingredient for direct human consumption. This positive effect was attributed to the increase in digestibility of the meal due to the enzymatic treatment; free amino acids released in the process might also act as attractants for consumption.[5] For further improving the quality of FPH, it is important to use a specific enzyme and choose a defined set of hydrolysis conditions, such as time, pH, and temperature, to partially hydrolyze the proteins to the desired extent. The production and nutrition of FPH from fish muscle of *C. niveatus* have not been investigated.

Given the reasons mentioned above, the objective of this study is to investigate the proximate composition and the enzymatic hydrolysis conditions on the properties of protein hydrolysate from fish muscle of a popular fish species (*C. niveatus*). Response surface methodology was used to explore the effect of hydrolysis conditions (temperature, pH, and E/S ratio) on the degree of hydrolysis, as well as the level of sweet and umami taste amino acids (SUA) produced from the hydrolysate of muscle protein. Finally, amino acid composition of the hydrolysate was examined to determine by AccQ·Tag derivatization and the HPLC−PDA method.

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MATERIALS AND METHODS

Materials and Reagents. The experimental fish (*C. niveatus*) with an average weight of ~50 g/fish and body length ~12 cm were cultured in the Hangzhou Bay (an inlet of the East China Sea), caught in the middle of October, and authenticated by Zhejiang Research Institute of Marine Fisheries (Zhejiang, China). Alcalase and Neutrase were purchased from Novo Nordisk's Enzyme Business (Wuxi, China), and Protamex and Flavourzyme were purchased from Wuxi Enzymes Co. (Wuxi, China). Amino acid mixture standards (containing 18 kinds of amino acids) were purchased from BBI Co., Ltd. (Boston, MA, USA). β-Mercaptoethanol (β-ME) was purchased from Sigma Chemicals Co. (St Louis, MO). HPLC grade methanol (MeOH) and acetonitrile (ACN), used as the LC mobile phase, were purchased from Merck (Darmstadt, Germany). High purity water with a resistivity of 18.2 MΩ cm^{-1} was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All other chemicals and solvents used in this study were of analytical or HPLC grade.

Sample Preparation. The fresh fish (*C. niveatus*), off-loaded within one day after capture, were placed in ice at a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Zhejiang Gongshang University, Hangzhou. Upon arrival, fish samples, with no evident signs of parasites as described by Mazorra–Manzano,[6] were collected, and the ordinary muscle from both sides of the fish sample was obtained by means of a mechanical deboner after removing the internal organs, heads, and tails of fish. Then, the muscle sample was rinsed with cold distilled water and ground to uniformity by a grinder (Micromill grinder, Shanghai Pingcheng Co., China). A portion of mince (100 g) was placed in a polytetrafluoroethylene tube and freeze-dried (LGJ-18, RuibangXinye Co., Beijing, China) under vacuum. Lyophilized muscle powder was sieved through a 120-mesh screen and was kept in sealed glass jars at 4 °C until used.

Chemical Analysis. Moisture and ash content were determined according to the AOAC (1995) standard methods to be 930.15 and 942.05, respectively. The content of crude protein was determined from representative samples of lyophilized muscle powder through the determination of the total Kjeldahl nitrogen ×6.25. Fat was determined gravimetrically after Soxhlet extraction of dried samples with hexane. Carbohydrate was determined by the phenol sulfuric acid method. All measurements were performed in triplicate. The protein and fat contents were expressed on a dry weight basis.

Hydrolysis of *C. niveatus* Protein. A modified enzymatic hydrolysis method was used for hydrolyzing the fish muscle protein of *C. niveatus*.[7] Muscle protein was dissolved as a 5% (w/v) solution in 100 mL of distilled water and homogenized with ultra-turrax (IKAT–18 basic) for 10 min. The pH of the mixture and temperature activity values of each enzyme, including Alcalase, Neutrase, Protamex, and Flavourzyme, was adjusted to the optimum condition recommended by the manufacturers. The reaction was initiated by the addition of each enzyme at the same activity levels (10.103 U) to compare the hydrolytic efficiencies in a 250 mL reaction vessel, and the solution was maintained at constant temperature when it was being agitated by magnetic stirrer. The pH of the mixture was kept constant by continuous addition of 0.2 M NaOH whose amount was recorded. After 4 h of hydrolysis, the solution was heated to 90 °C and kept for 15 min to inactivate the enzyme, and then cooled down quickly to room temperature (about 25 °C) by ice-cold water. Then, the resultant slurry was centrifuged at 8000*g* for 15 min to remove the insoluble residue. The supernatant was freeze-dried and stored at −20 °C before further analysis.

