Determination of glycosaminoglycan in sea cucumber by HPLC with post column derivatization

Xin Gao, Zhaohui Zhang*, Wenjing Sheng, Changhu Xue

* College of Food Science and Engineering, Ocean University of China, Qingdao, China

Abstract: A fast and sensitive method was developed for simultaneous determination of neutral sugars, amino sugars and uronic acids in glycosaminoglycan extracted from sea cucumbers using a reverse-phase HPLC with PMP derivatization and Variable Wavelength Detection. The calibration curve for each monosaccharide was linear within the range of 0.5 – 10 mg/L ($R^2 > 0.996$). This optimized method was applied to analyze the monosaccharide composition of the glycosaminoglycan extrcated from sea cucumber *Apostichopus japonicus*, which is best hydrolysed under the condition of with 2M TFA at 110 ℃ for 8 h. The average CI95 global recovery for all monosaccharides ranged from 96.3 % to 104.4 %, and the relative standard deviations were less than 8.19 %. This method can be applied for the analysis of all the reducing sugars in glycochemistry and glycobiology.

Key word: sea cucumber, glycosaminoglycan, HPLC, PMP derivatization

1 Introduction

As life science develops, more and more attention is paid on the function and structure of the glycosaminoglycan (GAG) in animal bodies. The determination of the monosaccharide content of GAGs provides basic information for the structure elucidation, much as amino acid analysis provides a characteristic property of a protein. Thus the determination of the monosaccharides in GAG becomes necessary for all the glycochemistry and glycobiology researchers.

Derivatization with 1-phenyl-3-methyl-5-pyrazolone has more recently been proposed as a suiTablele way to lable the reducing sugars for its facile preparation, high UV absorption, and ease of separation on reverse-phase HPLC systems [1-5]. Lots of research has been carried out on this [6-11]. Strydom DJ [12] has successfully separated

fifteen monosaccharides with PMP derivatization, Fu et al. [13] have reported a PMP-HPLC method for complete monosaccharide composition analysis of oligosaccharides or glycoproteins, and many other laboratories have also employed this mothodology. But none of them provides simultaneous separation and determination of neutral sugars, amino sugars and uronic acids in sea cucumber GAGs.

In this study, a fast and sensitive reverse-phase high-performance liquid chromatography (HPLC) with 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization and Variable Wavelength Detection (VWD) were applied to determine neutral sugars, amino sugars and uronic acids in GAG extracted from the sea cucmber *Stichipus japonicus*. The obtained results were to test the possibility of finding useful indicators for aquatic products.

2. Material and Methods

2.1 Materials

Sea cucumber *Stichipus japonicus*, harvested in Qingdao, Shandong Province, China (average weight was 138.3g.) were purchased at a retail store. Individual sea cucumber was removed the entrails and prepared to glycosaminoglycan sample (SJ-GAG) with Mourano's method [14].

2.2 Chemicals

Acetonitrile and methanol were of HPLC grade (Burdick & Jackson, Muskegon, USA). Ultrapure water was obtained from a Milli Q-System (Millipore, Bedford, MA, USA). D-mannose (Man) and L-fucose (Fuc) were obtained from Fluka (Germany). L-arabinose (Ara), D-galactose (Gal), D-(+)-lactose（Lac）, D-（+）-galactosamine（GalN）, D-（+）-glucosamine (GlcN), D-glucuronic acid（GlcUA）, D-(+)-galactuionic acid (GalUA) were purchased from Sigma Chemical (St. Louis, Missouri, USA). 1-phenyl-3-methyl-5-pyrazolone（PMP）was from Sinopharm Chemical Reagent Co.,Ltd (Shanghai, China). All other chemicals and reagents were of analytical grade and were commercially available.

2.3 Hydrolysis

Three parallels of glycosaminoglycan extracted from the sea cucumber *Stichipus japonicus* (SJ-GAG, 2 mg for each) were dissolved in 1ml of different acids in the hydrolysis vials, respectively, including 2 M trifluoroacetic acid (TFA), 2 M sulfuric

acid (H_2SO_4), and equally mixed acids of 2 M TFA and 2 M H_2SO_4 [13,15]. The vials were then fulfilled with N_2, sealed and placed at 110 for the required time. Hydrolysates were dried under vacuum and neutralized by 0.3 M sodium hydroxide (NaOH), ready for derivatization.

