2-Step Optimization of the Extraction and Subsequent Physical Properties of Channel Catfish (*Ictalurus punctatus*) Skin Gelatin

HONGSHUN YANG, YIFEN WANG, MINGKANG JIANG, JUN-HYUN OH, JOSH HERRING, AND PENG ZHOU

ABSTRACT: To optimize the extraction of gelatin from channel catfish (*Ictalurus punctatus*) skin, a 2-step response surface methodology involving a central composite design was adopted for the extraction process. After screening experiments, concentration of NaOH, alkaline pretreatment time, concentration of acetic acid, and extraction temperature were selected as the independent variables. In the 1st step of the optimization the dependent variables were protein yield (YP), gel strength (GS), and viscosity (V). Seven sets of optimized conditions were selected from the 1st step for the 2nd-step screen. Texture profile analysis and the 3 dependent variables from the 1st step were used as responses in the 2nd-step optimization. After the 2nd-step optimization, the most suitable conditions were 0.20 M NaOH pretreatment for 84 min, followed by a 0.115 M acetic acid extraction at 55 °C. The optimal values obtained from these conditions were YP = 19.2%, GS = 252 g, and V = 3.23 cP. The gelatin obtained also showed relatively good hardness, cohesiveness, springiness, and chewiness. The yield of protein and viscosity can be predicted by a quadratic and a linear model, respectively.

Keywords: catfish, fish skin, gelatin, response surface methodology, texture profile analysis

Introduction

Gelatin is a soluble polypeptide derived from insoluble collagen. Procedures to derive this soluble polypeptide involve the breakdown of cross-linkages between polypeptide chains of collagen along with some amount of breakage of polypeptide chain bonds. When tissues that contain collagen are subjected to mild degradative processes (for example treatment with alkali or acid followed or accompanied by heating in the presence of water), the systematic fibrous structure of collagen is broken down irreversibly and gelatin is formed (Ward and Courts 1977). To our best knowledge, it is the only food material that gels and melts reversibly below the normal human body temperature (37 °C). Gelatin's unique and outstanding functional properties, along with its reasonable cost, make it one of the most widely used food and pharmaceutical ingredients.

The gelatin industry primarily uses cattle hides, beef bones, and pork skin as raw materials to obtain collagen, which can be transformed into gelatin through partial hydrolysis. However, pork gelatin is not permitted to be used for both Muslims and Jews for religious reasons, while nonreligiously slaughtered beef gelatin is also generally a problem. Furthermore, bovine spongiform encephalopathy (BSE) and other food safety problems are perceived by some consumers as a concern and provide an opportunity to market alternative materials.

Extraction of gelatin from fish skins may provide an alternative to cattle and pork gelatin. The byproducts from fish processing after filleting account for a large percentage of the total catch weight. The yield of catfish fillets is only 45% of the total weight and the byproducts account for 55%. Processing data from a major catfish processor in Alabama indicate that the frame and skin are 25% and 6%, respectively, of the initial fish weight (Prinyawiwatkul and others 2002). Also fish gelatin has been shown to have a better release of a product's aroma and flavor with less inherent off-flavor and off-odor than a commercial pork gelatin. Thus fish gelatin can offer new opportunities to product developers (Choi and Regenstein 2000). In the past decade, fish gelatin extraction has been reported in the scientific literature for sole (*Solea solea*) (Devictor and others 1995), cod (*Gadus morhua)* (Gudmundsson and Hafsteinsson 1997), hake (*Merluccius merluccius*) (Montero and others 1999), blue shark (*Prionace glauca*) (Yoshimura and others 2000), megrim (*Lepidorhombus boscii*) (Montero and Gómez-Guillén 2000), black tilapia (*Oreochromis mossambicus*) and red tilapia (*O. nilotica*) (Jamilah and Harvinder 2002), yellowfin tuna (*Thunnus albacares*) (Lefebvre and others 2002; Cho and others 2005), Alaska pollock (*Theragra chalcogramma*) (Zhou and Regenstein 2004, 2005), horse mackerel (*Trachurus trachurus*) (Badii and Howell 2006), and skate (*Raja kenojei*) (Cho and others 2006). The amount of gelatin obtained commercially from fish and other species increased consistently from 2003 to 2005. Over this period, the percent of gelatin from fish and other marine species increased from 0.7% to 1.3% of total world production (GME 2006).

Generally, fish gelatins have lower gel strength than mammalian gelatins. Previous researchers indicated that warm-fish gelatin has physical properties more like beef and pork than that of cold-water gelatin (Cho and others 2005). In the southern regions of the United States, such as Alabama, Louisiana, and Mississippi, catfish is a common farm-raised, warm-water fish that can supply fish skin as a stable and year-round gelatin source.

In 2005, the amount of catfish processed by the main processors in the United States was over 272000 metric tons with a stable

MS 20060539 Submitted 09/26/2006, Accepted 2/13/2007. *Authors Yang, Wang, and Jiang are with the Biosystems Engineering Dept., Auburn Univ., 200 Tom E. Corley Building, Auburn, AL 36849-5417. Author Oh is with the Dept. of Food and Nutritional Sciences, Tuskegee Univ., Tuskegee, AL 36088. Author Herring is with Dept. of Food and Animal Sciences, Alabama A & M Univ., Normal, AL 35762. Author Zhou is with the Dept. of Food Science and Nutrition, Univ. of Minnesota–Twin Cities, St. Paul, MN 55108. Direct inquiries to author Wang (E-mail: wangyif@auburn.edu).*

doi: 10.1111/j.1750-3841.2007.00319.x

monthly quantity of around 22700 tons (NASS 2006). Thus, the total amount of gelatin from catfish skin could be approximately 2450 tons annually in the United States, which would comprise about 0.8% of the world's gelatin market. The sales of catfish produced in the United States in 2005 earned $462 million (out of the aquacultured food fish total of $672 million (http://www.nass.usda.gov/Census˙of˙Agriculture/2002/Aquaculture/index.asp).
However, gelatin obtained from the skin of catfish, the main farm-raised fish in the United States, has not been systematically studied as a raw material for edible gelatin. Only a patent referring to an acid process for the preparation of catfish gelatin (Lefebvre and others 2002) and a poster at the IFT annual meeting in 2005 (Olsen and others 2005) reported on the tensile and puncture properties of mammalian and fish gelatin films, with catfish gelatin being mentioned.