Degree of Hydrolysis. The degree of hydrolysis, defined as the percentage of the number of peptide bonds cleaved divided by the total number of peptide bonds in a protein, was calculated from the consumption of base (NaOH) by the pH-stat method of Adler–Nissen.[8] The percent DH was calculated by the following equation:

$$DH\ (\%) = \frac{h}{h_{tot}} \times 100 = \frac{BN_b}{\alpha M_p h_{tot}} \times 100 \quad (1)$$

where B is the base consumption in mL; N_b is base normality; α is the average degree of dissociation of the α-NH_2 groups in the protein substrate; M_p is the mass (g) of protein ($n \times 6.25$); and h_{tot} is the total number of peptide bonds available for proteolytic hydrolysis (7.8 meq·g^{-1})

Hydrolysis Conditions Optimization. RSM was utilized to optimize the hydrolysis conditions of the muscle protein by using Neutrase, which could produce the hydrolysate with the highest content of SUA. A three-level-three-factor Box–Behnken design using Design-Expert6 software (Stat-Ease Inc., Minneapolis, USA)[9] was employed in this study, requiring 15 experiments as shown in Table 1. On the basis of the preliminary experiments, the three levels for the selected variables were pH (7.0, 7.5 and 8.0), temperature (35, 40, and 45 °C), and E/S ratio (0.7, 0.8, and 0.9), and the measured variable response was SUA (μg·mL^{-1}). Each enzymatic hydrolysis experiment was done according to the pH-stat procedure described above and replicated three times. The experimental data obtained (shown in Table 1) were fitted by the following regression equation:

Table 1. RSM Test Design and Values for the Response Variables SUA (μg·mL^{-1})

	independent variables[a]			response[b]	
run	*A*	*B*	*C*	experimental	predicted
1	35	7.0	0.8	73.41 ± 0.32	74.01
2	35	8.0	0.8	97.30 ± 0.86	96.38
3	35	7.5	0.7	89.02 ± 0.45	89.33
4	35	7.5	0.9	103.46 ± 1.22	103.47
5	45	7.0	0.8	77.75 ± 0.18	78.67
6	45	8.0	0.8	104.67 ± 1.32	104.07
7	45	7.5	0.7	100.51 ± 0.64	100.50
8	45	7.5	0.9	104.96 ± 0.36	104.65
9	40	7.0	0.7	74.87 ± 0.88	73.96
10	40	8.0	0.7	102.15 ± 1.02	102.76
11	40	7.0	0.9	88.64 ± 0.73	88.03
12	40	8.0	0.9	106.09 ± 1.22	107.00
13	40	7.5	0.8	115.05 ± 0.91	112.17
14	40	7.5	0.8	111.06 ± 0.86	112.17
15	40	7.5	0.8	111.83 ± 0.92	112.17

[a]Independent variables *A*, *B*, and *C* represent the temperature (*T*, °C), pH, and the enzyme/substrate ratio (E/S, %), respectively. [b]Response Y represents SUA (μg·mL^{-1}), which refers to the concentration of sweet and umami taste amino acids in muscle protein hydrolysate.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j \quad (2)$$

where Y is the measured response variable, β_0, β_i, β_{ii}, and β_{ij} is the constant, linear, quadratic, and cross-product regression coefficients of the model, respectively, and where X_i and X_j represent the hydrolysis parameters. The model's goodness-of-fit was evaluated by the coefficient of determination (R^2) and the analysis of variance. The response surface and contour plots were developed using the fitted full quadratic polynomial equations, obtained by holding one of the independent variables at a constant value and changing the levels of the other two variables.