2.4 Derivatization with PMP

The hydrolyzed glycosaminoglycans were derivatized with 450μl PMP (0.5 M in methanol) and 450μl 0.3 M NaOH at 70 for 30 min [15,16]. After cooled to room temperature, the derivatives were neutralized with 450μl 0.3 M hydrochloric acid (HCl), and extracted three times with 1ml chloroform to remove the excess reagent. The aqueous layer was analyzed directly by HPLC.

2.5 Chromatographic conditions

Analysis of the PMP-sugars was carried out on an Agilent 1100 Series (Palo Alto, CA,USA) liquid chromatograph equipped with an Agilent G1314A VWD. An Agilent ZORBAX Eclipse XDB-C18 column（4.6×150 mm,5 μm）optimized for the separation of PMP-sugars was used. The flow rate was set to 1 ml/min, the temperature was at 25 , and the wavelength for VW detection was 250 nm. The mobile phases were a mixture of buffers A and B, which were 0.05 M potassium dihydrogen phosphate (KH_2PO_4, PH 6.9) with 15 % and 40 % acetonitrile, respectively. A gradient of 8 % to 17 % buffer B in 25 min was used for separation.

2.6 Calculations

Calculation the content of each monosaccharide peptide residue in the test sample according to the formula (1).

$$X_i = \frac{c_i \times V \times 1000}{M \times 1000} \qquad \text{……(1)}$$

Where:

X_i - the residue content of peptide in the test sample, ug/kg; c_i - the peptiede concentration of test sample obtained from the standard working curve, ng/mL; V - the final volume of the sample solution, mL; M – mass of test sample in the final sample solution, g.

All statistical tests were performed by means of the the Statistical Software Package for Windows SPSS, version 10.5 (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1 Hydrolysis of SJ-GAG

Effect of three kinds of acid against time is studied in this part, and the results are shown in Figures 1, 2, and 3. As is shown in Figure.1, all the monosaccharides, except for Gal, were completely released at the 8th hour, since when Fuc, GlcUA suffered great degradation while GlcN, GalN and Man got little changes. Gal achieved its highest content at the 6th hour and degraded clearly after then. Figure.2 shows different changes in the hydrolysis of 2 M H_2SO_4. Fuc and GlcUA degraded greatly since the 8th hour, and Gal degraded at the 10th hour after reaching its highest content. Unlikely, GlcN and GalN showd constent increase in content until the 16th hour, which means the complete release of amino sugars requires harsher condition than the other sugars. Figure. 3 provides the hydrolysis changes of monosaccharides in equally mixed acids of 2 M TFA and 2 M H_2SO_4. Fuc and GlcUA underwent slighter degradation since the 8th hour, and Gal degradated at the 10th hour after reaching its highest content. The release of GlcN and GalN reached a plateau at the 10th hour, which continued for another 4 h.

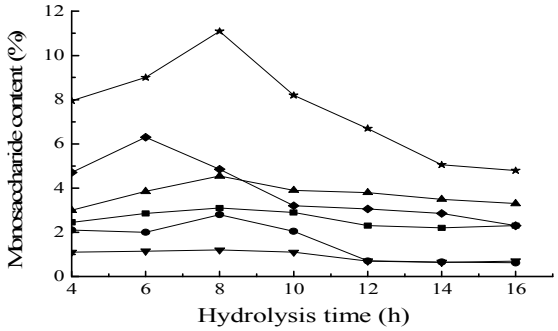

Fig. 1 Hydrolysis time course of SJ-GAG in 2 M TFA
Man (▼), GlcN (■), GlcUA (●), GalN (▲), Gal (◆), Fuc (★)

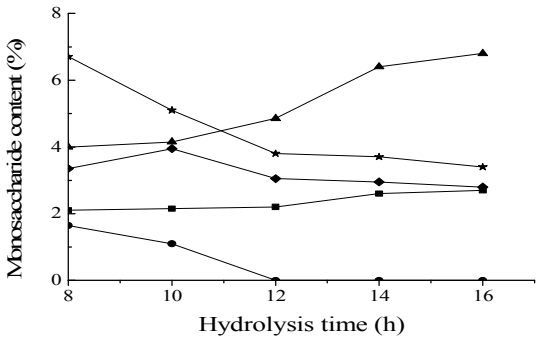

Fig. 2 Hydrolysis time course of SJ-GAG in 2 M H_2SO4
GlcN (■), GlcUA (●), GalN (▲), Gal (◆), Fuc (★)