Response surface methodology (RSM) is a set of mathematical and statistical techniques widely used to determine the effects of multiple variables and to optimize different processes. It is a mathematical modeling technique that relates independent and dependent variables and establishes regression equations that describe the interrelations between input parameters and output properties (Cho and others 2004). A 1-step optimization approach was reported for fish skin gelatin extraction optimization for several fish species (Gudmundsson and Hafsteinsson 1997; Cho and others 2004, 2005; Zhou and Regenstein 2004; Cho and others 2006). However, the 1-step optimization approach makes it difficult to obtain the best extraction conditions for gelatin production for 3 reasons: (1) during optimization, a large number of experiments are needed, and the experiments cannot therefore be done at the same time, which results in some deviation in the actual time versus the "reported" time during alkaline and acid pretreatment; (2) after optimization, several sets of results may have values close enough that it can be hard to determine whether the condition given by the software is the best one; and (3) it is impossible for the 1-step optimization approach to include all the important physical properties of gelatin as a response variable. One of the modified RSM, that is, the generalized RSM, can be used to solve such complicated situations but needs advanced mathematical knowledge (Schamburg and Brown 2004).

The aim of this work was to optimize the conditions for extracting gelatin from catfish skin using a set of preliminary screening experiments followed by a 2-step optimization approach. Several important factors were evaluated using the screening experiments. The advantages of the 2-step optimization approach preceded by screening showed that the optimization conditions could be selected from a larger candidate pool. Meanwhile, more physical properties can be considered as responses using this approach.

Materials and Methods

Gelatin extraction

Preparation of materials. Frozen catfish skins were obtained from the Harvest Select Inc. (Uniontown, Ala., U.S.A.) plant. The frozen skins were stored at −18 °C with a maximum storage of less than 2 months before use. All reagents were analytical grade. Cleaning of the fish skins used the procedures of Zhou and Regenstein (2004) with slight modifications. Frozen catfish skins were thawed at 4 °C for about 20 h, cut into small pieces (about 2 to 3 cm), and washed with tap water (1:6 w/v) at 4 °C for 10 min. Washing was repeated 3 times. The cleaned fish skins were drained using cheesecloth for 5 min, and the cheesecloth containing the skins were squeezed by hand to remove liquid.

Gelatin extraction. Based on preliminary experiments, a pretreatment with an alkaline solution followed by an acid solution was chosen for this project. After pretreatment, the fish skins were added to a flask, mixed with ion-free water (D4641 4 module Barnstead E-pure water system, Van Nuys, Calif., U.S.A.), and then the flask was heated in a water bath. The detailed steps were as follows: Cleaned skins (ca. 30.000 g) were added to a flask and treated with NaOH (1:6 w/v) for variable times. Then, the samples were drained using cheesecloth and rinsed with tap water. The above procedure was repeated 2 times. Afterwards the samples were treated with acetic acid (1:6 w/v) for variable times. The samples were then drained using cheesecloth and rinsed with tap water (1:6 w/v) 3 times. All the solutions used in the above steps were kept at 4 °C. After the above pretreatment, ion-free water was added to the flasks. Then Parafilm (Structure Probe, Inc/SPT Supplies, West Chester, Pa., U.S.A.) and aluminum foil were used to cover the flasks and samples were extracted in a water bath for variable times. Finally, the gelatin solutions were filtered through 4 layers of cheesecloth prior to further work.

Determination of physical properties of gelatin

Yield of protein. The soluble protein concentration of the extracted solutions was determined by the Biuret method (Gornall and others 1949) using a spectrophotometer (Milton-Roy Spectonic 20D+, Spectronic Instrument Inc, Rochester, N.Y., U.S.A.) with bovine serum albumin (BSA, standard grade, Equitech-Bio Inc., Kerrville, Tex., U.S.A.) as a standard. At low BSA concentrations a strong linear relation was not found, so the concentration of BSA was increased to create a standard curve for 5 mg/mL to 10 mg/mL.

The yield of protein (YP) was calculated using the following equation:

YP (%) = (protein concentration [g/mL] × volume of extract [mL]/weight of sample (wet skins after processing) [g]) × 100%.

Gel strength. The extracted solutions were diluted to a protein concentration of 3.3% using distilled water (if the concentration was below 3.3%, the solution was used as is). The solutions were heated in a 50 °C water bath for 30 min and then were added to small plastic bottles (the solution bottles were 31 mm dia × 25 mm height, flat bottom). After being matured at 4 °C for (17 ± 1) h, the gel strength was determined with The TA.XTPlus Texture Analyzer (Texture Technologies Corp., Scarsdale, N.Y./Stable Micro Systems, Godalming, Surrey, U.K.), using a 12.5-mm-dia flat plunger pressing 4 mm into the gelatin gel using a 5-kg load cell at a speed of 1 mm/s at 4 °C.

Texture profile analysis. The gelatin samples for texture profile analysis (TPA) were from the same samples as those previously used for gel strength. After being matured at 4 °C for 17 ± 1 h, the samples were taken out of the plastic bottles as 1 piece and texture profile analysis tests were performed with the TA.XTPlus Texture Analyzer using a 75-mm-dia plate.

