Amino acids profile changes of silver carp (*Hypophthalmichthys molitrix*) skin hydrolysate during hydrolyzing by Alcalase

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Abstract

Silver carp (*Hypophthalmichthys molitrix*) skin (SCS) is available as by-product in minced products manufacturing plants. Amino acids (AAs) profile changes of SCS hydrolysates, influenced by the time of hydrolysis by Alcalase, was studied. Different centrifuge cycles were used to clarify hydrolysates. Hydrolysis by Alcalase showed different effects on the AAs profile of SCS hydrolysates. As the hydrolysis time prolonged, the amount of total hydrophobic AAs decreased and total amount of hydrophilic AAs increased. Decreases in some AAs during 2 to 4 h and then from 4 to 6 h were significant (p<0.05). With the progression of hydrolysis (>2 h) the solution turned somewhat opaque and colored milky particles were observed. Increasing precipitation, possibly due to plastein formation, can change AAs profile and functional properties of hydrolysates. The results can be used to design enzymatic processes for the production of bioactive peptides with different functional properties and applications from fisheries by-products.

Keywords: Silver carp skin, Hydrolysis time, Alcalase, Amino acids profile

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Introduction

Silver carp (SC) is widely dispersed in different parts of Iran and consists up to 50% of total production of warm-water cultured carps (>100000 MT/y) (IFO, 2019). In recent years, with the construction of new fish processing plants, parts of cultured SC are processed to minced meat and ready-to-cook foods such as nuggets. Development of these plants has led to the production of by-products, which include considerable part of fish organs. The inadequate/inefficient use of these by-products, in addition to the loss of rich nutritional resources, has environmental and health problems. Fish skin, including silver carp skin (SCS), is a rich source of valuable connective tissue proteins available at a very low cost as processing waste. Total amount of skin and bones remaining after deboning of SC with an average weight of 1 kg is about 10%. Washed and cleaned skin is about 3.5% of total weight of the carcass (Jalili *et al*., 2019). Thus, considerable amount of SCS is discarded annually or, along with other by-products, is used exclusively for production of fish meal.

Amino acids composition and sequences determine the functional properties of peptides which depend on the protein source, method, and conditions for preparation and processing and the molecular weight distribution (MWD) of resulting hydrolysates (Gómez-Guillén *et al.,* 2011). Enzymatic hydrolysis of proteins found in processing co-products in the food industry can be used to produce foods with enhanced functional properties (Valencia *et al.,* 2014). Antioxidant activities have been reported for fish protein hydrolysates prepared from several sources, which include tuna skin (Gómez-Guillén *et al*., 2010), tilapia skin (Zhang *et al.,* 2012), SC processing by-products (Zhong *et al.,* 2011), common carp roe (Chalamaiah *et al.,* 2015), alaska pollock skin (Sun *et al.,* 2016), SCS collagen (Zhang *et al*., 2017) and grass carp skin (Yi *et al.,* 2017). Type of enzyme and hydrolysis conditions, such as enzyme/substrate ratio, substance/ solvent ratio, temperature, time and pH, can affect the chain length of peptides and the functional properties of protein hydrolysate (Chi *et al.,* 2014). Alcalase has been introduced as an appropriate candidate for industrial protein hydrolysis due to endopeptidase with a wide range of specificities, stability at a wide range of pH, and relatively low price (Doucet *et al.,* 2003). In different studies, Alcalase has been described as the best enzyme for the release of peptides that can inhibit oxidation of oils in various systems (Dong *et al*., 2008; Alemán *et al.,* 2011; Chi *et al.,* 2015; Yi *et al*., 2017). To the best of our knowledge, this is the first study reported on the Amino acids profile changes of SCS hydrolysis by Alcalase in defined conditions. The findings of this study can provide fundamental information for further study in this field.

Material and Methods

Enzyme and chemicals

Alcalase® 2.4 L from *Bacillus licheniformis*, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in this study were analytical grade.

