JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Enzymatic Activity and Flavor Compound Production in Fermented Silver Carp Fish Paste Inoculated with Douchi Starter Culture

Ladislaus M. Kasankala,[†] Youling L. Xiong,*[,‡] and Jie Chen[†]

[†]State Key Laboratory of Food Science and Technology and School of Food Science and Technology, Jiangnan University, Wuxi 214122, China

[‡]Department of Animal and Food Sciences, University of Kentucky, Lexington, Kentucky 40546, United States

ABSTRACT: Silver carp fish pastes inoculated with or without a douchi starter culture containing live *Aspergillus oryzae* were fermented for 30 days to produce two different fermented products, designated CulF and ConF, respectively. Protein degradation and flavor compound production during the course of fermentation were monitored. Proteolytic activity, generally higher in CulF than in ConF ($P < 0.05$) and dominated by acidic and serine proteases, declined to an overall minimum after 30 days. Myosin in the CulF and ConF extractives was completely degraded after 1 and 5 days, respectively. The content of free amino acids and low molecular weight (<1.3 kDa) peptides rose rapidly in CulF and progressively in ConF ($P < 0.05$). Ethanol, silanediol, pyrazine, phenol, and formic acid were prevalent volatile compounds in CulF, whereas butanol, butanoic acid, and acetic acid were abundant in ConF. Therefore, douchi-inoculated fermentation is an attractive process to produce savory fish pastes.

KEYWORDS: *douchi, fermented fish, proteases, silver carp, flavor*

INTRODUCTION

Silver carp (*Hypophthalmichthys molitrix*), a freshwater white-flesh fish species most cultivated in China and increasingly aquacultured worldwide, has attracted much attention in recent years due to its fast growth rate, low feed demand, and resistance to diseases.[1] Furthermore, feeding on plankton, silver carp contains very low levels of metal contaminants in the muscle tissue when compared with many other fish.[2] Silver carp was imported to the United States in the 1970s to control algae growth in aquaculture and municipal waters; it is now populating rivers and lakes along the Mississippi River and considered an invasive species.[3] To control overpopulation, thereby reducing its threat to U.S. native plankivorous species such as paddlefish, gizzard shad, and bigmouth buffalo, research incentives and initiatives have been proposed toward market development of silver carp and other Asian carp species.

In 2010, The Asian Carp Marketing Summit (ACMS), developed with efforts from the U.S. Fish and Wildlife Service, U.S. Geological Survey, Louisiana Sea Grant, Illinois Commercial Fishing Association, and Shedd Aquarium, identified the following three potential markets for Asian carp captured in the United States: (1) high-volume/low-price export for human consumption, (2) high-quality/higher price domestic for human consumption, and (3) byproduct.[4] Among them, processing to produce value-added products for human consumption is considered to be an attractive alternative. Currently, the potential products of silver carp in the United States focus on fresh fillets, whole fish, frozen patties, and canned fish.

Silver carp muscle hosts multiple enzymes engaged in protein degradation and synthesis.[5,6] The synthesis of proteins ceases when post-mortem muscle tissue loses its biological integrity while catabolism of proteins continues.[7,8] However, the rate of protein degradation depends on many factors, including species, physiological conditions of fish ante-mortem, and the season in which the fish is harvested.[9,10] The importance of post-mortem protein degradation is manifested by its profound influence on the functional properties and palatability of fish muscle. Whereas enzymatic hydrolysis is desirable for fermented fish products due to its role in flavor compound production,[11] the hydrolysis may be undesirable for other products such as filets and surimi because it could cause mushiness of muscle tissue or hinder texture formation of surimi.[12]

Although not yet widely consumed in the United States, fermented fish is a delicacy product enjoyed by many cultures in other parts of the world, and the enzymology involved has been extensively studied. During fermentation, both microbial proteases and endogenous proteases can degrade muscle proteins to their constitutive amino acids and peptides as well as ammonia, which are precursors of the unique flavor of fermented seafood.[13,14] Muscle endogenous proteases, including cysteine and lysosomal enzymes, catalyze the degradation of myofibrillar proteins at the initial phase of fish fermentation.[15] On the other hand, proteases released from halophilic bacteria that tolerate and thrive well at high salt levels are thought to play an active role at the ripening stage.[16] Furthermore, proteases of fungal origins stable and active over a broad range of pH could reinforce muscle protein degradation.[17] Alkaline proteases are another group of enzymes found to be involved in fish fermentation.[16] Despite numerous attempts made to identify and characterize proteases responsible for the production of flavor-eliciting compounds in fermented fish,

Received: September 24, 2011
Revised: December 12, 2011
Accepted: December 12, 2011
Published: December 12, 2011

Figure 1. Silver carp, fillet, douchi, and fermented fish paste with douchi starter culture.

the specific agent(s) is often difficult to establish due to the complexity of the fermented muscle system as well as simultaneous involvements of multiple enzymes.

A typical fish fermentation process entails the addition of high concentrations of salt to extract myofibrillar proteins and inhibit spoilage microorganisms. Free amino acids and peptides that accumulate in the extractive (liquor) serve as the basis for the production of various volatile and nonvolatile saporous components that collectively constitute fermented fish flavor profiles.[18] Moreover, the distinct aroma attributed to volatile compounds, such as alcohols, aldehydes, nitrogen compounds, and sulfur compounds, are derived from proteins and lipids through the actions of autolytic and microbial activity.[11]

Cao et al.[5] reported that silver carp myosin was susceptible to a myofibril-bound serine protease(s) and cathepsins at slightly acidic or neutral pH. In a previous investigation,[19] we introduced a novel microbial fermentation scheme to silver carp paste production. Specifically, a fermented silver carp mince with a high degree of organoleptic acceptability was produced when the fermentation was aided by douchi, a traditional Chinese fermented soybean. Douchi is an *Aspergillus oryzae*-cultured product made from steam-cooked whole or crushed soybean in the presence of high concentrations of salt. It is a popular condiment used to flavor or complement Chinese dishes such as rice, vegetables, meats, and seafoods.[19] As a novel starter culture to fish formation, douchi has a number of conceivable advantages over traditional nonculture fish fermentation. It can serve as a source of proteases, amylase, and lipases which act on different food components.[20] Thus, a product of improved nutritional value, taste, and aroma can be obtained. The objective of the present study was to identify the type of enzymes responsible for the quality improvements of this unique fermented fish product. Several key sensory quality parameters associated with enzymatic actions in the fermentation process were also examined.