On the basis of the preliminary experiment and other research (Demars and Ziegler 2001), a reasonable compression value for TPA was determined, that is, a 40% compression. The detailed test settings were Pretest speed: 1.0 mm/s; Test speed: 0.5 mm/s; Posttest speed: 0.5 mm/s; Target mode: Distance; Distance of compression: 10.0 mm (the height of the gel is 25 mm); Time: 10.0 s; Trigger type: Auto (Force); Trigger Force: 0.05 N; Tare Mode: Auto; and Advanced Options: On.

The specimens were lubricated on their top and bottom with mineral oil prior to measurement. To keep the temperature consistent between samples, the testing was done immediately after the sample was removed from the 4 °C refrigerator. The results were reported as the means of 5 replicates from same lot. Textural parameters such as hardness, cohesiveness, springiness, and chewiness were calculated from the TPA curve as shown in Figure 1. Hardness was

calculated from the peak force during the first compression cycle. Cohesiveness was defined as a ratio of the areas delimited by the curves of the second and the first compression. Springiness was defined as a ratio of the time measured between the start of the second area and the second compression direction reversal divided by the time measured between the start of the first area generation and the first compression's direction reversal. Chewiness (g) was calculated by multiplying hardness, cohesiveness, and springiness (Surówka 1997; Lau and others 2000; Bayarri and others 2005).

Viscosity. The concentrations of extracted gelatin solutions were adjusted to a 3.3% with distilled water. About 10 mL solutions were used to determine the viscosity (V, cP) using a Cannon-Fenske routine viscometer (Cannon Instrument Co., State College, Pa., U.S.A.) at 60 °C, a standard temperature for measuring viscosity of gelatin (Ward and Courts 1977). A stopwatch was used to record the efflux time. The density of the gelatin solution at 3.3% was calculated from the viscosity (V, cP), according to a certification manual of calibration for viscometer No. 100 that came with the viscometer.

Viscometer constant $= C_0(1 - B[T_T - T_F]) = 0.01434 \times (1 - 78 \times 10^{-6}[60 - 23]) = 0.01430$ cSt/s; where C_0 is a constant, 0.01434 cSt/s (mm^2/S^2); B is the coefficient of the thermal expansion of the viscometer, 78×10^{-6}/°C; T_T is test temperature, 60 °C; and T_F is the filling temperature for calibration of the instrument, 23 °C.

Kinematic viscosity (cSt) = Efflux time (s) × Viscometer constant (cSt/s);

Viscosity (cP) = Kinematic viscosity (cSt) × Density of the measured solution (g/mL).

Response surface methodology

Design-Expert, Version 7 software for Windows (Stat-ease Inc., Minneapolis, Minn., U.S.A.) was used for the RSM analysis (Design-Ease 2006). Three stages were used for the optimization of the gelatin extraction: screening, 1st-step optimization and 2nd-step optimization.

A factorial design (2^{7-3}) was used for screening the extraction factors. This 7-factor, 2-level fractional factorial screening design was used to select the 4 most important factors out of an original 7 factors (alkaline concentration and pretreatment time, acid concentration and pretreatment time, extraction temperature and time, and the skin/water ratio). This stage helped to determine which factors were significant for the gelatin extraction (Araujo and Brereton 1996).

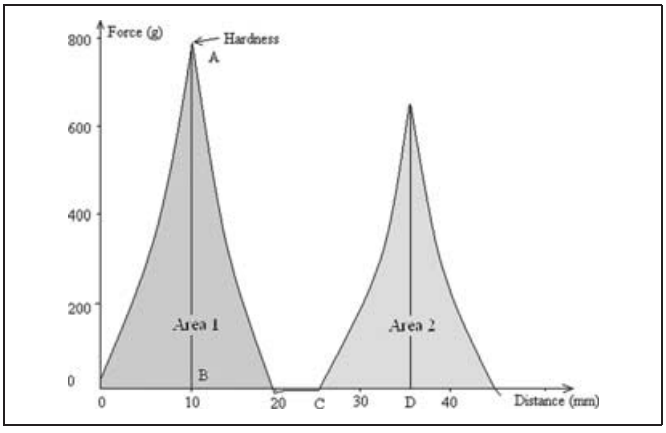

Figure 1—A schematic texture profile analysis curves of gelatin extracted from catfish skin Note: Hardness = BA (g); Cohesiveness = Area 2/Area 1; Springiness = CD/Original sample height × 100%; Chewiness = Cohesiveness × Hardness × Springiness (g).

The optimization process was divided into 2 steps. In the 1st step a central composition design (CCD) was adopted with yield of protein, gel strength, and viscosity as responses, with the experimental design for the CCD step consisting of 2^4 factorial points, 8 axial points ($\alpha = 2$, α indicates the number of axial point levels), and 7 replicates of the central point. Four factors from the screening experiments were chosen as independent variables with the range and center point value of the 4 independent variables also being based on the screening experiments. Randomized experiments were conducted to minimize the effects of unexpected variability in the observed responses (Cho and others 2005). After the conditions for the desired range for the independent and dependent variables were set up, the RSM software would supply many groups of optimized conditions during the 1st-step optimization.

In the 2nd-step optimization, several groups of optimized conditions from the 1st-step optimization were selected for further evaluation. TPA as well as the 3 dependent variables (responses) that were used in the 1st-step optimization was comprehensively evaluated with the selected groups. Also, experiments based on the conditions indicated by these groups were done to verify the 1st-step optimization results and to further evaluate these conditions during the 2nd-step optimization. At the end of the 2nd-step optimization, the best group of gelatin extraction conditions was determined.

