EXTRACTION OF COLLAGEN FROM FISH WASTE AND DETERMINATION OF ITS AMINO ACID COMPOSITION

VIKTORIIA PLAVAN[1], MAKSYM KOLIADA[1], WUYONG CHEN[2], VIACHESLAV BARSUKOV[1]

[1] *Kiev National University of Technology & Design, Kiev, Ukraine*

[2] *National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, China*

In this paper, the analysis of the most famous ways collagen-containing solid waste recycling of fish and leather industry was made. Their influence on the properties of the obtained hydrolysates was shown. The method for processing of collagen containing wastes from gutting mackerel (*Scomber*) is developed for the preparation of biopolymer materials for various purposes. The proposed method involves acid-enzymatic hydrolysis of waste in a solution of acetic acid in combination with the previous washing with alkali to remove soluble proteins. By means of ion-exchange chromatography using 339M automatic analyzer (Microtechna, the Czech Republic) have determined that the resulting hydrolyzate is balanced in amino acid composition and can be used to produce organic fertilizer and as growth promoter and as feed additive and after further modification as a component of biopolymers.

Keywords: collagen containing fish waste, acid-enzymatic hydrolysis, amino acid composition.

INTRODUCTION

Collagen is a general extracellular structural protein involved in the formation of connective tissues. Collagen occurs in genetically distinct forms identified as type I to type XIX. They vary considerably in amino acid composition and structure.

Industrial utilization of collagen is very wide. The main sources of industrial collagen are limited to those from pigskins and bovine hides and bones. Collagen is ductile and is used in different fields, such as leather and films, cosmetics, biomedical and pharmaceutical industries, and in food (Ratnasari *et al.*, 2013; Gaidau *et al.*, 2013; Bostaca and Crudu, 2013). A considerable proportion of collagen is consumed in the manufacture of food gelatins that have a number of functional properties as gel and mousse, thickening agent, emulsifier, stabilizer, protective colloid (Schrieber and Gareis, 2007).

The occurrence of bovine spongiform encephalopathy (BSE) and foot/mouth disease (FMD) along with religious constraints has resulted in an anxiety among users of collagen and collagen-derived products from land-based animals. In recent years, increasing attention has been paid to alternative collagen sources, such as fish skin, which comprise about 30% of the total fish weight available after fish fillet preparation (Gómez-Guillén *et al*., 2011).

Modern production of fish is accompanied by the formation of a large number of collagen wastes (bones, fins, skin, scales, viscera, etc.) that ranged from 30 to 70% by weight of the feedstock (Shahidi and Botta, 1994). Partial use of them, on one hand, leads to the loss of an important protein based product and other purposes, and to the other, to pollution

Several studies have focused on the characterization of different fish collagens. Most fish collagens consist of two α-chain variants, which are normally known as α1 and α2. In addition to differences in molecular types, fish collagens have been shown to vary widely in their amino acid composition. In particular, the physical properties of the

protein and the quantity of the amino acids proline and hydroxyproline vary significantly among fish species, and this is strictly correlated with the outside temperature of the animal's environment (Muyonga *et al*., 2004).

The greatest features of fish collagen are lower denaturation temperature and viscosity than collagens of land-vertebrated animals. These features distinguishing fish collagens from land-vertebrated collagens are very important for food processing. This led to a recent increasing interest in fish collagens (Kimura and Ohno, 1987; Leuenberger, 1991).

The shrinkage temperatures (T_s), values of fish-skin collagens range from about 35°C to 57°C, according to the mean temperature of the environment[1], while mammalian collagen has a T_s value of about 62°C. By an examination of the complete amino-acid analyses, fish-skin gelatin hydroxyproline and mammalian gelatin are 67 and 95 respectively (residues of amino acid per 1000 total residues); the lower the amount of hydroxyproline the lower the value of T_s (Rigby and Spikes, 1960). Interchain hydrogen bonding between hydroxyl groups of hydroxyproline and backbone carbonyl groups was, on this basis, suggested as an important stabilizing feature of the collagen structure.