MATERIALS AND METHODS

Materials. Live silver carp fish (Figure 1), weighing 0.9−1.2 kg, were purchased from a local market (Wuxi, China) in the months of May and June. Fish were stunned by a blow on the head, bled, and skinned. The dressed whole fish were placed in plastic bags, transported on ice to the laboratory, and immediately used. Black soybeans used for douchi preparation were also obtained from a local market. The douchi starter culture with live *A. oryzae* was provided by Wuxi Soybean Sauce Co. (Wuxi, China). Ethyleneglycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol (βME), L-tyrosine, bovine serum albumin, phenylmethanesulfonyl fluoride (PMSF), 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-*p*-tosyl-L-lysine chloromethyl (ketone) (TLCK), saturated alkane (C_7−C_{30}) standard mixture, and molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Douchi and Fermented Fish. Black soybeans were washed with tap water, soaked overnight, ground, and steam-cooked at 121 °C for 30 min. Cooked soybeans were cooled to 35 °C, mixed with 18% salt (NaCl), and then inoculated with 1×10^7 spores/mL of *A. oryzae*, followed by incubation at 30 °C for 5 days.[19] This starter culture is referred to as "douchi" hereafter (Figure 1). Fish sample preparation and fermentation were done as follows. Dressed fish were rinsed in tap water, filleted, and chopped into a fine mince. Triplicate muscle samples (160 g each) were packed into 500 mL Erlenmeyer flasks, and 40 g of douchi, together with 6.4 g of boiled rice, 30 g of salt, 6.4 g of sugar, and 160 g of boiled and chilled water, was added aseptically. The flasks were swirled to mix the content to homogeneity and then kept at 4 °C for 6 h to allow ingredient equilibration. Subsequently, the flasks were incubated at 35 °C for up to 30 days to allow fish fermentation.[19] The fish paste inoculated with douchi was designated CulF, whereas the fish paste incubated without douchi (ConF) was used as the control. For ConF, an appropriate amount of salt (7.2 g) was added in place of 40 g of douchi. Therefore, both CulF and ConF fish pastes contained 9.2% salt. The final products of fermented fish pastes (30 days) had a smooth texture and brownish color (Figure 1). The pH of the extractive liquid during fish fermentation was measured directly using a model 320-s pH-meter (Mettler Toledo, Columbus, OH).

Proteolytic Activity Assay. Proteolytic activity was determined according to the method of Klomklao et al.[21] with minor modifications. The liquid portion obtained by natural settlement was centrifuged at 10000*g* for 20 min and then filtered through Whatman no. 4 filter paper before use. The substrate for the enzyme assay (1% casein) was prepared in 0.2 M sodium acetate buffer for pH 4.5 and in sodium phosphate buffer for pH 6.0−7.5. A mixture consisting of 200 μL of casein solution, 200 μL of water, and 625 μL of buffer solution was preincubated at 35 °C for 5 min before the addition of 200 μL of enzyme extract and incubated for 20 min. The reaction was terminated by adding 400 μL of 10% trichloroacetic acid (TCA). After setting for 20 min at 35 °C, the mixture was centrifuged at 10000*g* for 10 min at 4 °C and thereafter stored at 4 °C for 30 min. A blank was run in the same manner except that 10% TCA was added before incubation at 35 °C. TCA-soluble peptides in the filtrate were determined according to

Lowry's method.[22] One unit of protease (U) was defined as the amount of enzyme that yields the color equivalent to 1 μmol of tyrosine in 1 mL of the reaction mixture per minute at 30 °C.

Effects of Inhibitors on Protease Activity. The effect of inhibitors on protease activity was determined according to the method of Klomklao et al.[21] Specifically, the extractives of intermediate fermented fish pastes (day 20) were incubated at 35 °C for 30 min with an equal volume of protease inhibitor solutions at the following assay concentrations: 0.1 mM E-64, 1 mM PMSF, 0.1% soybean trypsin inhibitor, 5 mM TLCK, 1 mM pepstatin A, or 2 mM EDTA. The remaining enzyme activity was measured at 35 °C using 1% casein as substrate as described above at pH 7.0. The relative percent inhibition was calculated.

Molecular Weight (MW) Distribution. Proteins and peptides in the extractive liquids were separated by MW through gel permeation chromatography using a Waters 1525 high-performance liquid chromatography (HPLC) system (Waters Corp., Milford, MA). A liquid sample (20 μL) was eluted through a Shodex PROTEIN KW-804 column (8 mm i.d. × 300 mm) (Showa Denko KK, Japan) with 0.05 M sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl at a flow rate of 1 mL/min with the UV water detector (wavelength at 220 nm). A MW calibration curve was prepared by plotting the MW of individual markers (vitamin B12, 1350 Da; aprotinin, 6500 Da; ribonuclease A, 13700 Da; adenylate kinase, 32000 Da; BSA, 67000 Da; aldolase, 158000 Da; thyroglobulin, 669000 Da) against their retention times.

SDS-PAGE of Myofibrillar Proteins. Mixed myofibrillar proteins (MP) were isolated from fish pastes according to the method of Park and Xiong[23] and immediately subjected to SDS-PAGE according to the standard procedure of Laemmli.[24] The stacking and resolving gels contained 5 and 12% acrylamide, respectively. Protein samples were run under nonreducing conditions (without βME).

Particle Size Measurements. The particle size of the fish extractives was measured by a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). An aliquot of 1 mL of extractive liquid was centrifuged at 10000*g* for 15 min. The intensity of light scattering of the supernatant was determined at the wavelength of 633 nm. The average particle size was calculated using Dispersion Technology software version 4.2 (Malvern Instruments).