Statistical analysis

A factorial design (2^{7-3}) was applied for screening, then a CCD consisting of 2^4 factorial points was conducted as the 1st-step optimization, and then one of the optimized groups was selected after the 2nd-step optimization. If there are no special notations, all the experiments were conducted in triplicate and the average results were reported. Average results along with the standard deviations were reported in the verification tests. Statistical analysis of the verification results were evaluated using ANOVA ($P < 0.05$) and Duncan's multiple range test using SAS (Version 9.1, Cary, N.C., U.S.A.).

Results and Discussion

Screening factors for gelatin extraction

To study a large number of factors efficiently, reduced factorial designs were employed. With this design the important factors can be efficiently evaluated using a small fraction of the experiments required for a full factorial design (Araujo and Brereton 1996). Low temperature is often set within a narrow range for fish by-product processing because of the need to prevent spoilage. Therefore, a temperature of 4 °C was used in our experiments. The other 7 factors and their ranges between model levels described as −1 and 1 were selected. Alkaline concentration (mol/L), A (0.1 to 0.3); Alkaline pretreatment time (min), B (30 to 90); Acid concentration (mol/L), C (0.06 to 0.12); Acid pretreatment time (min), D (30 to 90); Extraction temperature (°C), E (40 to 60); Extraction time (min), F (120 to 240); and skin/water ratio (w/w), G (1/3 to 1/4) were evaluated. A total of 16 groups of extraction experiments were conducted using different combinations of these 7 factors and the results are shown in Table 1. Note that experimental results were for certain properties of the extracted catfish gelatin. For the convenience of the analyses and evaluations, Table 1 is summarized and rearranged into Table 2.

In Table 2, the effects of different factors on the responses can be ranked in order. For protein yield, the order is E>C>F>A>G>D>B; for gel strength: E>B>A>C>F>G>D; and for viscosity: C>E>A>D>B>G>F. From the orders above, the factors

E (extraction temperature), C (acid concentration), and A (alkaline concentration) were selected as the 3 most important factors, and the 4th one could be selected from F (extraction time), B (alkaline pretreatment time), or D (acid pretreatment time). To select one of these 3 factors, the importance of the responses was considered. The gel strength is a major commercially important physical property of gelatin gels (Zhou and others 2006), which suggests that the strength of gels may be a limiting factor for applications of fish gelatin. The higher the value, the stronger the gel (Grossman and Bergman 1992). Viscosity is a commercially important property of gelatin. Low viscosity of a gelatin solution usually results in gels with a short and brittle texture, while high viscosity provides tough and extensible gels (Zhou and others 2006). Therefore, high viscosity gelatin is preferred and fetches a higher commercial price (Badii and Howell 2006). On the basis of the values of GS and V, the factor B: alkaline pretreatment time was selected as the 4th factor. Thus the 4 factors selected for optimization were E: extraction temperature (°C); C: acid pretreatment concentration (mol/L); A: alkaline pretreatment concentration (mol/L); and B: alkaline pretreatment time (min), other factors were set on the basis of the preliminary experiment with a pretreatment temperature at 4 °C, acid pretreatment time at 60 min and the skin/water ratio at 1:4 for all the experiments conducted thereafter.

The screening experiments can provide the information as to which steps are crucial to the efficiency of extraction. Table 2 shows that the degree of conversion of collagen into gelatin (yield of protein) is related to the severity of both the pretreatment (such as A: alkaline pretreatment concentration, B: alkaline pretreatment time, and C: acid pretreatment concentration) and the extraction processes (such as E: extraction temperature). Our observations were in good agreement with the report of Montero and Gómez-Guillén (2000).

1st-step optimization of the gelatin extraction

The experimental design for the 1st-step optimization of the process was based on the CCD to decrease the number of experiments. The 4 independent variables and their range were set (Table 3) and 31 groups of extraction experiments were conducted according to the CCD design (Table 4). Both the experimental results and the predicted values determined by the software are shown in Table 4.

Yoshimura and others (2000) reported that alkaline attacks predominantly the telopeptide region of the collagen molecule during pretreatment; thus an alkaline solution can be used to solubilize collagen. The amino acid composition of the solubilized product shows that the noncollagenous protein is released at the early stage of alkaline extraction (Yoshimura and others 2000). Another possible explanation of the results is the inability to recover protein as gelatin when the alkaline concentration is above a certain concentration (Zhou and Regenstein 2004). Therefore, long-time, high concentration alkaline pretreatment would decrease the yield of protein. This statement was verified by our experiments. In Table 4, for example, group 2 has a higher alkaline concentration than group 1 when the rest of the conditions stayed the same. This resulted in a lower yield

Table 1—Fractional factorial screening design (2^{7-3}) in coded units and experimental results

	Independent variables							Dependent variables		
No.	A	B	C	D	E	F	G	YP (%)	GS (g)	V (cP)
1	−1	−1	−1	−1	−1	−1	−1	6.62	146.9	1.40
2	1	−1	−1	−1	1	−1	1	11.09	290.7	1.77
3	−1	1	−1	−1	1	1	−1	17.63	239.0	1.96
4	1	1	−1	−1	−1	1	1	3.38	19.8	0.71
5	−1	−1	1	−1	1	1	1	17.62	244.6	2.86
6	1	−1	1	−1	−1	1	−1	13.03	255.0	2.91
7	−1	1	1	−1	−1	−1	1	13.10	221.7	2.42
8	1	1	1	−1	1	−1	−1	14.96	235.3	3.00
9	−1	−1	−1	1	−1	1	1	10.76	194.8	1.69
10	1	−1	−1	1	1	1	−1	14.49	254.2	2.75
11	−1	1	−1	1	1	−1	1	15.31	299.3	2.80
12	1	1	−1	1	−1	−1	−1	2.80	17.4	0.70
13	−1	−1	1	1	1	−1	−1	13.55	287.3	3.85
14	1	−1	1	1	−1	−1	1	14.41	220.9	2.35
15	−1	1	1	1	−1	1	−1	14.16	205.0	2.86
16	1	1	1	1	1	1	1	18.46	167.0	2.68