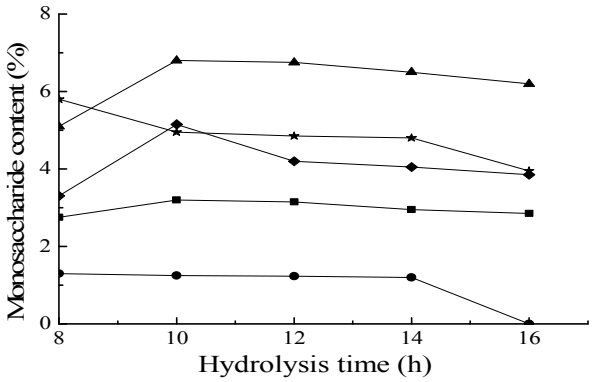

Fig. 3 Hydrolysis time course of SJ-GAG in mixed acids
GlcN (■), GlcUA (●), GalN (▲), Gal (◆), Fuc (★)

3.2 Determination of standard PMP-sugars

A mixture of standard monosaccharides, 100 μl each of Man, GlcN, GlcUA, GalUA, Lac, GalN, Gal, Ara and Fuc, prepared in 2 mM, was derivated with PMP as described in above. The separation of standard PMP-sugars is presented in Figure. 4. The broad reagent peak separates well from all the PMP derivatives, and the peaks of all the neutral, acidic and basic PMP-sugars were sharp and symmetrical. Lac was chosen for the internal standard (IS) for its good separation and retention position.

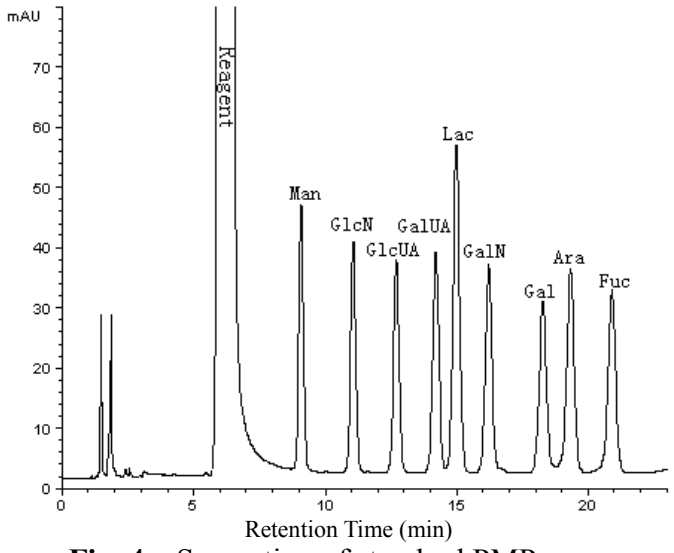

Fig. 4 Separation of standard PMP-sugars

The corresponding calibration curves of the response factor (peak area ratio of the standard PMP-sugars and the internal standard) against concentration (mg/L) generated linear functions (coefficient of determination ≥ 0.996) for all the analytes within a range

from 0.5 mg/l to 10 mg/l. The relative standard deviation (RSD) of the response factors was less than 5.0 % in all cases, ranging from 0.90 %-4.76 %.

Six determinations of a standard solution of all PMP-sugars (5.0 mg/l) were performed using the same reagents and equipments on the same day to evaluate method reproducibility. The RSDs obtained from all determinations indicated less than 5 % (Man = 1.94 %, GlcN = 1.81 %, GlcUA = 4.23 %, GalUA = 2.54 %, GalN = 2.53 %, Gal = 4.65 %, Ara = 2.93 %, Fuc = 4.58 %). These results demonstrated that the method was reproducible for the detection of all PMP-sugars.

The detection limit was calculated from the amount of the standard monosaccharides required to give a 3 folds of signal-to-noise ratio, and was found to be between 47.08 and 61.53 μg/l for all the standard monosacharides. The quantification limit was esTablelished at 10 folds of signal-to-noise ratio, and the result was between 0.47-0.62 mg/l, as seen in Table. 1.