AccQ·Tag Derivatization and Amino Acids Analysis. For derivation of the amino acids with AccQ·Tag reagents, 10 μL of either a standard amino acid mixture or pre-purified hydrolysate was mixed with 70 μL of AccQ·Tag Ultra borate buffer, and 20 μL of AccQ·Tag reagent previously dissolved in 1.0 mL of AccQ·Tag Ultra reagent diluent was added. The reaction was allowed to proceed for 10 min at 55 °C.

Chromatographic analysis was performed on a Waters 2695 LC system (Waters, Milford, MA, USA) which was equipped with a

quaternary pump, an autosampler, a vacuum degasser, a PDA detector, and a LC workstation. The separation column was a Waters AccQ·Tag Ultra column (3.9 mm i.d. × 150 mm, 4 μm particles). The column heater was set at 55 °C, and the mobile phase flow rate was maintained at 1.0 mL·min^{-1}. Eluent A was 10% AccQ·Tag Ultra concentrate solvent A, and eluent B was 100% ACN. The nonlinear separation gradient was 0−0.54 min (99% A), 18.00 min (95% A), 19.00 min (91% A), and 29.5 min (83% A); during rest time, the column was cleaned, readjusted to the initial conditions, and equilibrated. Five microliters of sample was injected for analysis. The PDA detector was set at 254 nm, with a sampling rate of 20 points·s^{-1}.

RESULTS AND DISCUSSION

Proximate Composition. The proximate composition of fish mince of *C. niveatus* in this study had 79.72 ± 2.32% moisture, 16.76 ± 1.24% crude protein, 1.77 ± 0.01% lipids, 1.25 ± 0.08% ash, and 0.17 ± 0.01% carbohydrate. According to Tzikas et al.,[10] seafood is usually classified as fatty (>8% fat), moderately fat (3−8%), and lean (<3%). The fish species studied was found to be a lean fish because its fat content was lower than 3% by weight. About 50−80% of its fat is triacylglycerol stored in the liver, which is a source of fat soluble vitamins, such as vitamins A and D.[11] According to Osman et al.,[12] low fat fish have higher water content, and as a result, their flesh is white in color. The results obtained in the present study confirmed it well since high moisture content was found in *C. niveatus* at 77.72% with 1.77% fat. Meanwhile, crude protein content is high also (classified as high when greater than 15%);[13] thus, it is suitable for enzymatic hydrolysis.

Screening of Efficient Proteases. The type of enzyme used had great effect on the yield and properties of the final product. Therefore, the muscle protein of *C. niveatus* was hydrolyzed with different enzymes (Alcalase, Neutrase, Protamex, and Flavourzyme) for screening the most efficient one. To evaluate the hydrolysis efficiency, the degree of hydrolysis (*DH*) value is a wildly used criterion since it is an important factor highly related with the hydrolytic process yield. Higher *DH* usually produces lower molecular weight polypeptides, thus increasing the solubility of the hydrolysate. Additionally, it influences the other functional properties, such as emulsifying and foaming properties.[14,15] However, a very high degree of hydrolysis can have enormously negative effects on the functional properties.[16] After enzymatic hydrolysis of the muscle protein of *C. niveatus*, the hydrolysate was full of amino acids, polypeptides, and nucleotides, while free amino acids contribute most to the taste. Although a higher *DH* value produces more sweet and umami taste amino acids, more bitter tasting amino acids are produced simultaneously as byproducts. Thus, appropriate hydrolysis conditions for satisfactory hydrolysis degrees and SUA content need to be studied. Finally, three sweet and umami amino acids (alanine, aspartic acid, and glutamic acid) and one bitter amino acid (phenylalanine) were selected as the main criteria for screening of efficient proteases. The hydrolyzing results of each protease are summarized in Table 2.

The degree of hydrolysis of the hydrolysate catalyzed by Alcalase was highest, with the DH value at 17.03%, while that of Flavourzyme is lowest, with a DH value of only 5.82%. For Neutrase and Protamex, their DH values are 15.04% and 12.98%, respectively. Such a glaring discrepancy is related to various factors, including the protein source and the enzyme used. Since the sweet, umami and bitter tasting amino acids influence the properties a lot, much attention was paid to the SUA and phenylalanine contents. The hydrolysate catalyzed by

Table 2. DH, Sweet and Umami Taste Amino Acids, and Phenylalanine Content of Hydrolysates Catalyzed by Four Different Proteases

proteases	DH (%)	SUA[a] (μg·mL^{-1})	phenylalanine (μg·mL^{-1})
Alcalase	17.03	65.129	35.149
Neutrase	15.04	88.259	33.202
Protamex	12.98	40.796	27.005
Flavourzyme	5.82	49.172	25.316

[a]Sweet and umami taste amino acids, alanine, aspartic acid, and glutamic acid were taken as representative amino acids.