Raw Material

Fresh/pre-rigor Silver Carp (*Hypophthalmichthys molitrix*), 1295-930 g/fish, was purchased from domestic farm (Rasht city in Gilan province of Iran) and transported on ice within 4 h to the National Fish Processing Technology Research Center (in Anzali port). Upon arrival, scaled skins were removed manually and washed with cold tap water to remove residual flesh. The sample were cut into approximately 5×5 cm pieces and stored at refrigerator before treatments.

Skin pretreatment

SCS pieces were treated before hydrolysis as described previously by Jalili *et al*. (2019). NaOH (4 g/L) was used for soaking the skin (skin/solution: 1/8, w/v) at 4±1ºC for 3 h with every 10 min gentle stirring. Then washed thoroughly with cold tap water until the washed water reached pH<7.5. Washed skin was soaked in 6.1 g/L acetic acid (skin/solution: 1/8, w/v) for 2 h at room temperature (25±2ºC). The acid-treated skin was washed as previously described. The swollen white colored SCS was freez-dried (OPERON, OPR-FDB-5503, Korea) to <10% moisture content, and kept cool and dry.

Enzymatic hydrolysis

Pretreated SCS was soaked in distilled water (skin/water: 1/20, w/v) at refrigerator overnight. Soaked skin was homogenized using warring blender, at room temperature. pH was adjusted >7.0 by adding NaOH (4 g/L) and then warmed to 50ºC before enzyme addition. Proteolysis using Alcalase (E/S: 1/100, w/w) was carried out at 50ºC for 6 h in a water-bath with continuous gentle stirring. pH was not adjusted during hydrolysis and enzyme was re-added after 3 h. Samples were withdrawn before hydrolysis as a control and at 0.25, 0.5, 0.75, 1, 2, 4 and 6 h intervals during hydrolysis process. The enzymatic reaction was immediately terminated by heating the samples in a boiling-water bath for 15 min to inactivate Alcalase. After centrifugation (4500-13000×g, 20 min), resulting clarified supernatant containing peptides was collected, freez-dried and used for determination of antioxidative activities.

Proximate composition and pH

Moisture content was determined by drying samples at 105ºC until a constant weight was reached. Ash content was estimated by charring in a crucible at 550ºC until the ash had a white appearance. Total nitrogen content of the substrate was determined by using the Kjeldahl method (Behr, S4, Germany). Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. Lipids were determined gravimetrically after Soxhlet extraction of dried samples with hexane (AOAC,

2000). pH was measured directly in solution during hydrolysis using pH meter (WTW, pH 7110, Germany).

Amino acid composition

Preparation of SCS hydrolysates samples were performed using HCl 218.8 g/L at 110°C for 24 h. prior to injection into high performance liquid chromatography (HPLC) (Knauer, Germany), amino acids derivatization were similar to those reported by Ovissipour *et al*. (2010) with o-phthaldehyde. The column C18 (Knauer, Germany) was used at a flow rate of 1 mL/min with a fluorescence detector (RF-530, Knauer, Germany).

Statistical analysis

Statistical analysis was performed using SPSS software version 17.0 (SPSS Statistical Software, Inc., Chicago, IL, USA). Data were reported based on mean±standard deviation (n=3). Duncan's multiple range test was used at the significance level of $p \leq 0.05$.

Results and discussion

Proximate composition and pH

Our previous results showed that pretreatment with acetic acid and NaOH can effectively reduce lipid and ash content of SCS (Table 1) (Jalili *et al.*, 2019).