Table 1 Determination of standard PMP-sugars

Standard PMP-sugars	Calibration curves	Coefficient of determination	RSD (%)	Detection Limit (μg/l)	Quantification limit (mg/l)
Man	Y=0.1108x+0.0047	0.9998	2.20	51.25	0.51
GlcN	Y=0.1036x+0.0035	0.9995	2.73	59.44	0.60
GlcUA	Y=0.1142x-0.0284	0.9960	1.53	60.42	0.60
GalUA	Y=0.1211x-0.0068	0.9994	0.90	61.53	0.62
GalN	Y=0.1285x+0.0116	0.9997	2.84	61.11	0.61
Gal	Y=0.1119x+0.008	0.9994	4.34	47.08	0.47
Ara	Y=0.1399x-0.0004	0.9962	4.76	51.53	0.52
Fuc	Y=0.1317x-0.0148	0.9988	3.36	49.72	0.50

3.3 Determination of PMP-sugars in SJ-GAG

Figure.5 displays a separation of PMP-sugars in GAG extracted from sea cucumber *Stichipus japonicus*. Man, GlcN, GlcUA, GalN, Gal and Fuc were detected with sharp and symmetrical peaks. A PMP-sugar separated between PMP-Man and PMP-GlcN which has not been reported before was found, the confirmation of the sugar will be studied in future research.

Recovery was tested by the standard addition procedure using two addition levels (2.67 mg/l and 6.51 mg/l on average) for each monosaccharide, shown in Table.2. Six replicas were carried out for each addition level. The low concentration (level I) recovery was between 86.46-110.58 %, and the RSD (n = 6) was between 2.48 % -8.19 %. The high concentration (level II) recovery was between 85.65- 110.61 %, and

the RSD (n = 6) was between 1.70-7.86 %. The 95% confidence interval of global recovery for all the PMP-sugars is satisfactory except for Ara, which is possibly caused by the uncomplete separation with Gal in the recovery determination.

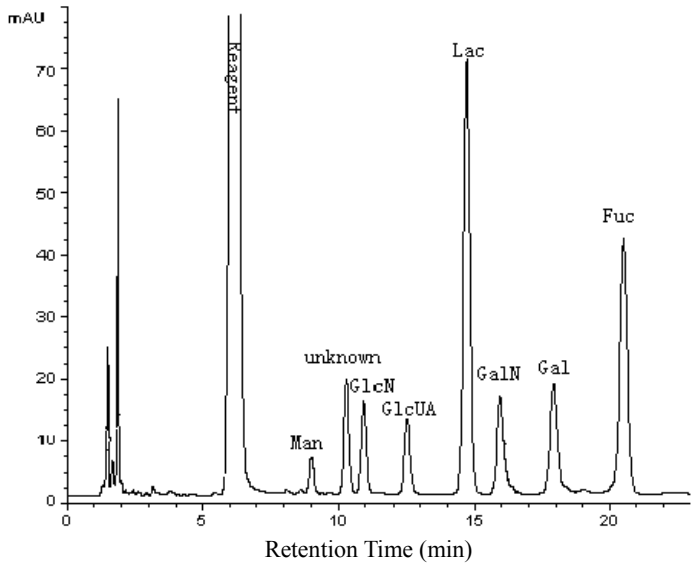

Fig. 5 Separation of PMP-sugars in SJ-GAG

Table 2 Precision and recovery for determination of monosaccharides in SJ-GAG

	Addition level				Addition level				CI95* global recovery (%)
	Initial content (μg)	Content after additon (μg)	RSD (%) n=6	Recovery (%)	Initial content (μg)	Content after additon (μg)	RSD (%) n=6	Recovery (%)	
Man	0.64	8.02	8.19	96.84	0.64	29.52	3.96	102.15	93.6-103.6
GlcN	1.93	10.49	3.78	99.91	1.93	27.61	3.47	101.10	95.4-102.5
GlcUA	1.65	8.81	7.20	86.46	1.65	23.13	2.43	85.65	84.03-92.27
GalUA	nd**	8.86	4.53	109.41	nd	26.58	4.01	97.98	99.3-108.0
GalN	2.91	11.71	2.93	100.97	2.91	29.31	1.69	100.81	98.6-103.2
Gal	2.97	6.78	3.03	98.10	2.97	20.34	1.70	105.48	99.5-104.1
Ara	nd	7.42	7.42	110.58	nd	22.26	7.86	110.61	102.3-118.9
Fuc	8.42	15.58	2.48	102.22	8.42	29.9	2.66	98.34	97.8-102.8

*CI95:confidence interval (95%) of the mean recovery from both addition levels.
**nd: not detected.