Free Amino Acids and TCA-Soluble Peptides. The liquid portion formed by natural settlement, which contained extractives of fermented fish, was analyzed. Free amino acids were measured according to the method of Itou et al.[25] as detailed by Kasankala et al.[19] Liquid samples containing free amino acids were treated two times with 5% (final) perchloric acid (PCA). The combined supernatants after centrifugation (10000*g* for 20 min) were subjected to an Agilent 1100 amino acid analyzer (Agilent Technologies, Palo Alto, CA) using a Zorbax 80A C18 column (4.6 × 180 mm). The results were processed with ChemStation for LC 3D software (Agilent Technologies). To measure the concentration of TCA-soluble peptides, the procedure of Benjakul et al.[26] was followed. To 1 mL of liquid extracts was added 9 mL of 5% TCA with vortexing for 30 s to precipitate unhydrolyzed oligopeptides. The TCA-soluble content in the supernatant was estimated according to the method of Lowry et al.[21] and expressed as milligrams of bovine serum albumin (BSA) released per gram of protein.

Gas Chromatography–Mass Spectrometry (GC-MS). GC-MS was used for the identification of volatile compounds in the headspace of final fish pastes. Flavor compounds were extracted by means of solid-phase microextraction (SPME), where a fiber with a 70 μm coating of polydimethylsiloxane (PDMS)/Carboxen (Supelco, Bellefonte, PA) was used for the extraction at 50 °C for 30 min. The adsorbent volatiles on the fiber were thermally desorbed at 250 °C into GC injection by holding the splitless mode for 5 min. Volatiles were separated using a capillary column VF17 (30 m × 0.25 mm i.d., 0.25 μm film thickness; Varian Inc., Palo Alto, CA). Helium was used as carrier gas at a flow rate of 1 mL/min. The initial holding time was 2 min; thereafter, the column temperature was programmed from 40 to 120 °C at the rate of 5 °C/min, raised to 250 °C at the rate of 10 °C, and then held at this temperature for 5 min. A mass spectrometer 1200 L (Varian Inc., Palo Alto, CA) was used to acquire mass spectra using the electron impact ionization method at 70 eV. The interface temperature was maintained at 200 °C. A mixture of straight-chain alkanes (C_7–C_{30}) was injected under the same conditions to establish the sizes of volatile compounds in samples. Retention indices were calculated by using a Kovats index. These aroma compounds were subsequently identified by comparing their mass spectra with the published authentic spectra database in the GC-MS libraries (NIST05a.L, Agilent Technologies, Inc., Santa Clara, CA).

Statistical Analysis. Three independent fish fermentation processes were conducted at different times to obtain three experimental replications (n = 3) for all fermentation treatments. Data were analyzed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). Comparisons between fermentation treatments were made by analysis of variance (ANOVA) with post hoc comparisons of the means using Duncan's multiple-range test. Differences between means were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

pH. Because a true fermentation "time zero" was difficult to obtain due to the lengthy sample preparation, data for "day 0" were not collected. During fermentation, the pH of the fish samples decreased by almost 2 units in the first 10 days and rose slightly thereafter (Figure 2). Specifically, the pH of the

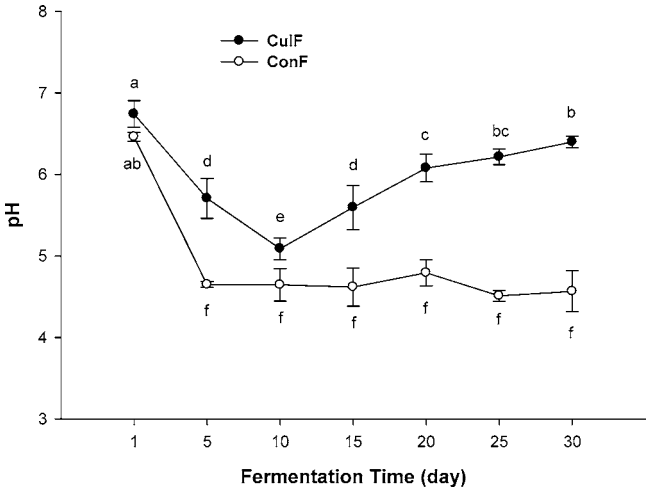

Figure 2. Changes in the pH of the extractive liquid during silver carp fish fermentation with (CulF) or without (ConF) douchi culture. Means (± standard deviations, n = 3) between samples without a common letter differ significantly ($P < 0.05$).

liquid in CulF fish declined from 7.0 to 5.1 ($P < 0.05$) after 10 days and then increased to 6.3 at the end of fermentation (30 days). For ConF, the ultimate pH of 4.6 was reached within 5 days and no further change was observed. The low pH is practically important for the release of muscle enzymes, for example, cathepsins.[27] Kakio et al.[28] suggested that fungi may consume amino acids and peptides as a carbon source for their growth and consequently hasten the release of ammonium compounds, resulting in the rise of pH as seen in CulF contrary to ConF. Moreover, *A. oryzae* possesses multiple genes expressing secretory proteases that function in acidic pH, including aspartic protease, pepstatin-insensitive protease, serine-type proteases, and carboxypeptidase.[20] Some of the enzymes can hydrolyze muscle proteins, resulting in the accumulation of ammonia compounds and peptides.[29]

Proteolytic Activity. Proteolytic activity was detected over the entire wide range of pH (4.5–7.5) tested, suggesting the

dx.doi.org/10.1021/jf203887x | J. Agric.Food Chem. 2012, 60, 226–233

presence of acidic, neutral, and alkaline proteases in all fish pastes (Figure 3). Because the amount of exudate (extractive)