Table 1: Proximate composition (g/100g) of silver carp skin (SCS) and pretreated SCS with NaOH and acetic acid [a]

	Raw SCS	Pretreated SCS
Moisture	76. 20 ± 0.54[a]	85.63 ± 0.57[b]
Protein	19.36 ± 0.49[a]	13.62 ± 0.43[b]
Lipid	2.47 ± 0.21[a]	0.18 ± 0.07[b]
Ash	1.69 ± 0.16[a]	0.57 ± 0.03[b]
pH	-	7.1 ± 0.11

[a] Different letters indicated significant differences ($p<0.05$)

pH not adjusted during hydrolysis and decrease in pH was observed (Fig. 1). The initial pH of the SCS solution was 7.1 which, after 4 h hydrolysis, reached a final pH of 5.7 and then remained constant. pH change during the initial 2 h was significant at different times of sampling ($p<0.05$). The rate of pH change at different times of hydrolysis was not constant. pH drop was very fast initially, and its acceleration slowed down gradually. The cleavage of peptide bonds by protease enzymes released H+ ions due to freed carboxyl and amino groups, thereby reducing pH. It can be concluded that while the rate of hydrolysis of SCS proteins in the early stages of hydrolysis by Alcalase was very fast, it slowed down during 4h.

Amino acids composition

Hydrolysis of SCS by Alcalase for 6 h showed different effects on the amount of some amino acids as well as the total hydrophobic and hydrophilic amino acids (Table 2). Results indicated that hydrolysis for ≤2 h does not significantly change the weight ratio of different amino acids.

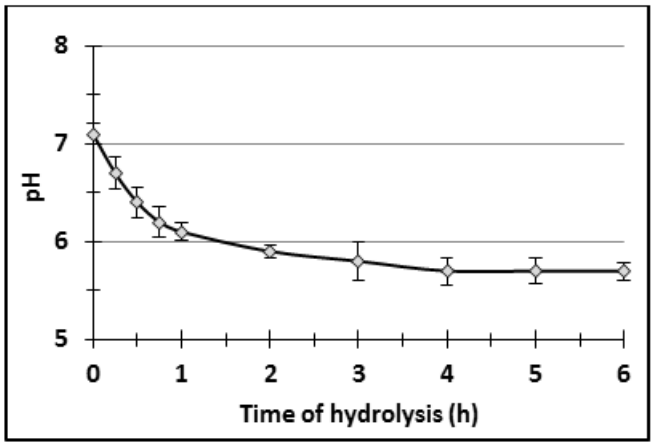

Figure 1: pH changes of silver carp skin during hydrolysis by Alcalase (Mean±SD).

Table 2. Comparative amino acid profiles of peptide fractions from silver carp skin hydrolyzed by Alcalase at different hydrolysis time (g/100 g protein).[1]

Amino acids	0.25 h	1 h	2 h	4 h	6 h
Gly	24.79±1.49^{a2}	24.60±1.23^{a}	24.03±1.20^{a}	23.56±1.41^{a}	22.73±0.97^{a}
Pro	11.55±0.69^{a}	11.45±0.57^{a}	10.83±0.54^{a}	9.54±0.39^{ab}	8.99±0.36^{b}
Ala	9.49±0.57^{a}	9.91±0.50^{a}	9.64±0.48^{a}	9.61±0.58^{a}	8.52±0.34^{a}
Val	1.95±012^{a}	1.93±0.10^{a}	1.66±0.08^{a}	1.37±0.07^{a}	1.31±0.05^{a}
Met	2.40±0.14^{a}	2.32±0.12^{a}	2.19±0.11^{ab}	1.95±0.12^{ab}	1.83±0.07^{b}
Ile	1.36±0.08^{a}	1.47±0.07^{a}	1.43±0.07^{a}	0.89±0.05^{b}	0.65±0.03^{c}
Leu	3.07±0.18^{a}	2.89±0.14^{a}	2.88±0.15^{a}	1.92±0.12^{b}	1.76±0.07^{c}
Phe	2.26±0.12^{ab}	2.81±0.14^{a}	2.69±0.13^{a}	2.11±0.12^{bc}	1.82±0.09^{c}
Hyp	7.38±0.44^{a}	7.09±0.35^{a}	7.11±0.36^{a}	6.83±041^{a}	6.74±0.27^{a}
Asp	12.54±0.75^{a}	12.07±0.60^{a}	13.18±0.66^{a}	15.52±0.93^{ab}	16.41±0.61^{b}
Glu	3.59±0.22^{a}	3.52±0.18^{a}	3.73±0.19^{a}	5.76±0.35^{b}	6.63±0.27^{b}
Ser	2.43±0.18^{a}	2.75±0.14^{ab}	2.79±0.14^{ab}	2.91±0.17^{ab}	3.10±0.12^{b}
Lys+Hyl	4.33±0.26^{a}	4.18±0.22^{a}	4.38±0.25^{a}	4.52±0.28^{a}	4.97±0.20^{a}
Arg	8.52±0.51^{a}	8.58±0.47^{a}	8.65±0.43^{a}	8.59±0.52^{a}	9.35±0.39^{a}
Cys	0.35±0.04^{a}	0.49±0.02^{ab}	0.58±0.03^{ab}	0.61±0.04^{bc}	0.72±0.03^{c}
Thr	2.93±0.19^{a}	2.81±0.16^{a}	3.10±0.18^{a}	3.07±0.20^{a}	3.17±0.14^{a}
His	0.57±0.03^{a}	0.54±0.04^{a}	0.55±0.03^{a}	0.63±0.04^{a}	0.65±0.05^{a}
Tyr	0.49±0.03^{a}	0.59±0.04^{a}	0.58±0.04^{a}	0.61±0.07^{a}	0.65±0.06^{a}
THAAs[3]	32.1±1.97^{a}	32.8±1.65^{a}	31.3±1.57^{a}	27.4±1.75^{ab}	24.9±1.08^{b}
TMHAAs[4]	31.4±1.43^{a}	31.1±1.38^{ab}	32.7±1.65^{ab}	37.3±1.73^{bc}	40.5±1.90^{c}