Neutrase had significantly higher SUA content, 88.3 μg·mL^{-1}, and comparatively lower phenylalanine content, 33.2 μg·mL^{-1}. The hydrolysate catalyzed by Alcalase gave the highest content of phenylalanine 35.1 μg·mL^{-1}. On the one hand, it was related to the high degree of hydrolysis; on the other hand, short peptides and amino acids with hydrophobic groups, which contribute to the bitter taste, were generated during the hydrolysis process since the active site of Alcalase was usually formed at the hydrophobic amino acids. SUA and phenylalanine contents of Protomex (40.8 μg·mL^{-1} and 27.0 μg·mL^{-1}) and Flavourzyme (49.2 μg·mL^{-1}, 25.3 μg·mL^{-1}) catalyzed hydrolysates had similar values. On the basis of these, Neutrase was chosen for hydrolyzing the muscle protein of *C. niveatus*. Its high efficiency and low cost will provide an incentive for using it in commercial operations.

Effect of Individual Factors on Products. Hydrolysis time is an important factor that would influence the hydrolyzing efficiency and SUA content generation in the fluid. Longer hydrolysis time usually presents more positive effects since more chances were provided for molecule collision. Enzymatic hydrolysis was carried out at different time conditions, while the other parameters, like pH value and E/S ratio, were kept constant, and the influence of time on hydrolysis of protein from *C. niveatus* is shown in Figure 1A. When the hydrolysis time varied from 1 to 4 h, the rate of SUA production was relatively more rapid. The yield of SUA reached a maximum at 4 h, and no obvious change was observed as the hydrolysis proceeded. As a result, 4 h was ensured as the optimum hydrolysis time.

During enzymatic catalysis, temperature can affect the reaction speed of molecules, the activation energy of the catalytic reaction, and the thermal stability of the enzyme and substrate. The effect of hydrolysis temperature from 30 to 70 °C on the amount of total SUA of hydrolysate was determined with pH at 7.5 and hydrolysis time 4 h (Figure 1B). As expected, the results demonstrate that temperature has obvious effect on the reaction efficiency. The SUA content sharply increased along with the increase of temperature and reached the maximum level at 40 °C. When the temperature was above 40 °C, the inflection point appeared, and the total amount of the products started to decrease. This is because at relatively lower temperature, the rate of enzymatic reaction is slower, the molecules have lower kinetic energies, and the collisions between them are less frequent. At a higher temperature, the force maintaining the shape of the molecule is disrupted, and the enzyme molecules are gradually denatured. Finally, 40 °C was chosen as the optimum temperature for the hydrolysis of protein from *C. niveatus* by Neutrase.

The effect of pH ranging from 6.0 to 8.5 on the SUA production of hydrolysate from fish muscle protein of *C. niveatus* was determined at 40 °C, with a hydrolysis time of 4 h,