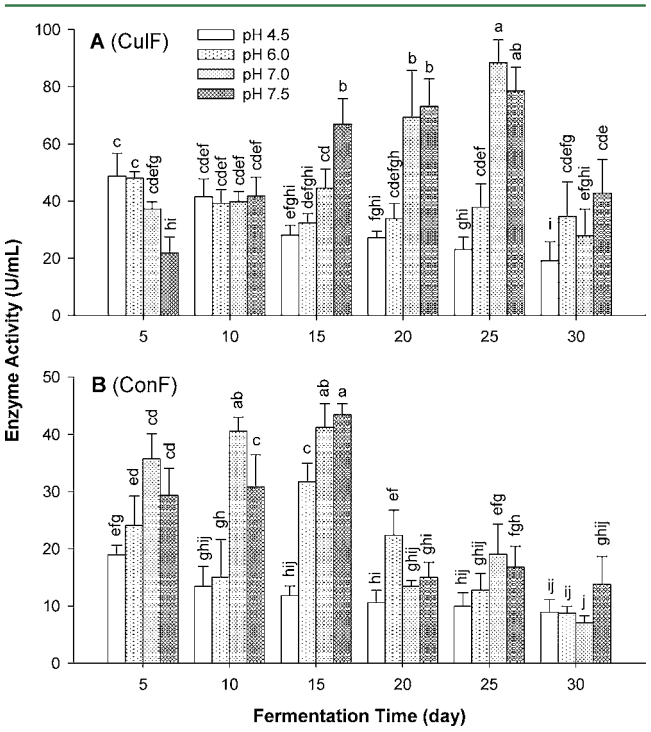

Figure 3. Changes in total proteolytic activity at various pH levels of the extractive liquid during silver carp fish fermentation: (A) fish paste inoculated with douchi culture (CulF); (B) fish paste without douchi as control (ConF). Means ± standard deviations (n = 3) for the same fish paste without a common letter differ significantly (P < 0.05). Note the y-axis scale difference (2-fold) between A and B.

on day 1 was too small to run protease activity tests at all of the pH values, there was no data collection for day 1 samples. A decline in proteolytic activity on the acidic side with the progress of fermentation was observed in both types of fish pastes (CulF and ConF). This activity was attributed to acidic proteases, which were also seen in other fish species, such as herring.[30] On the other hand, protease activity in the slightly acidic, neutral, and slightly alkaline pH range (6.0−7.5) in CulF samples rose during fermentation for up to 25 days and declined sharply afterward (Figure 3A). For ConF, the enzymes active around the neutral pH had an initial high activity value and became more active with fermentation until 15 days (Figure 3B). The activity dropped thereafter.

The maximum activity recorded between 20 and 25 days for CulF was related to *A. oryzae* proteases. García-Gómez et al.[29] found that proteases extracted from comminuted fish inoculated with *A. oryzae* were active over a broad range of pH (6.0−9.0) and temperature (30−60 °C). Despite its initial low activity, CulF exhibited up to 4-fold (P < 0.05) higher activity than ConF as fermentation time reached 25 days. *A. oryzae* in douchi starter culture apparently boosted the release of proteases. These were stable enzymes active at high ionic strengths,[31] including aspartic and serine proteases and carboxypeptidase.[28] The enhanced activity of *A. oryzae* enzymes in CulF during fermentation supported the previous finding that CulF fish pastes fermented for 30 days contained 3 times as much free amino acids as ConF fish without douchi.[19] The initial higher enzyme activity detected at pH 4.5 and 6.0 than at pH 7.5 for CulF can be attributed to cathepsins and calpain, which were found to be nearly optimal in the pH 5.0−6.2 range.[6,15] However, the decreased protease activity (P < 0.05) in ConF after 15 days was probably caused by the persistent low pH (<5.0, Figure 2), which mostly favors acidic proteases but not others.[15]

Effect of Inhibitors on Protease Activity. To further understand the nature of the proteases involved in the chemical changes in fermented fish pastes, the extractive liquids from day 20 of the fermentation were subjected to enzyme inhibitor assays at neutral pH 7. This particular sampling time was chosen because the enzyme activity in both CulF and ConF seemed to have reached a stable period (Figure 3). Proteases in CulF were strongly inhibited by pepstatin and PMSF and moderately inhibited by EDTA, suggesting the presence of acid proteases, serine-like proteases, and metallo proteases, respectively (Table 1). Moderate inhibitions up to 48% were

Table 1. Effects of Various Enzyme Inhibitors on Protease Activity in the Extractive Liquid of Fish Pastes Sampled on Day 20 of Fermentation[a]

			relative activity (%)	
enzyme inhibitor	targeted protease	final concentration	CulF	ConF
none			100	100
PMSF	serine proteases	1 mM	6.72	4.59
E-64	cysteine proteases	0.1 mM	47.84	45.97
EDTA	metalloproteases	2 mM	30.86	11.49
TLCK	trypsin-like proteases	5 mM	41.92	52.87
soybean trypsin inhibitor	trypsin-like proteases	1.0 g/L	37.03	41.38
pepstatin A	acid proteases	1 mM	1.00	16.09

[a]CulF, fermented fish with douchi culture; ConF, fermented fish without douchi culture.

rendered by E-64 (cysteine protease inhibitor), TLCK (trypsin-like protease inhibitor), and soybean trypsin inhibitors (Table 1). Sinsuwan et al.[32] reported that serine proteases and metalloproteases are common in moderate halophiles, and the inhibition of protease activity by PMSF is recognized as a typical feature of serine proteases.[16] Despite the presence of similar types of enzymes in CulF and ConF fish pastes, the degree of inhibition of trypsin, trypsin-like, and acid proteases was slightly less for ConF, whereas the inhibition of several other types of proteases was more pronounced. Therefore, douchi culture was a significant source of different proteolytic enzymes.

The findings coincided well with enzyme activities tested at different pH levels (Figure 3) and supported previous reports for *A. oryzae* proteases that exhibited activity under slightly acidic to alkaline pH conditions, namely, pH 2.5−6.5[33] and pH 6.0−10.0.[29] Furthermore, the presence of myofibril-bound serine proteases in silver carp muscles[3] as well as halotolerant serine protease in fish sauce[15] has been documented.