The aim of this work is to develop a method to use collagen-containing materials after the production of mackerel (*Scomber*), and to determine a rational way of using the obtaining collagen-based materials.

MATERIAL AND METHODS

As an object of our investigation was used waste mackerel (offal) obtained after butchering of the fish. Typically, these wastes are not reused and disposed to the landfills. Wastes were preserved using salt (100% v/w salt). The chemical composition of the fish waste is presented in Table 1.

Table 1. Physical-chemical properties of the salted fish waste

Content in the waste, %	
– moisture	43.5
– mineral substances	38.6
– fatty substances	5.9
– total nitrogen content	7.9

Obtaining of Collagen Hydrolysates

Collagen hydrolysate is denatured and partly hydrolysed protein. Collagen hydrolysates display properties that depend on the raw material source and processing method. Collagen hydrolysates may be prepared through acid hydrolysis (mostly dilute H_2SO_4, HCl or H_3PO_4), alkaline hydrolysis (for example NaOH, KOH or $Ba(OH)_2$), enzymatic hydrolysis or microbial breakdown.

The use of enzymes in combination with acids or alkalis and high temperatures to obtain hydrolysates is the best method found to create collagen hydrolysates preventing breaking down of amino acids, carbohydrates and other nutrients contained in the waste (Nam *et al.*, 2008).

In this work alkali-enzymatic and acid-enzymatic methods were used. The level of hydrolysis was determined by total nitrogen content.

Alkali-enzymatic hydrolysis of fish waste was carried out for 6-8 hours at 40°C as follows: offals were washed with running water, crushed to the consistency of stuffing, loaded into the reactor, 50% of water was added; 1.6% v/w H_2O_2; 2% v/w NaOH. The enzymatic hydrolysis was then carried out for 4 hours, enzyme demand was 3%. The resulting mixture was adjusted to pH 6.8 with a solution of sodium carbonate. After separation of the layers of the hydrolysate, it was evaporated to the desired concentration.

The level of hydrolysis was monitored by total nitrogen content of the hydrolysis product, it was 12.2 g /l. The disadvantage of the obtained product was a dark brown color and an unpleasant "fishy" smell.

To intensify the process of hydrolysis, further treatment with hydrogen peroxide was used. The effectiveness of this treatment was confirmed in previous studies using waste from the leather industry (Plavan *et al*., 2013). Unfortunately, in the case of fish waste, such treatment was not effective. Although the total nitrogen content in the final product was 15.4 g /l. The unpleasant smell of the product was still present as result of the formation of peroxides via partial oxidation of the fats.

Relative high fat content in the collagen-containing waste of the fish industry affects negatively the properties of hydrolysates. Fat undergoes oxidation, which leads to a rapid breaking down of the resulting products, it is also the source of the unpleasent "fishy" smell. Therefore, it is necessary to carry out degreasing, the essence of which is to free the pores, capillaries and extracellular space of the fat contained.

The most rational way to degrease collagen-containing wastes from the fish industry is the use of enzyme pretreatments. Enzymes break down proteins and the structure of the tissue, thus as a result – the release of the fat.

For the acid-enzymatic hydrolysis, solutions with different concentrations of acetic acid and enzyme were used. The enzyme was Zime SB: activity 1500 U/g, the optimal pH 3.5-6.5. Consumption of enzyme was 1.3%. The degree of hydrolysis was determined by the total nitrogen content in the final product (Table 2).

Table 2. Characteristics of the methods and products of hydrolysis

Variant	Washing with alkali	Carrying hydrolysis				Content, g/l		
		alkali	enzyme	acid	H_2O_2	Nitrogen	Dry matter	Ash
1	-	+	+	-	-	12.2	218.9	124.4
2	-	+	+	-	+	15.4	215.3	120.2
3*	+	-	+	+	-	11.2	15.2	4.9
4**	+	-	+	+	-	14.3	22.3	9.0

*Alkali washing duration 24 hrs. Alkali-enzymatic hydrolysis duration 4 hrs, at 40°C.