Independent variables and their ranges (–1–1). A: Alkaline concentration, 0.1 to 0.3 mol/L; B: Alkaline pretreatment time, 30 to 90 min; C: Acid concentration, 0.06 to 0.12 mol/L; D: Acid pretreatment time, 30 to 90 min; E: Extraction temperature, 40 to 60 °C; F: Extraction time, 120 to 240 min; G: Skin/water ratio, 1/3 to 1/4 w/w. YP = yield of protein; GS = gel strength; V = viscosity. The density of the 3.3% gelatin solution at 60 °C is 1.020 g/mL.

Table 2—Effect of the factors on the responses during screening experiments

Result	Level	A	B	C	D	E	F	G
YP(%)	−1	108.75	101.57	82.08	97.43	78.26	91.84	97.24
	+1	92.62	99.80	119.29	103.94	123.11	109.53	104.13
	Range	16.13	1.77	37.21*	6.51	44.85*	17.69	6.89
GS(g)	−1	1838.7	1894.5	1462.1	1653.1	1281.6	1719.7	1640.2
	+1	1460.4	1404.6	1836.9	1646	2017.4	1579.4	1658.9
	Range	378.3	489.9	374.8	7.1	735.8*	140.3	18.7
V(cP)	−1	19.83	19.57	13.78	17.03	15.04	18.28	19.42
	+1	16.87	17.13	22.92	19.67	21.65	18.41	17.28
	Range	2.96	2.44	9.14*	2.64	6.61	0.13	2.14

* indicates significant ($P < 0.05$) differences among the 2 levels.
Independent variables and their ranges (–1–1), A: Alkaline concentration, 0.1 to 0.3 mol/L; B: Alkaline pretreatment time, 30 to 90 min; C: Acid concentration, 0.06 to 0.12 mol/L; D: Acid pretreatment time, 30 to 90 min; E: Extraction temperature, 40 to 60 °C; F: Extraction time, 120 to 240 min; G: Skin/water ratio, 1/3 to 1/4 w/w. YP = yield of protein; GS = gel strength; V = viscosity.

of protein for group 2 than for group 1; however, it resulted in a higher purity gelatin.

One of the main difficulties in using fish skin to produce gelatin is the dark color and strong odor of the skin of most fish species (Montero and Gómez-Guillén 2000). For certain fish where the ventral part of the fish skin is white, for example, some flat fish, skin could be pretreated with alkaline only (Yoshimura and others 2000; Cho and others 2004). However, in this study, the catfish skin gelatin extraction with pretreatment with alkaline only resulted in a dark-colored gelatin. Pretreatment with acid only has also been reported (Devictor and others 1995; Gómez-Guillén and others 2002; Giménez and others 2005). However, in this study the acid only extraction resulted in some fish oil being left in the gelatin. So, the alkaline pretreatment followed by acid pretreatment was indispensable for catfish skin gelatin extraction, which consisted of the process required for tilapia (Jamilah and Harvinder 2002). Moreover, catfish skin gelatins from alkaline and acid pretreatment showed more elastic behavior than viscous behavior. On the contrary, gelatins from only alkaline pretreatment showed a stronger viscous behavior than elastic behavior, which was similar to shark skin gelatin from alkaline pretreatment only as reported by Yoshimura and others (2000).

The correlation matrix of the experimental conditions and the response variables showed that a high correlation exists between responses. The correlation coefficient between YP and V was 0.86 and $P < 0.001$. Acid pretreatment concentration showed a significant correlation with YP (0.48 and $P < 0.01$) and V (0.55 and $P < 0.01$). However, a high correlation was not found between alkaline pretreatment concentration or time and the response variables.

On the basis of the experimental data, the software generated prediction equations for YP, GS, and V in several formats such as linear, quadratic, cubic, etc. It then compared the formats and automatically underlined at least one "Suggested" model (Design-Ease 2006). At least 1 significant model was obtained for YP and V, but no significant model for GS was obtained. This indicated that the relationship between the independent variables and GS was very complicated, which could not be accurately described by any of the commonly used models derived by the software system.

Table 3—Independent variables and their levels in the 4-factor, 5-level central composite rotatable design for optimizing the extraction condition of catfish gelatin

	Symbol						
Independent variable	coded	uncoded			Level		
Alkaline concentration (mol/L)	x_1	X_1	0.1	0.15	0.2	0.25	0.3
Alkaline pretreatment time (min)	x_2	X_2	30	45	60	75	90
Acid concentration (mol/L)	x_3	X_3	0.060	0.075	0.090	0.105	0.120
Extraction temperature (°C)	x_4	X_4	40	45	50	55	60

Both the alkaline and following acid pretreatment were conducted at 4 °C.