[1]Determinations were performed in triplicate and data correspond to mean±SD; 2. Mean values within each row with different superscript letters are significantly different (p<0.05); 3. Total hydrophobic amino acids (THAAs) (Pro+Ala+Val+Met+Leu+Ile+Phe); 4. Total major hydrophilic amino acids (TMHAAs) (Asp+Glu+Ser+Lys+Hyl+Arg).

As the hydrolysis time prolonged, the amount of total hydrophobic amino acids (THAAs) decreased and total amount of major hydrophilic amino acids increased. THAAs, which were 32.8% at 0.25 h, decreased to 24.9% after 6 h of hydrolysis. Among the hydrophobic amino acids, Ile, Leu, and Val, with 52.2, 42.7, and 32.8%, had the highest decrease rate after 6 h

hydrolysis. Then, Met, Pro, Phe, and Ala showed 23.8%, 22.2%, 19.5%, and 10.2% decrease respectively. Gly and Hyp also had a slight decrease (≈8%). In contrast, total hydrophilic amino acids increased more than 17% after 6 h. Cys had the highest increase: it more than doubled after 6 h hydrolysis. The variation of each amino acid was also different at different sampling time intervals. Changes in amino acid profile of samples were noticeable between 2–4 h and then between 4–6 h. Pro, Met, Val, Ile, Leu, Asp, Glu, and His had the highest changes after 4 h as compared to 2 h of hydrolysis. Gly, Ala, Phe, Ser, Cys, Tyr, Arg, and Thr showed the highest changes after 6 h, compared to 4 h and initial times. Dong *et al*. (2008) reported that hydrolysis by Alcalase and Flavourzyme at 0.25, 0.5, 1.5, and 4 hours did not significantly change the ratio of most amino acids. Sulfur containing amino acids (Met and Cys) increased 1 to 3 times, and a slight increase in the amount of Pro was also reported. During experiments, it was visually observed that the hydrolysates recovered from ≤2 h hydrolysis were completely clear, free of opacity, and suspended particles after centrifugation (4500×g). However, after 4 h hydrolysis, the solution remained somewhat opaque and colored milky particles were observed. With the progression of hydrolysis after 4 h, the amount of suspended particles increased. Using a higher centrifuge cycle resulted in the clarification of hydrolysate. Based on recent observations, it can be concluded that a part of the resulting peptides forming gel gets removed from solution with increase in hydrolysis time. Change in the color of the protein solution to brownish yellow was also reported during hydrolysis after 3 h by Dong *et al*. (2008). Alcalase has high specificity towards the production of peptides, with the ends of aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) amino acids residues with often no charge, at pH about 6, and some amount of hydrophobicity. This causes intermolecular attractive forces, such as hydrophobic interaction, resulting in the aggregation of these peptides and, finally, the formation of gel (Doucet *et al*., 2003). Plastein formation reactions occur at pH 3 to 11 and are related to several factors, including substrate concentration, amino acid composition, and size of peptides derived from protease hydrolysis. It has been suggested that the presence of hydrophobic amino acids in the peptide chain would enhance plastein formation (Gong *et al.,* 2015). Doucet *et al.* (2003) observed that aggregates in WPI hydrolysate are made of small molecular mass (<2 kDa) peptides. Visual observations and the results of amino acid profiles reinforce the possibility of plastein formation in SCS hydrolysates solution for a longer period of hydrolysis (>2 h) by Alcalase. It seems that the formation of plastein and its removal from resulting hydrolysates decreases THAAs and increases the proportion of hydrophilic