Molecular Weight (MW) Distribution and Particle Size Reduction. Because of the high concentration of salt present in processed whole fish pastes (9.2%, w/w), the extractive liquid of freshly prepared fish pastes would contain both sarcoplasmic and myofibrillar proteins. Within the first day of fermentation, most of the native muscle proteins (>29 kDa) were degraded into intermediate MW fragments (1.3−29 kDa)

dx.doi.org/10.1021/jf203887x | J. Agric.Food Chem. 2012, 60, 226−233

that represented 73 and 61% of all protein fractions in CulF and ConF, respectively (Figure 4). Within the same

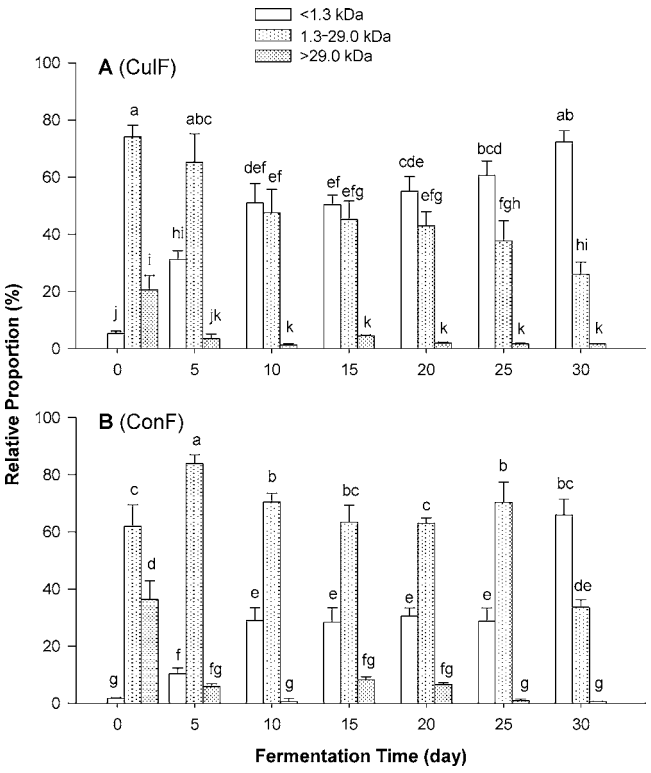

Figure 4. Changes in the molecular weight distribution of the extractive liquid during silver carp fish fermentation: (A) fish paste inoculated with douchi culture (CulF); (B) fish paste without douchi as control (ConF). Means (± standard deviations, $n = 3$) between samples without a common letter differ significantly ($P < 0.05$).

fermentation period, the amount of oligopeptides (<1.3 kDa) was minimal. The particularly high proportion of intermediate MW polypeptides in CulF when compared with ConF was due to the high activity of acidic (pH 4.5, 6.0) and neutral (pH 7.0) proteases introduced by douchi (Figure 3).

A prominent proteolytic event during the fermentation was the rapid hydrolysis of the high MW fraction (>29 kDa) within the first 5 days and the concomitant increase of the low MW peptide fraction (<1.3 kDa), notably for CulF. Enzymatic hydrolysis of myosin must be a major contributor to the rapid and initial rise in the intermediate MW and, subsequently, low MW fractions (Figure 5, inset). Myosin in CulF was completely degraded after 1 day, whereas myosin in ConF, largely degraded after 1 day, completely disappeared after 5 days (result not shown). The change of the intermediate MW fraction was dynamic (Figure 4), reflecting simultaneous formation and degradation throughout the fermentation process.

The production of low MW short peptides, which was rapid in CulF and progressive in ConF, corroborated the results of protease activity assays (Figure 3). The gradual rise of pH during fermentation to a final level of 6.5 (Figure 2) in CulF samples promoted the activity of neutral proteases (pH 7.0 and 7.5) (Figure 3), including trypsin or trypsin-like enzymes and likely also calpain, a cysteine protease.[14] These neutral enzymes, as well other proteases derived from *A. oryzae* in douchi, allowed a more efficient conversion of intermediate MW protein fragments into short peptides and amino acids that dominated in the final stage (leading to day 30) of fermentation of CulF fish pastes when compared with the control. As reported previously,[19] free glutamic acid together with aspartic acid, alanine, lysine, and leucine was abundant (>100 mg/mL) in fish pastes fermented for 30 days. The lower MW peptides and free amino acids are desirable because they serve as substrates or reactants in the production of various volatile and nonvolatile flavor components.[18]

Proteins extracted from fish muscle tissue or peptides generated during fermentation might form aggregates under the high salt concentration condition due to charge depletion.

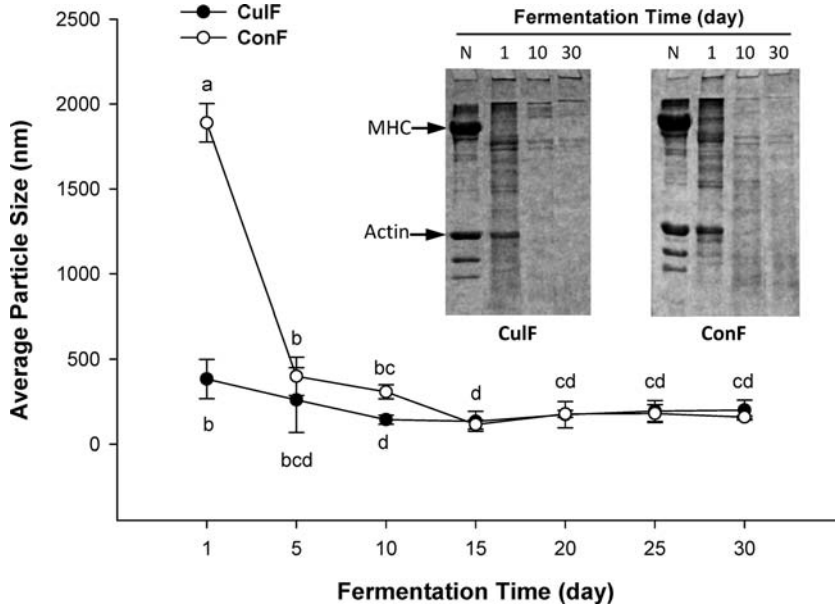

Figure 5. Changes in the size of particles in the extractive liquid during silver carp fish fermentation with (CulF) or without (ConF) douchi culture. Means (± standard deviations, $n = 3$) between samples without a common letter differ significantly ($P < 0.05$). (Inset) SDS-PAGE of myofibrillar proteins from fermented fish pastes. MHC, myosin heavy chain.

dx.doi.org/10.1021/jf203887x | J. Agric.Food Chem. 2012, 60, 226–233

Hence, particle size measurement provided further information about molecular degradation as well as aggregation. At the beginning of fermentation (day 1), the average particle size in the extractive of CulF was 450 nm, compared to 1912 nm for ConF (Figure 5). The particle size of ConF dropped sharply from day 1 to day 5 and reached a minimum of 115 nm by day 15, whereas that of CulF decreased to a minimum in 5 days. After 15 days, there was no difference between CulF and ConF. Proteins, myofibrils, and other protein aggregates were effectively hydrolyzed into small entities, particularly peptides, thereby reducing the size of particles suspended in the extractive liquid. These findings supported the results shown in Figure 4 as well as the inset in Figure 5 that low MW molecules had an increasing proportion and myosin disappeared during early fermentation.