Table 4—Predictive and experimental results of the central composite design for gelatin extraction from catfish skin

Group	x_1	x_2	x_3	x_4	YP (Exp)	YP (Pred)	GS (Exp)	GS (Pred)	V (Exp)	V (Pred)
1	−1	−1	−1	−1	14.58	12.58	427.2	315.0#	2.18	1.73
2	1	−1	−1	−1	8.20	7.89	186.3	150.1	1.18	1.14
3	−1	1	−1	−1	15.04	13.31	384.8	291.7#	2.50	2.05
4	1	1	−1	−1	9.60	7.71	226.5	173.5	1.50	1.31
5	−1	−1	1	−1	16.69	16.18	287.2	287.6	3.08	2.67
6	1	−1	1	−1	17.59	15.72	240.9	203.7	3.03	2.52
7	−1	1	1	−1	17.08	16.81	277.7	260.4	2.98	2.99
8	1	1	1	−1	17.48	15.44	268.1	223.1	3.22	2.69#
9	−1	−1	−1	1	16.47	18.10	314.7	301.4	2.20	2.45
10	1	−1	−1	1	15.89	14.70	291.9	263.5	2.81	2.25#
11	−1	1	−1	1	17.36	17.77	299.5	291.0	2.58	2.54
12	1	1	−1	1	13.36	13.46	358.3	299.7	2.05	2.18
13	−1	−1	1	1	18.23	18.66	234.1	241.4	3.22	2.86
14	1	−1	1	1	18.17	19.49	249.7	284.5	2.92	3.10
15	−1	1	1	1	18.34	18.24	249.1	227.1	3.18	2.95
16	1	1	1	1	17.63	18.16	250.3	316.8	3.13	3.03
17	−2	0	0	0	20.57	20.71	235.8	313.2	2.21	2.64
18	2	0	0	0	14.21	15.94	211.5	238.0	1.75	2.14
19	0	−2	0	0	17.69	18.01	305.3	345.7	2.13	2.67#
20	0	2	0	0	15.85	17.40	291.1	354.7	2.63	2.92
21	0	0	−2	0	8.27	9.82	77.6	227.2#	1.17	1.44
22	0	0	2	0	17.80	18.12	262.6	216.9	2.67	3.23#
23	0	0	0	−2	2.57	6.94#	0.0	144.8#	0.70	1.57#
24	0	0	0	2	17.67	15.17#	265.8	224.9	2.68	2.63
25	0	0	0	0	12.60	12.45	230.1	195.7	1.58	1.52
26	0	0	0	0	12.31	12.45	224.1	195.7	1.55	1.52
27	0	0	0	0	14.83	12.45	279.9	195.7	1.92	1.52
28	0	0	0	0	11.13	12.45	143.8	195.7	1.37	1.52
29	0	0	0	0	12.10	12.45	164.9	195.7	1.38	1.52
30	0	0	0	0	11.96	12.45	160.4	195.7	1.48	1.52
31	0	0	0	0	12.19	12.45	166.9	195.7	1.39	1.52

The superscript of # denotes that there is a relatively large deviation between the experimental result (Exp) and the predicted (Pred) value.
x = variables in coded. x_1 = alkaline pretreatment concentration (mol/L); x_2 = alkaline pretreatment time (min); x_3 = acid pretreatment concentration (mol/L); x_4 = extraction temperature (°C).

Taking YP as an example, the suggested quadratic format model is $YP = 12.45 - 1.19A - 0.15B + 2.07C + 2.06D - 0.23AB + 1.06AC + 0.32AD - 0.023BC - 0.26BD - 0.76CD + 1.47A^2 + 1.31B^2 + 0.38C^2 - 0.35D^2$. Then, individual terms with a probability value that was > 0.10 were removed as insignificant terms (Design-Ease 2006). The simplified model is then

$$YP = 12.45 - 1.19A + 2.07C + 2.06D + 1.06AC + 1.47A^2 + 1.31B^2 \quad (1)$$

With the same method, the linear and quadratic formats were suggested for V, which after removing insignificant ($P > 0.1$) variables gave

$$V(\text{Linear}) = 2.21 + 0.45C + 0.27D \quad (2)$$

$$V(\text{Quadratic}) = 1.52 + 0.45C + 0.27D + 0.22A^2 + 0.32B^2 + 0.20C^2 \quad (3)$$

During the optimization, the range of the independent and dependent variables should be given. Cho and others (2005) found that extraction temperature was the most important factor for gelatin extraction from yellowfin tuna. However, gel strength decreased as extraction temperature increased from 60 to 75 °C. The high extraction temperature caused protein degradation and denaturation, which, in turn, produced small protein fragments and a lower gelling ability. Many low-molecular proteins were extracted at higher extraction temperatures, and a lower molecular weight gelatin is known to have a lower gel strength value than a high molecular weight gelatin (Badii and Howell 2006). Grossman and Bergman (1992) also reported that extracting tilapia skin gelatin with water at temperatures at or below 55 °C resulted in a higher-quality product (for example, the absence of a fishy smell). This lower temperature extraction is also beneficial for reducing energy cost. High extraction temperatures invariably resulted in a poorer quality product with a stronger fishy smell. Also alkaline pretreatment of collagen might lead to a lower denaturation temperature (Yoshimura and others 2000).

Therefore, the extraction temperature for optimization should be set in a narrow range (D, 45 to 55 °C). For other independent variables, a relative wider level can be used: Alkaline concentration (A, 0.1 to 0.3 M), alkaline treatment time (B, 30 to 90 min), and acetic acid concentration (C, 0.06 to 0.12 M). Considering that gel strength and viscosity are probably the most important functional properties, fish gelatins are generally different from mammalian gelatin, and this can affect final product quality (Choi and Regenstein 2000). Higher gel strength and viscosity could extend the application of fish gelatin (Zhou and others 2006). For the dependent responses, removing the noncollageous protein would decreased the yield of protein (Yoshimura and others 2000) but not necessarily to the deteriment of the final product; thus YP should only be optimized after the viscosity and gelatin strength are maximized. In the optimization, YP was set in range of 15% to 20% with a weighing factor of 1 and an importance of " + + +". The number of "+" signs indicates the degree of importance of the variable, the more pluses, the more important the variable; GS was maximized in the range of 290 to 450 g with a weighing factor of 1 and importance of "+++++" and V was maximized in the range of 3.2 to 3.5 with weighing factor of 1 and importance of +++++ (Design-Ease 2006). After these limitations were set, the optimization tests were evaluated by the Design-Ease software and 15 groups of optimization conditions for further evaluation were obtained.