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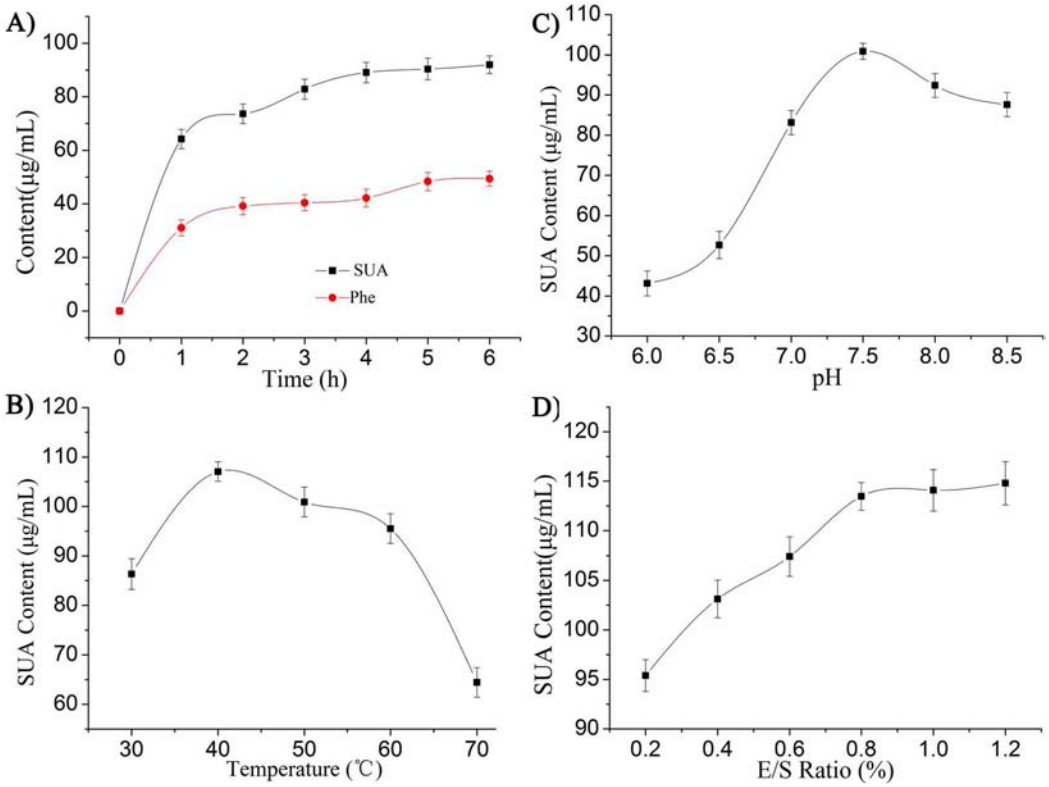

Figure 1. Effect of hydrolysis time (A), temperature (B), pH (C), and E/S ratio (D) on the SUA yield of hydrolysate. Results are presented as means ± standard deviations (n = 3).

as shown in Figure 1C. Too high or low pH values generally result in the loss of activity for enzyme since the active site will be progressively distorted, and the enzyme function will be negatively influenced. The optimum for the hydrolysis of protein by the selected Neutrase was pH 7.5. Therefore, pH 7.5 was chosen as the central point with a step change of 0.5.

As shown in Figure 1D, the increase of E/S ratio from 0.2 to 0.8 resulted in the increase of SUA content in the hydrolysate. The results could be due to greater hydrolysis of the protein when more Neutrase were added. Considering the cost, the central point for E/S ratio was 0.8 with a step change of 0.1.

Interactive Effect of Temperature, pH, and E/S Ratio. RSM was used to evaluate the interactive effect of reaction pH (A), temperature (B, °C), and time (C, h) on the hydrolysis of muscle protein from *C. niveatus* by Neutrase. Table 1 shows the experimental data and predicted values of the SUA content under different treatment conditions. The yield of SUA content ranged from 73.41 $\mu g \cdot mL^{-1}$ to 115.05 $\mu g \cdot mL^{-1}$, and the maximum value was found at the 13th run, with the following conditions: reaction temperature, 40 °C; pH 7.5; and E/S ratio, 0.8. The predicted values agreed well with the experimental ones obtained from the RSM design. Multiple regression analysis was applied to the experimental data, and the response variable and the test variables yielded the following second-order polynomial equation:

$$Y = 112 + 3.09A + 11.94B + 4.58C - 8.91A^2 - 15.46B^2 - 4.25C^2 + 0.76AB - 2.50AC - 2.45BC \quad (3)$$