Amino Acids and TCA-Soluble Peptides. The extractive components were analyzed to quantify flavor-eliciting compounds. Both free amino acids and peptides are saporous substances; their presence in the liquid portion accounted for the taste of the fish paste. As shown in Figure 6A, the amount

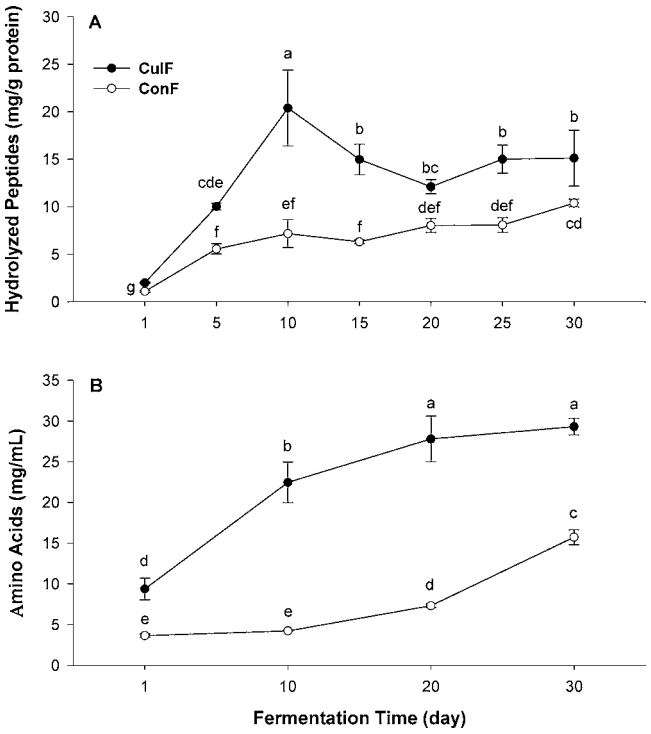

Figure 6. Changes in the concentrations of TCA-soluble peptides (A) and free amino acids (B) in the extractive liquid during silver carp fermentation with (CulF) or without (ConF) douchi culture. Means (± standard deviations, $n = 3$) between samples without a common letter differ significantly ($P < 0.05$).

of peptides resulting from fish protein hydrolysis (measured as TCA-soluble peptides) in CulF rose sharply ($P < 0.05$) in the first 10 days and declined thereafter until day 20. On the other hand, ConF showed a gradual but steady increase ($P > 0.05$) of soluble peptides through the entire 30 day fermentation period. In comparison with ConF, CulF extractives consistently showed high amounts of peptides except for day 1.

The production of free amino acids displayed a trend similar to that of soluble peptides during fermentation except that the maximum amount for CulF was obtained on day 20 (Figure 6B) instead of day 10 for peptides. Moreover, the amino acid content in the CulF extractive, which increased 3-fold after 30 days, was always greater than that in the ConF extractive. The increases in α-amino groups and TCA-soluble peptides were attributed to proteolytic activity during the course of fermentation, in agreement with previous findings.[34] From these results, it can be inferred that CulF had greater ($P < 0.05$) hydrolytic potential than ConF. Indeed, this was shown in protease assays (Figure 3; Table 1). The slight decline in TCA-soluble peptides after day 10 can be attributed to fungi, which tend to utilize them for their growth and protein synthesis when carbohydrates are exhausted.[27,35] The continuous enzymatic degradation of peptides to free amino acids would also contribute to the drop in the TCA-soluble peptides.

Volatile Compounds. In addition to taste-eliciting compounds such as short peptides and amino acids, aroma substances generated during fermentation contributed to the distinct flavor of fish pastes. Representative volatile compounds determined from the headspace of final fish pastes are presented in Table 2. The volatiles of the fish pastes fermented with the douchi inoculum (CulF) were largely composed of alcohols (predominantly ethanol), ketones, aromatic hydrocarbons (mostly phenol and pyrazine), aldehydes, and short-chain fatty acids, particular formic acids. In comparison, the fish pastes fermented without douchi (ConF) had mixed alcohols, fatty acids (particularly acetic acid and butanoic acid), and ketones in substantial amounts. These volatiles were largely derived from lipids but can be from proteins (amino acids) as well. The influence of individual aldehydes and ketones on the flavors in fermented fish products is well documented.[9] According to Yongsawatdigul et al.[11] and Adamiec et al.,[36] amino acids can undergo Strecker degradation generating flavor-active compounds, including volatile aldehydes and subsequently alcohols, acetals, acids, esters, and other compounds that contribute to flavor in fish sauce. Alcohols, although abundant, may not have a major impact because of their high detection threshold.[11]

Other volatile compounds in appreciable amounts in CulF but not detectable in ConF included phenol, which has a special sweet smell, and pyrazine, which has cocoa, coffee, and soybean aromas.[37] A low amount of dimethyl trisulfide, which contributes to fishy, sweaty, and fecal notes, was detected in CulF. Notwithstanding, their presence might be masked by the high concentrations of desirable aroma compounds such as pyrazine and phenol. Presumably, these compounds, along with several others that were detected only in CulF, were derived from soybeans used in the douchi starter culture manufacturing. On the other hand, several undesirable volatile compounds, such as butanoic acid and butanone, were notably abundant in ConF, whereas the aforementioned desirable volatiles were relatively deficient. These aroma profile differences between CulF and ConF, coupled with lower levels of soluble peptides and free amino acids produced, explained why CulF fish pastes were much more acceptable and considered more desirable than ConF when subjected to sensory evaluation.[19] The present results suggested that douchi starter culture could contribute to the formation of distinctive and unique volatile compounds essential for the attractive aroma of the final product.