Table 5—Values of the independent variables for the 2nd-step optimization

No.	Alkaline concentration (mol/L)	Alkaline pretreatment time (min)	Acid concentration (mol/L)	Extraction temperature (°C)
1	0.21	83	0.116	55
2	0.21	81	0.117	55
3	0.20	84	0.115	55
4	0.19	84	0.114	55
5	0.17	85	0.110	55
6	0.20	81	0.118	53
7	0.23	72	0.120	55

Table 6—Experimental and predicted results in the 2nd-step optimization

		YP (Pred)					V (Pred)		
No.	YP (Exp)	Sw	Eq (1)	GS (Exp)	GS (Pred) Sw	V (Exp)	Sw	Eq (2)	Eq (3)
1	19.14 ± 0.43^{bc}	20	21.20	229.6 ± 4.6^{b}	317.5	3.05 ± 0.00^{d}	3.32	3.25	3.90
2	19.41 ± 0.06^{b}	20	21.12	244.7 ± 7.2^{a}	317.7	2.86 ± 0.02^{f}	3.32	3.28	3.88
3	19.20 ± 0.56^{bc}	20	21.25	251.7 ± 4.7^{a}	317.4	3.23 ± 0.01^{a}	3.32	3.23	3.88
4	19.83 ± 0.13^{b}	20	21.28	247.3 ± 2.3^{a}	314.8	3.13 ± 0.00^{c}	3.31	3.20	3.90
5	21.95 ± 1.01^{a}	20	21.39	211.5 ± 1.7^{c}	300.3	2.57 ± 0.00^{g}	3.26	3.09	3.75
6	19.28 ± 0.13^{b}	20	20.10	247.0 ± 9.6^{a}	299.5	3.15 ± 0.00^{b}	3.26	3.19	3.81
7	18.42 ± 0.06^{c}	20	20.60	248.5 ± 3.7^{a}	293.9	2.97 ± 0.01^{e}	3.33	3.36	3.75

The conditions of experiment nr 1 to 7 were shown in Table 5.
Sw = predicted by the Design-Ease (2006) software; Exp = experimental value; Pred = predicted value. Eq (1) to Eq (3): simulated data by Eq (1) to Eq (3). The results are shown as mean ± standard deviation of 3 replicated samples. Different letters (a, b, c, d, e, f, g) in the same column indicate significant ($P < 0.05$) differences among different extraction temperatures. YP = yield of protein, %; GS = gel strength, g; V = viscosity, cP.

Table 7—Hardness, cohesiveness, springiness, and chewiness of the 7 groups in the 2nd-step optimization

No.	Hardness	Cohesiveness	Springiness	Chewiness
1	673.2 ± 38.0^{d}	0.91 ± 0.01^{a}	0.96 ± 0.01^{a}	584.2 ± 31.3^{c}
2	765.8 ± 48.9^{abc}	0.91 ± 0.00^{a}	0.95 ± 0.01^{ab}	664.8 ± 45.4^{b}
3	835.8 ± 29.9^{a}	0.91 ± 0.00^{a}	0.95 ± 0.00^{ab}	728.7 ± 25.0^{a}
4	762.1 ± 82.9^{bc}	0.92 ± 0.01^{a}	0.96 ± 0.02^{a}	668.7 ± 73.8^{ab}
5	644.9 ± 41.6^{d}	0.92 ± 0.01^{a}	0.96 ± 0.01^{a}	572.2 ± 32.4^{c}
6	709.1 ± 61.1^{cd}	0.90 ± 0.03^{a}	0.95 ± 0.01^{ab}	602.5 ± 37.3^{c}
7	834.4 ± 66.5^{ab}	0.91 ± 0.01^{a}	0.94 ± 0.01^{b}	714.9 ± 59.3^{ab}

The conditions of experiment nr 1 to 7 were shown in Table 5. The results shown are mean ± standard deviation for 5 replicated samples. Different letters (a, b, c, d) in the same column indicate significant ($P < 0.05$) differences among different extraction temperatures.

Verification of predicted values and the second step of the optimization of the gelatin extraction

In the 15 groups given by the Design-Ease software at the end of the 1st-step optimization, several groups with low-response values or very similar values of the independent variables were not considered for further verification. Thus, only 7 out of the 15 groups of optimization conditions were selected (Table 5). Seven groups of extraction experiments were conducted according to these 7 groups of optimization conditions for both further verification and to complete the 2nd-step optimization.

Table 6 shows the experimental and corresponding predicted values of YP, GS and V. The 2 sets of predicted values were calculated by the Design-Ease software and the equations obtained during the 1st-step optimization, respectively. For YP, the 3 groups matched well. For V, the values predicted by Equation 2 matched the experimental values better than those suggested by the software. The linear model was better than the quadratic model for V. Groups 3 and 6 showed higher values in terms of the 3 responses than the other groups.