Plus sign in front of the terms indicates a synergistic effect, whereas the minus sign indicates an antagonistic effect. Statistical test of the model was performed by analysis of variance (ANOVA), which is required for testing the significance and adequacy of the model. The ANOVA for the response surface model of enzymatic hydrolysis conditions is given in Table S-1 of the Supporting Information. The fit of the model was measured by the coefficient of determination R^2, which was calculated to be 0.9946, indicating that the model eq 3 was adequate to explain the influence of the independent variables studied on the SUA content of the protein hydrolysate and represent the actual relationship between the reaction parameters. In the model, the effect of each coefficient on the respective response variable was determined using the F-test and p-value. The corresponding variables would be more significant if the absolute F-value becomes greater and the p-value becomes smaller.[17] As shown (see Table S-1 of the Supporting Information), the variable with the largest effect on SUA yield was the linear term of pH (F = 408.39, $p < 0.0001$), followed by the quadratic term of pH (F = 315.71, $p < 0.0001$), quadratic term of temperature (F = 104.82, $p < 0.001$), and the linear term of E/S ratio (F = 59.97, $p < 0.001$). The model F-value is 101.92 ($p < 0.0001$), implying that the degree of fit was better on the border of the independent variables. In addition, the lack of fit of this model was nonsignificant (p = 0.7869), which would give a relative better fit to the mathematic model in eq 3.

The graphical representation of the regression eq 3, called response surfaces, and the contour plots were generated by varying two of the independent variables within the experimental range while holding the other two constant at the central point. Response surface plots provide a method to predict the yield of SUA for different values of the tested variables and the contours of the plots help in the identification of the types of interactions between these variables. As shown in Figure S-1 of the Supporting Information, the maximum

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predicted value indicated by the surface was confined in the smallest ellipse in the contour diagram, and the elliptical contours can be obtained when there is a perfect interaction between each independent variable. Figure S-1A (Supporting Information) illustrates the response surface and contour plot for the effect of temperature and pH on the yield of SUA at a fixed E/S ratio 0.8. Yield of SUA was very sensitive to the changes of the solution pH. At a given temperature, the SUA content increased sharply when the pH of the substrate increased from 7.0 to 7.6, while it declined along with the further increase of pH. It is important to note that the optimum pH for SUA yield is ~7.7, whereas under highly acidic and moderate basic conditions, little hydrolysis occurred since such extreme conditions inhibit the activity of Neutrase. The interactive effect of temperature and E/S ratio on the yield of SUA is shown in Figure S-1B (Supporting Information). It indicated that under lower E/S ratio, the SUA yield increased with the rise of temperature obviously, due to the elevation of enzyme activity at a center temperature. Afterward, SUA content decreased since Neutrase is less active at higher temperatures and may even be deactivated. At appropriate temperature (~42 °C), the increase of E/S ratio would cause the increase of SUA content and hydrolyzing power. But at the high E/S ratio value area, the contour plot became flattened. From an economic view, the optimum value (~0.85) of E/S ratio was selected when the turning point appeared. Figure S-1C (Supporting Information) is the response surface and contour plots showing the effect of pH and E/S ratio on SUA yield at the fixed temperature of 40 °C. The E/S ratio behaved in a manner similar to that in Figure S-1B (Supporting Information). The optimum pH is predicted to be ~7.7, which confirmed the results in Figure S-1A (Supporting Information). Among the three enzymatic hydrolysis parameters studied, pH is the most significant factor to affect the yield of SUA, followed by temperature according to the gradient of slope in the 3D response surface plots.

The optimal conditions were extracted by Design Expert software. The optimum values of the process variables for the maximum decolorization efficiency are temperature, 40.67 °C; pH 7.68; and E/S ratio, 0.84. The results of the optimized parameters generally agree well with that of Zhang et al.[18] Under the above optimized conditions, the hydrolysate of muscle protein from *C. niveatus* contains maximum production of SUA, estimated to be 115.49 $\mu g \cdot mL^{-1}$. To ensure the predicted result was not biased toward the practical value, experimental rechecking was performed using this deduced optimal condition. After verifying by a further experimental test with the predicted values, the result indicated that the maximal SUA content, 116.07 $\mu g \cdot mL^{-1}$, was obtained when the values of each parameter were set as the optimum values, which was in good agreement with the value predicted from the model. It implies that the strategy to optimize the enzymatic hydrolysis conditions and to obtain the maximal SUA content by RSM for improving the flavor and taste of muscle of *C. niveatus* in this study is successful.