In conclusion, acidic, neutral, and alkaline proteases that coexisted during silver carp fermentation were responsible for the degradation of main muscle proteins into short peptides and free amino acids that dominated in the extractive liquid of fermented fish pastes. Enzymes released from *A. oryzae*-

dx.doi.org/10.1021/jf203887x | J. Agric.Food Chem. 2012, 60, 226–233

Table 2. Volatile Compounds Identified in Fish Pastes during Fermentation at 35 °C for 30 Days[a]

compound	RI	peak area % CulF	peak area % ConF	compound	RI	peak area % CulF	peak area % ConF
alcohols				**acids**			
ethanol	919	10.31	6.05	formic acid	1472	10.07	1.94
butanol	1030	2.82	2.96	pentanoic acid	1336	0.28	4.79
silanediol	946	6.19	1.60	acetic acid	1437	2.17	10.58
hexanol	1469	1.77	1.74	butanoic acid	1740	TR	14.14
1-decanol	1516	TR	0.17	propanoic acid	1046	0.13	1.02
ketones				1,2-benzenedicarboxylic acid	2259	0.16	0.01
hexanone	1033	0.35	TR	**aldehydes**			
1-butanone	1347	0.13	14.14	decanal	1340	1.26	0.56
5,9-undecadien-2-one	1612	0.91	ND	nonanal	1375	0.08	0.76
2-undecanone	1297	4.10	ND	butanal	912	2.82	2.96
2-furanone	1780	TR	0.01	hexanal	1168	TR	2.06
2,4-pentanedione	1264	TR	0.03	hepatanal	1178	0.4	0.26
aromatic hydrocarbons				2-octenal	1356	TR	0.07
benzene alcohol	2103	0.41	0.32	pentanal	928	0.02	0.15
pyrazine	1261	13.41	ND	propanal	825	TR	0.15
4-ethanoxy-2-hydroxyoctadecanophenone	1708	0.88	0.04	**others**			
phenol	2026	17.83	ND	hexatriacontane	2656	2.82	ND
benzaldehyde	1499	1.22	2.04	tetracosane	2646	1.28	1.15
quinoline	1957	7.78	5.53	dimethyl trisulfide	1386	0.37	ND
				epicedrol	1921	0.61	ND

[a]RI, retention index; CulF, fermented fish pastes with douchi culture; ConF, fermented fish pastes without douchi culture; TR, trace (concentration <0.01%); ND, not detected.

cultured douchi served as a powerful source, or extra catalyst, for the generation of taste- and aroma-eliciting compounds, enabling the production of fish pastes that were superior to those from traditional fermentation in the flavor profile.

AUTHOR INFORMATION

Corresponding Author

*Phone: (859) 257-3822. Fax: (859) 257-5318. E-mail: ylxiong@uky.edu.

Funding

The study was supported by the Earmarked Fund for Modern Agro-industry Technology Research System (Grant NYCYTX-49-22) and the State Key Laboratory of Food Science and Technology, Jiangnan University (Grant SKLF-MB-200803).

ACKNOWLEDGMENTS

We thank Wuxi Soybean Sauce Co. (Wuxi, China) for providing the douchi starter culture (*Aspergillus oryzae*).

REFERENCES

(1) Yuan, C. Culture and processing of silver carp, a plankton feeder fish: for sustainable development of freshwater fisheries in China. Presented at the International Symposium on "Sustainability Science on Seafood and Ocean Ecosystem Conservation", Nov 7, 2009, Hakodate, Japan, 2009.

(2) Rogowski, D. L.; Soucek, D. J.; Levengood, J. M.; Johnson, S. R.; Chick, J. H.; Dettmers, J. M.; Pegg, M. A.; Epifanio, J. M. Contaminant concentrations in Asian carps, invasive species in the Mississippi and Illinois Rivers. *Environ. Monit. Assess.* **2009**, *157*, 211−222.

(3) Hoff, M. *U.S. Fish and Wildlife Service*. http://www.fws.gov, 2006.

(4) ACMS. Proceedings of the Asian Carp Marketing Summit, Sea Grant Publication IISG-11-04; http://www.iiseagrant.org/catalog/ais/asian_carp_summit.html, 2010.

(5) Cao, M.-J.; Shao, W.; Li, Y.; Hara, K.; Wang, X.-C.; Su, W.-J. Identification of a myofibril-bound serine protease in the skeletal muscle of silver carp. *J. Food Biochem.* **2004**, *28*, 373−386.

(6) Liu, H.; Yin, L.; Li, S.; Zhang, N.; Ma, C. Effect of endogenous cathepsins B and L on degradation of silver carp (*Hypophthalmichthys molitrix*) myofibrillar proteins. *J. Muscle Foods* **2008**, *19*, 125−139.

(7) Iacobuzio-Donahue, C. A.; Shuja, S.; Cai, J.; Peng, P.; Murnane, M. J. Elevations in cathepsin B protein content and enzyme activity occur independently of glycosylation during colorectal tumor progression. *J. Biol. Chem.* **1997**, *272*, 29190−29199.

(8) Platt, M. O.; Ankeny, R. F.; Jo, H. Laminar shear stress inhibits cathepsin L activity in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **2006**, *26*, 1784−1790.

(9) Wang, P. A.; Martinez, I.; Olsen, R. L. Myosin heavy chain degradation during post mortem storage of Atlantic cod (*Gadus morhua* L.). *Food Chem.* **2009**, *115*, 41228−41233.

(10) Yuan, C.; Kaneniwa, M.; Wang, X.; Chen, S.; Cheng, Y.; Qu, Y.; Fukuda, Y.; Konno, K. Seasonal expression of 2 types of myosin with different thermostability in silver carp muscle (*Hypophthalmichthys molitrix*). *J. Food Sci.* **2006**, *71*, 39−43.

(11) Yongsawatdigul, J.; Rodtong, S.; Raksakulthai, N. Acceleration of Thai fish sauce fermentation using proteases and bacterial starter cultures. *Food Sci.* **2007**, *72*, 382−390.

(12) Ramírez, J. A.; García-Carreño, F. L.; Morales, O. G.; Sánchez, A. Inhibition of modori-associated proteases by legume seed extracts in surimi production. *J. Food Sci.* **2002**, *67*, 578−581.