** Alkali-enzymatic hydrolysis duration 4 hrs., at 40°C and 8 hrs. Room temperature (left overnight). Alkali washing duration decreased to 1,5 hrs. Alkali was dosed in 3-4 receptions after 30 min.

The Method of Ion-Exchange Liquid-Column Chromatography

To conduct qualitative and quantitative analyses of amino acid composition of the collagen-containing material of the resulting hydrolyzate, ion-exchange liquid-column chromatography with the 339 M automatic analyzer (Microtechna, the Czech Republic) was employed.

RESULTS AND DISCUSSION

Decreasing the alkali washing time leads to an increase in mineral content in the final product (Table 2). The increasing of the acid-enzymatic hydrolysis reaction speed has positive effect on the quality of the final product, indeed the content of total nitrogen increases.

As a result of hydrolytic decomposition of fish collagen, the number of basic amino acids increased due to breaking down of the peptide bonds (Table 3), arginine content increased to 9.59%. Hydrolysate content of the essential amino acid histidine in fish collagen is 1.37%. Very important amino acids for the nutrition of young animals are: isoleucine and leucine (1.72 and 5.04%), methionine (2.03%), threonine (3.92%), and phenylalanine (2.84%), the latest is higher in fish collagen hydrolysate than in hydrolysate from cattle skins.

Table 3. Amino acid composition of fish and cattle hide hydrolyzate (%)

Amino acid	Nativecattle hide collagen (Heidemann, 1993)	Cattle hide collagen hydrolyzate (Plavan *et al*., 2013)	Native fish collagen (mackerel) (Sun Young Lim, 2012)	Fish collagen hydrolyzate
Glycine	33.5	7.10	3.4	17.68
Proline	12.2	6.86	3.8	6.64
Alanine	12.0	6.76	5.4	7.79
Hydroxyproline	9.4	8.25	5.4	1.76
Glutamic acid	7.6	6.13	14.8	15.16
Arginine	5.2	7.11	5.6	9.59
Aspartic acid	4.6	4.21	10.3	7.66
Serine	3.1	2.35	4.3	5.92
Leucine	2.2	1.77	8.4	5.04
Lycine	2.5	5.94	10.5	4.73
Valine	1.7	4.05	6.1	2.63
Threonine	2.0	3.74	4.9	3.92
Isoleucine	1.1	1.55	5.0	1.72
Phenylalanine	1.1	2.19	4.6	2.84
Methionine	1.0	0.68	3.0	2.03
Histidine	0.3	1.02	6.6	1.37
Tyrosine	0.0	1.34	1.2	1.64
Total	100	100	100	100

The presence of reactive capable amino groups, gives us the ability to change the properties of hydrolysates (Zhongkai Zhang, 2006). The most reactive capable groups of proteins are those containing the amino acids serine (whose primary group is the -OH group), hydroxyproline (secondary -OH), threonine (secondary -OH), tyrosine (phenolic -OH), aspartic and glutamic acids containing the group –COOH and lysine and arginine containing alkaly groups (Mokrejs *et al*., 2010). Hydrolysates can be chemically modified (Bucevschi *et al.*, 1999) by the application of crosslinking reagents (in particular aldehydes, starch, enzymes).

Thus, the amino acid composition of the obtained hydrolysate is balanced. It can be used as an organic fertilizer and as growth promoter in animals food, after further modifications as a component of composite materials and biopolymers.

CONCLUSION

A method was developed for the disposal of collagen-containing mackerel (S*comber*) waste to get collagen-based biomaterials for various purposes. The method involves the acid-enzymatic hydrolysis of the waste in a solution of acetic acid and enzyme in conjunction with the previous washing with carried out with alkali to remove soluble proteins. Reducing the duration of alkali washing leads to an increase in mineral content in the final product. The increasing of the acid-enzymatic hydrolysis has positive effects on the quality of the final product and the content of total nitrogen increases. The amino acid composition of the resulting hydrolysate is balanced and it can be used to produce organic fertilizers and growth promoters in animals food, after further modifications as a component of composite materials and biopolymers.