Table 3 Determination of PMP-sugars in SJ-GAG

	Neutral sugars			Amino sugars			Uronic Acid
	Man	Gal	Fuc	GlcN	GalN	Total	GlcUA
Content (%)	1.41	5.05	11.18	2.70	3.91	6.61	2.78
Molar ratio	0.21	0.76	1.85	0.41	0.59	1.00	0.39

As seen in Table.3, SJ-GAG consists of three neutral sugars, Man, Gal and Fuc, two amino sugars, GlcN and GalN, and one uronic acid, GlcUA. The molar ratio of the total amino sugar, uronic acid and Fuc is 1.00: 0.39: 1.85, where the uronic acid molar ratio is obviously lower than those presented by Xu et al. [17] and Moura˜o et al. [14]. The possible reason maybe the uncomplete detection of uronic acids due to the lack of the standard sugars. In Figure.5, there is an unknown

peak between Man and GlcN, which is suspected to be the mannuronic acid or guluronic acid according to Strydom DJ. [12]. Further confirmation will be achieved in our future research.

4. Discussion

Accuracy in monosaccharide composition analysis of GAG relies to a large extent on effective hydrolysis. But due to their defferences in hydrolysis rate and in the acid lability, monosaccharides are not easily get complete release in a single hydrolysis condition. The kind and the content of each monosaccharide differ greatly with the hydrolysis conditions. GlcN, GlcUA, GalN, Gal and Fuc were released in all three hydrolysis conditions, while Man was released only in the hydrolysis of 2 M TFA. Each monosaccharide reached the highest content in the hydrolysis of 2 M TFA at the 8^{th} hour, except for Gal, which required the hydrolysis of 2 M TFA at the 6^{th} hour. The universal hydrolysis condition for composition analysis of most glycoprotein samples that Fu et al.[13]. provided is the hydrolysis of 2 h with 4 M TFA at 121 . According to our results, we recommended the hydrolysis of 8 h with 2 M TFA at 110 , which agrees well with the studies of Yang et al.[15], for its mild condition of complete release of the monosaccharides.

Compared with other methods [12,13,15,16], this method provided much superior separation of neutral sugars, amino sugars and uronic acids with a single chromatographic step, and allowed accurate quantitation with Lac as the internal standard. The retention time was shortened half an hour in comparison with the method reported by Fu et al. [13] and the separation of uronic acids was improved over the method presented by DJ Strydom. [12]. However, the separation and quantitation of other common neutral sugars, such as Glc, Xyl, Rha, and N-acetyl-amino sugars has not been achieved. Further study is needed to present a separation of more PMP-sugars.

In conclusion, we have demonstrated in this study that simultaneous separation and determination of neutral sugars, amino sugars and uronic acids in SJ-GAG based on reverse-phase HPLC. This simple and rapid method demonstrated good linearity, precision, recovery, and sensitivity, indicating as a reliable procedure for the analysis of monosaccharide composition in sea cucumber GAGs. According to the principle of derivatization with PMP explained by Honda et al. [18], this method can be applied for the determination of all the reducing sugars, and thus can be conveniently employed in

glycochemistry and glycobiology research.

Acknowledgements

The study was partly Supported by grants from the Natural Science Foundation of China (No. 31071631), and 948 of Chinese Ministry of Agriculture (2011-Z26).