TPA results were used as another response for evaluating groups of conditions in the 2nd-step optimization. TPA tests were intended to simulate the action exerted upon the gel by the tongue and teeth, and, therefore, differ from the simpler gel strength test. Figure 1 shows a typical experimental curve for the TPA test of gelatin from catfish skin. A gentle compression on the gel by a plunger of relatively large surface produced recordings resembling isosceles triangles, similar to those obtained for the gel strength test. The second compression shape in Figure 1 was almost the same as that of the first one, suggesting that the gel surface had not been broken during the first compression (Surówka 1997). Table 7 shows the TPA results of gelatins obtained at the conditions indicated for these 7 groups. Hardness is related to the strength of the gel structure under compression. Cohesiveness is a measurement of the degree of difficulty in breaking down the gel's internal structure. Springiness (also called "elasticity") is a perception of gel "rubberiness" in the mouth, and is a measurement of how much the gel structure is broken down by the initial compression. High springiness results from the gel structure being broken into a few large pieces during the first TPA compression while low springiness results from the gel breaking into many small pieces (Lau and others 2000). Chewiness is related to the work needed to masticate a solid food to a state ready for swallowing, and the value can be obtained from the product of hardness, cohesiveness, and springiness. The higher these values, the better the gelatin qualities. Thus, group 3 had the best results among all groups. Table 8 shows the correlation matrix of YP, GS, V, hardness, cohesiveness, springiness, and chewiness. The results showed that GS had a high correlation with V, hardness, and chewiness (0.800, 0.833 and 0.781, respectively, $P < 0.05$). It is also interesting that chewiness shows a very significant correlation with hardness ($r = 0.991$, $P < 0.001$).

From Table 6 and 7, it can be seen that group 3 provided the best conditions for gelatin extraction. The corresponding extraction condition was 0.20 M NaOH for 84 min, 0.115 M acetic acid for 60 min, and extraction in 55 °C water bath for 180 min. The pretreatment was done at 4 °C. With these conditions the yield of protein was 19.2%, the gel strength was 252 g, and the viscosity was 3.23 cP.

Lefebvre and others (2002) reported a catfish skin gelatin with a protein yield of 11.2%, gel strength of 217 bloom (10 °C gel strength), and a viscosity of 1.7 cP. Table 9 shows comparisons with gelatins from other fishes and some mammals. The data suggested that the gelatin's physical properties in our study were promising, even though the experimental conditions were a little different in determining these properties. The yield was also relatively high when compared with other kinds of gelatin extractions, which were 15% for tilapia (Grossman and Bergman 1992); 8.3% for sole, 7.4% for megrim, 7.2% for cod, and 5% for hake (Gómez-Guillén and others 2002). It is difficult to compare the results with the pollock gelatin reported by Zhou and Regenstein (2004) because the temperatures for determination of the physical properties were different, and the viscosity was determined at 25 °C, while it was 60 °C in this work. High temperature results in a lower viscosity.

Table 8—Correlation matrix of gelatin physical properties during 2nd-step optimization

	YP	GS	V	Hardness	Cohesiveness	Springiness	Chewiness
YP	1.000						
GS	−0.809*	1.000					
V	−0.722	0.800*	1.000				
Hardness	−0.665	0.833*	0.515	1.000			
Cohesiveness	0.626	−0.472	−0.480	−0.157	1.000		
Springiness	0.650	−0.613	−0.206	−0.730	0.548	1.000	
Chewiness	−0.581	0.781*	0.469	0.991***	−0.037	−0.653	1.000

The results were from Table 6 and 7 and the experiment conditions were shown in Table 5.
*, **, *** indicate significance at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.
YP = yield of protein; GS = gel strength; V = viscosity.

Table 9—Comparison the gelatin from different fishes and mammals

Gelatin	References	Protein yield (%)	Gel strength (g)
Catfish	This report	19.2	252
Catfish	Lefebvre and others 2002	11.2	217
Tilapia	Grossman and Bergman 1992	15	280
Sole	Gómez-Guillén and others 2002	8.3	350
Megrim	Gómez-Guillén and others 2002	7.4	340
Cod	Gómez-Guillén and others 2002	7.2	75
Hake	Gómez-Guillén and others 2002	5	103
Pollock	Zhou and Regenstein 2004	18	460
Tilapia	Jamilah and Harvinder 2002	5.39—7.81	128–180
Pork skin	Choi and Regenstein 2000	—	100–300
Pork bone	Choi and Regenstein 2000	—	230

The extraction and determination conditions were different, thus it cannot be compared directly from the values given.

Most commercial gelatins have a GS that varies from less than 100 to more than 300 bloom (Zhou and others 2006). Bloom value is about 150 for a typical commercial standard catfish gelatin (Croda Colloids Ltd. Dilton, Cheshire, U.K.) and 280 for tilapia fish skin gelatin (Badii and Howell 2006). Most commercial general gelatins have a viscosity between 1.5 and 7.5 cP (Zhou and others 2006). In this study, the determination of gel strength was done at a temperature of 4 °C and with a gel concentration of 3.3%. The magnitude of gel strength and viscosity determined would increase greatly when the physical properties are determined at a gel concentration of 6.67%, which should be used for future study.

Conclusion

The 2-step RSM was used to optimize the extraction of gelatin from channel catfish (*Ictalurus punctatus*) skin. It was found that alkaline concentration, alkaline treatment time, acid concentration, and extraction temperature showed significant effects on yield, gelatin strength, and viscosity. The optimization solution showed that in 0.20 M NaOH for 84 min treatment, 0.115 M acetic acid for 60 min, pretreatment at 4 °C, and extraction using a 55 °C water bath for 180 min, the corresponding responses were YP = 19.2%, GS = 252 g, and V = 3.23 cP. With these production conditions, the gelatin extracted also showed relatively good hardness, cohesiveness, springiness, and chewiness. Quadratic and linear models can be used to predict the yield of protein and viscosity properties of the gelatin, respectively. However, no significant model can be obtained from the software system to predict gel strength.

Acknowledgments

This research project was supported by the Alabama Agricultural Land Grant Alliance (AALGA) and the Alabama Agricultural Experiment Station (AAES) for making this project possible. The authors are thankful for the advice and editorial assistance provided by Dr. Joe Regenstein with his expertise and knowledge on the extraction of fish skin gelatin.

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