Acknowledgements

Authors are very grateful to the State Agency of Science, Innovation and Informatization of Ukraine for its financial support towards the research project.

REFERENCES

Bostaca, Gh. and Crudu, M. (2013), "Considerations on fish skin processing", *Revista de Pielarie Incaltaminte* (*Leather and Footwear Journal*), 13(3), 211-220.

Bucevschi, M.D., Chirita, G., Colt, M. *et al.* (1999), "Chemical modification of collagen hydrolyzates", *J. Amer. Leather. Chem. Ass.*, 94 (2), 89-95.

Gaidau, C., Maereanu, M., Foiasi, T., Adiguzel Zengin, C., Karavana, H.A., Mutlu, M.M., Bitlisli, B.O., Basaran, B. (2013), "Sturgeon skins - a valuable resource or luxury leather industry", *Revista de Pielarie Incaltaminte* (*Leather and Footwear Journal*), 13(4), 311-320.

Gómez-Guillén, M.C., Giménez, B., López-Caballero, M.E. and Montero, M.P. (2011), "Functional and bioactive properties of collagen and gelatin from alternative sources: A review", *Food Hydrocolloids*, 25(8), 1813-1827.

Heidemann, E. (1993), *Fundamentals of Leather Manufacture*, Eduard Roether KG., Darmstadt.

Kimura, S. and Ohno, Y. (1987), "Fish type I collagen: Tissue-specific existence of two molecular forms $(\alpha 1)_2$ $\alpha 2$ and $\alpha 1\alpha 2\alpha 3$ in Alaska Pollack", *Comp. Biochem. Physiol*, 88(B), 409-413.

Leuenberger, B.H. (1991), "Investigation of viscosity and gelation properties of different mammalian and fish gelatins", *Food Hydrocolloids*, 5(4), 353-362.

Mokrejs, P., Janacova, D., Svoboda, P. and Vasek, V. (2010), "Modelling the Solubility of Films Prepared from Collagen Hydrolysate", *Journal of the Society of Leather Technologists and Chemists*, 94 (6), 231-239.

Muyonga, J.H., Cole, C.G.B. and Duodu, K.G. (2004), "Characterisation of acid soluble collagen from skins of young and adult Nile perch (Lates niloticus)", *Journal of Food Chemistry*, 85(1), 81-89.

Nam, K.A., You, S.G., Kim, S.M. (2008), "Collagens and biological properties of their enzymatic hydrolysates", *J. Food Sci.*, 73(2), 249-255.

Plavan, V., Kovtunenko, O., Koliada, M. (2013), "Extraction of Collagen from Phosphonium Tanned Leather Waste and Research of its Properties", Abstract of 2013 CAS - TWAS Symposium on Green Technology (SGT2013), Beijing.

Ratnasari, I., Yuwono, S.S., Nusyam, H. and Widjanarko, S.B. (2013), "Extraction and characterization of gelatin from different fresh water fishes as alternative sources of gelatin", *International Food Research Journal*, 20(6), 3085-3091.

Rigby, B.J. and Spikes, J.D. (1960), "Hydroxyproline and the Shrinkage Temperature of Collagen", *Nature*, 187, 150-151.

Schrieber, R. and Gareis, H. (2007), *Gelatin Handbook. Theory and Industrial Practice*, Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA.

Shahidi, F. (1994), "Seafood processing by-products", in: F. Shahidi and J.R. Botta, eds., *Seafoods chemistry, processing, technology and quality*, Blackie Academic and Professional, Glasgow.

Sun Young Lim (2012), "Effect of catch areas on chemical composition and heavy metals concentration of chub mackerel (Scomber japonicus)", *Life Science Journal*, 9(3), 1276-1280, http://www.lifesciencesite.com

Zhang, Z.K., Li, G.Y., Shi, B. (2006), "Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes", *Journal of the Society of Leather Technologists and Chemists*, 90 (1), 23-27.