(13) Kilinc, B.; Cakli, S.; Tolasa, S.; Dincer, T. Chemical, microbiological and sensory changes associated with fish sauce processing. *Eur. Food Res. Technol.* **2006**, *22*, 604−613.

(14) Klomklao, S.; Benjakul, S.; Viessaguan, W.; Kishimura, H.; Simpson, B. K. Proteolytic degradation of sardine (*Sardinella gibbosa*) proteins by trypsin from skipjack tuna (*Katsuwonus pelamis*) spleen. *Food Chem.* **2006**, *98*, 14−22.

(15) Chéret, R.; Delbarre-Ladrat, C.; Lamballerie-Anton, M. D.; Verrez-Bagnis, V. Calpain and cathepsin activities in post mortem fish and meat muscles. *Food Chem.* **2007**, *101*, 1474−1479.

(16) Toyokawa, Y.; Takahara, H.; Reungsang, A.; Fukuta, M.; Hachimine, Y.; Tachibana, S.; Yasuda, M. Purification and characterization of a halotolerant serine protease from thermotolerant *Bacillus licheniformis* RKK-04 isolated from Thai fish sauce. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1867−1875.

(17) Ichishima, E.; Yamane, A.; Nitta, T.; Kinoshita, M.; Nikkuni, S.; Oka, T.; Yokoyama, S. Production of a new type of acid carboxypeptidase of molds of the *Aspergillus niger* group. *Appl. Microbiol.* **1973**, *26*, 327−331.
(18) Candogan, K.; Acton, J. C. Proteolytic activity of bacterial starter cultures for meat fermentation. *J. Muscle Foods* **2004**, *15*, 23−34.
(19) Kasankala, L. M.; Xiong, Y. L.; Chen, J. The influence of douchi starter cultures on the composition of extractive components, microbiological activity, and sensory properties of fermented fish pastes. *J. Food Sci.* **2011**, *76*, 154−161.
(20) Machida, M.; Asai, K.; Sano, M.; Tanaka, T.; Kumagai, T. Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **2005**, *l 43*, 1157−1161.
(21) Klomklao, S.; Kishimura, H.; Benjakul, S. Endogenous proteases in true sardine (*Sardinops melanostictus*). *Food Chem.* **2008**, *107*, 213−220.
(22) Lowry, O. H.; Rosebrough, N. J.; Fan, A. L.; Randall, R. J. Protein measurement with folinphenol reagent. *J. Biol. Chem.* **1951**, *193*, 256−275.
(23) Park, D.; Xiong, Y. L.; Alderton, A. L.; Ooizumi, T. Biochemical changes in myofibrillar protein isolates exposed to three oxidizing systems. *J. Agric. Food Chem.* **2006**, *54*, 4445−4451.
(24) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(25) Itou, K.; Kobayashi, S.; Ooizumi, T.; Akahane, Y. Changes of proximate composition and extractive components in narezushi, a fermented mackerel product, during processing. *Fish. Sci.* **2006**, *72*, 1269−1276.
(26) Benjakul, S.; Morrissey, M. T. Protein hydrolysates from Pacific whiting solid wastes. *J. Agric. Food Chem.* **1997**, *45*, 3423−3430.
(27) Riebroy, S.; Benjakul, S.; Visessanguan, W.; Rustad, U. E. T. Comparative study on acid-induced gelation of myosin from Atlantic cod (*Gardus morhua*) and burbot (*Lota lota*). *Food Chem.* **2008**, *109*, 42−53.
(28) Kakio, M.; Kawai, Y.; Kunimoto, M.; Yamazaki, K.; Inouei, N.; Shina, H. Chemical and microbiological characteristics of sardine meal fermented with *Aspergillus oryzae* IFO 4202. *Food Sci. Technol. Int. Tokyo* **1997**, *3*, 61−68.
(29) García-Gómez, M. J.; Huerta-Ochoa, S.; Loera-Corral, O.; Prado-Barragán, L. A. Advantages of a proteolytic extract by *Aspergillus oryzae* from fish flour over a commercial proteolytic preparation. *Food Chem.* **2009**, *112*, 604−608.
(30) Nielsen, L. B.; Nielsen, H. H. Purification and characterization of cathepsin D from herring muscle (*Clupea harengus*). *Comp. Biochem. Physiol. Part B* **2001**, *128*, 351−363.
(31) Zhang, J.-H.; Tatsumi, E.; Fan, J.-F.; Li, L.-T. Chemical components of *Aspergillus*-type douchi, a Chinese traditional fermented soybean product, change during the fermentation process. *Int. J. Food Sci. Technol.* **2008**, *42*, 263−268.
(32) Sinsuwan, S.; Rodtong, S.; Yongsawatdigul, J. Purification and characterization of a salt-activated and organic solvent-stable heterotrimer protease from *Virgibacillus* sp. SK33 isolated from Thai fish sauce. *J. Agric. Food Chem.* **2010**, *58*, 248−256.
(33) Vishwanatha, K. S.; Appu Rao, A. G.; Singh, S. A. Characterisation of acid protease expressed from *Aspergillus oryzae* MTCC 5341. *Food Chem.* **2009**, *114*, 402−407.
(34) Yongsawatdigul, J.; Choi, Y.; Udomporn, S. Biogenic amines formation in fish sauce prepared from fresh and temperature abused Indian anchovy (*Stolephorus indicus*). *J. Food Sci.* **2004**, *69*, 312−319.
(35) Russi, J. P.; Wallace, R. J.; Newbold, C. J. Influence of the pattern of peptide supply on microbial activity in the rumen simulating fermenter (RUSITEC). *Br. J. Nutr.* **2002**, *88*, 73−80.
(36) Adamiec, J.; Cejpek, K.; Roossner, J.; Velisek, J. Novel strecker degradatiom products of tyrosine and dihydroxyphelalanine. *Czech. J. Food Sci.* **2001**, *19*, 13−18.
(37) Zhang, Y.; Li, X.; Lo, C.-K.; Guo, S.-T. Characterization of the volatile substances and aroma components from traditional soy paste. *Molecules* **2010**, *15*, 3421−3427.