amino acids (Fig. 2).

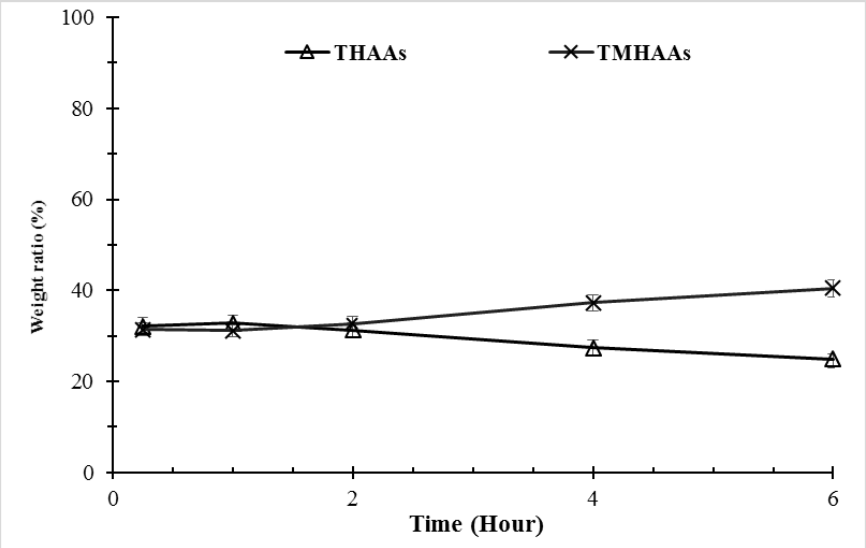

Figure 2: Total Hydrophobic and Hydrophilic amino acids changes of silver carp skin during 6 hours hydrolysis by Alcalase (%).Total hydrophobic amino acids (THAAs) (Pro+Ala+ Val+Met+Leu+Ile+Phe); Total major hydrophilic amino acids (TMHAAs) (Asp+Glu+ Ser+Lys+Hyl+Arg).

Conclusion

Enzymatic hydrolysis of proteins from fisheries by-products can be used to produce value-added products with enhanced functional properties. The present study revealed that prolonging the hydrolysis time of SCS by Alcalase more than 2 hours changes the color of the hydrolysate solution and increases the aggregation. Increased precipitation, possibly due to plastein formation, changes the amino acid profile, especially the relative decrease in the total hydrophobic amino acids, can alter the biological activities of the resulting peptides. Therefore, optimizing the enzymatic hydrolysis time to achieve the maximum desired biological activity, in addition to the peptide chain length, will be inevitable. Further research on determining the efficiency and chemical structure of precipitate (plastein) formed at different concentrations of fish skin protein, different times of hydrolysis and identification of their biological functions are recommended.

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