AccQ·Tag Based Amino Acids Quantization. Multiple analytical methods for amino acid analysis in various matrices had been reported, including gas chromatography–flame ionization detector (GC–FID), micellar electrokinetic chromatography (MEKC), capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), and an enzyme based biosensor.[19,20] Because of the simplicity, HPLC methods are widely used for the determination of amino acids. In addition, many kinds of chemical derivatization reagents have been implemented for amino acid detection. Among various derivatization methods, the AccQ·Tag technology continues to gain widespread acceptance.[21] An attractive feature of this technology is that the derivatization reaction is straightforward and can be completed within 10 min.

Sensitive and efficient methods for amino acid quantization in the hydrolysate of *C. niveatus* generated under optimum conditions were AccQ·Tag HPLC–PDA. The AccQ·Tag regent, *N*-hydroxysuccinimide–activated heterocyclic carbamate, was utilized to react with primary and secondary amines, yielding stable fluorescent derivatives.[22] In order to guarantee complete conversion and high stability of the formed derivatives, 10 min is allowed for proceeding in the derivatization step, although the reaction takes place within seconds. Figure 2A shows a typical chromatogram for the

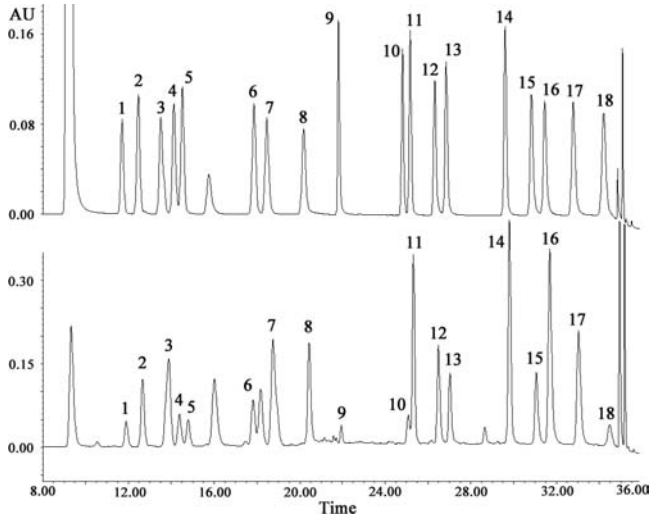

Figure 2. HPLC–PDA chromatograms of (A) 18 amino acid standards and (B) tested amino acids in hydrolysate of muscle protein of *C. niveatus*. On the basis of the order of retention time, the peaks are 1, Asp; 2, Ser; 3, Glu; 4, Gly; 5, His; 6, Arg; 7, Thr; 8, Ala; 9, Pro; 10, Cys; 11, Tyr; 12, Val; 13, Met; 14, Lys; 15, Ile; 16, Leu; 17, Phe; and 18, Trp.

HPLC analysis of a standard mixture of AccQ·Tag-derivatized amino acid standards using a PDA detector. All of the 18 tested amino acids were observed in the chromatogram, with sharp and symmetric peaks, designated from 1 to 18. The peak observed at ~1.25 min corresponded to the major hydrolysis product (6-amino quinoline, AMQ) of unreacted AccQ·Tag reagent.

The linearity of the calibration curves, limits of detection for AccQ·Tag-derivatized physiological amino acid standards, and amino acid content in the hydrolysate of *C. niveatus* are summarized in Table 3. Values reported in this table were obtained using the external standardization method, in which absolute peak areas are correlated to amino acid concentration. Each calibration curve was linear in a concentration ranging from the quantification limit to 1000 $ng \cdot mL^{-1}$ for each amino acid, with satisfactory average correlation coefficients (0.9982–0.9997), which indicated good linearity between the peak area and the concentration of the investigated amino acids. The LOD was considered as the analyte minimum concentration that can be confidently identified by the method. It was determined by analyzing a blank sample at levels that provided

Table 3. Parameters of Linearity, Limit of Detection (LOD), Retention Time, and Amino Acid Content

names	R^2	RT[a]	LOD[b]	content[c]	percent[d]
Asp	0.9992	11.85	51.2	32.1	1.91
Ser	0.9996	12.66	43.1	56.5	3.36
Glu	0.9984	13.70	57.8	182.2	10.85
Gly	0.9984	14.36	33.2	33.8	2.01
His	0.9995	14.77	64.3	32.4	1.93
Arg	0.9995	18.10	45.2	97.2	5.79
Thr	0.9990	18.71	47.3	183.4	10.92
Ala	0.9997	20.38	35.9	87.6	5.21
Pro	0.9988	21.92	47.6	12.1	0.72
Cys	0.9983	24.81	38.4	41.9	2.49
Tyr	0.9998	25.18	54.8	165.7	9.86
Val	0.9982	26.30	46.2	86.0	5.12
Met	0.9984	26.83	52.1	70.4	4.19
Lys	0.9996	29.49	42.1	164.0	9.76
Ile	0.9996	30.70	45.3	71.2	4.24
Leu	0.9995	31.32	62.1	122.4	7.29
Phe	0.9987	32.58	57.4	181.2	10.79
Trp	0.9996	34.40	80.2	59.7	3.55

[a]Retention time, min. [b]Limit of detection, ng·mL^{-1}. [c]Measured in ng·mL^{-1}. [d]Amount of total amino acids, %.

signals at three times above the background noise. LOD of the tested amino acids ranged from 33.2 ng·mL^{-1} to 80.2 ng·mL^{-1}, which indicated that the analytical method has excellent sensitivity. The amino acid composition of the muscle of *C. niveatus* (Figure 2B) indicates that the major amino acids were Thr, Glu, Phe, Tyr, and Lys, accounting for 10.92%, 10.85%, 10.79%, 9.86%, and 9.76%, respectively. Thr is important for *C. niveatus* because it is related to some mechanisms of functionality, such as the antioxidant activities and the tyrosinase inhibitory effect.[23] Glu (10.85%), Ala (5.21%), and Gly (2.01%) are found to be responsible for flavor and taste amino acids in marine fish.[24] Glu and Asp, accounting for 12.76% of total amino acid content, contributed most to the umami taste, especially their sodium salts, which could cooperate with AMP and IMP to enhance the strength of flavor. Thr and Ala are important sweet taste amino acids, and their total content is up to 16.13%. Moreover, the flavor was further enhanced by the buffer capacity of His when coexisting with lactic acid and KH_2PO_4. In bitter tasting amino acids, they are also abundant in the hydrolysate, especially Phe (10.79%). However, most of them did not reach the bitter taste threshold and will not cause much effect on the flavor. Although Lys and Met were beyond individual thresholds (data are not shown), their bitter taste could be inhibited by adding limited NaCl to the hydrolysate. The total content of essential amino acids was calculated to be 970.7 ng·mL^{-1}, while that of nonessential amino acids was 907.1 ng·mL^{-1}. The essential/nonessential amino acid ratio was 136.8%, which was obviously higher than the FAO/WHO reference model with a value of 60%. In other words, under the optimized hydrolysis condition, the generated hydrolysate from *C. niveatus* has higher sweet taste amino acid content and essential/nonessential amino acid ratio value.

In summary, this study has shown that the analyzed fish species, *C. niveatus*, is quite nutritious in terms of its low fat (1.77 ± 0.01%) and high protein (16.76 ± 1.21%) contents. RSM results gave the optimum value of temperature, pH, and enzyme/substrate ratio (E/S ratio) at 40.7 °C, 7.68, and 0.84%, respectively. Finally, amino acid composition of the hydrolysate was determined, and high SUA content (116.07 μg·mL^{-1}) and abundant essential amino acids (136.8%) were obtained. The results suggest that the fish muscle and its protein hydrolysate from *C. niveatus* could be utilized to develop potentially functional foods. The hydrolysate provides a versatile supply of the benefits and can be incorporated as a supplement in health-care food. Further studies will focus on the bioactivity and medicinal properties of hydrolsate of *C. niveatus*.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

ANOVA results of SUA content as affected by temperature, pH, and E/S ratio, during optimization experiments using Neutrase; response surface plots and contour plots for the effects of variables on SUA content. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

RSM, response surface methodology; E/S, enzyme/substrate; HPLC−PDA, high−performance liquid chromatography−photo diode array; LOD, limit of detection; SUA, sweet and umami taste amino acids

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