References

1. S Honda, E Akao, S Suzuki, M Okuda, K Kakehi, J Nakamura, High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and electrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives. *J. Anal. Biochem*. (1989) **180**: 351-357.
2. K Kakehi, S Honda, Analysis of carbohydrates in glycoproteins by high-performance liquid chromatography and high-performance capillary electrophoresis. *Appl. Biochem. Biotechnol*. (1993) **43**(1): 55-71.
3. LY Zhang, J Xu, LH Zhang, WB Zhang, YK Zhang, Determination of 1-phenyl- 3-methyl -5-pyrazolone-labeled carbohydrates by liquid chromato-graphy and micellar electrokinetic chromatography. *J Chromatogr B* (2003) **793** (1): 159-165.
4. WJ Zhang, The Biochemical Technology of Carbohydrate MeTableolism. Zhejiang University Press, Hangzhou, 1999. (in Chinese).
5. B Zhou, CM Zhang, CH Zhang, Determination of Tableacco carbohydrate by High-Performance Liquid Chromatography with 1-phenyl- 3-methyl -5-pyrazolone-labeled derivative. *Chinese Journal of Chemical Industry*. (2006) **33**(6): 40-44.
6. RH Angeletti, Techniques in Protein Chemistry III. Academ Press, San Diego, CA.1992.
7. S Honda, T Ueno, K Kakehi, High-performance capillary electrophoresis of unsaturated oligosaccharides derived from glycosaminoglycans by digestion with chondroitinase ABC as 1-phenyl-3-methyl-5-pyrazolone derivatives. *J. Chromatogr. A*. (1992) **608**:289-295.
8. T Yoshiaki, U Hidemitsu, H Kenkoh, Novel fucose-rich glycosaminoglycans from squid ink bearing repeating unit of trisaccharide structure (-6GalNAcα1-3GlcAβ1-3Fucα1-). *J. Biochem Biophys Res Commun.*(1994) **198**(2): 560-567.
9. RM Albano, PA Mourao, Isolation, fractionation, and preliminary characterization of a novel class of sulfated glycans from the tunic of Styela plicata (Chordata Tunicata). *J. Biol. Chem.* (1986) **261**(2): 758-765.
10. ML Ana, RMA Karin, SA Rafael, ALF Silva, L Meis, PAS Mourão, A sulfated polysaccharide from the sarcoplasmic reticulum of sea cucumber. *Glycobiology.* (2000) **10**(8): 773-779.
11. Y Kariya, S WaTablee, M Kyogashima, M Ishihara, T Ishii,Structure of fucose branches in the glycosaminoglycan from the body wall of the sea cucumber *Stichopus japonicus*. *Carbohydrate Research*. (1997) **297**(39): 273-279.
12. DJ Strydom, Chromatographic separation of 1-phenyl-3-methyl-5-pyrazolone derivatized neutral, acidic and basic aldoses. *J. Chromatogr. A*. (1994) **678**(11):17-23.
13. DT Fu, RA O'Neill, Monosaccharide composition analysis of oligosaccharides and glycoproteins by high-performance liquid chromatography. *J. Anal. Biochem.*(1995) **227**(2):377-384.
14. PS Mourão, MS Pereira, MG Pavão, B Mulloy, DM Tollefsen, MC Mowinckel, U Abildgaard, Structure and anticoagulant activity of a fucosylated chondroitin sulfate from echinoderm. *J. Biol. Chem*. (1996) **271** (39): 23973-23984.
15. XB Yang, Y Zhao, HF Wang, An composition analysis of angelica polysaccharide by High-Performance Liquid Chromatography with Post column Derivatization. Chinese *J. Anal. Chem*. (2005) **33**(9):1287-1290.

16. AC Lines, High-performance liquid chromatographic mapping of the oligosaccharides released from the humanized immunoglobulin, CAMPATHTM^{1}H. *Journal of Pharmaceutical Biomedical Analysis*. (1996) **14**:601-608.
17. JD Xu, HZ Fang, Isolation of acid glycosaminoglycan from sea cucumber. *Chinese Journal of Marine Drugs*. (1994)**13**(1):24-26.
18. S Honda, S Suzuki, A Taga, Analysis of carbohydrates as 1- phenyl-3-methyl-5- pyrazolone derivatives by capillary/microchip electrophoresis and capillary electrochro-matography. *J Pharm Biomed Anal.* (2003)**30**(6): 1689-1714.

Academic backgrounds:

Name: Gao Xin

09/1987 – 07/1991	Undergraduate course of Graduate School of Ocean University of Qingdao, Qingdao, China
09/1991 – 03/1996	Researcher of Food Institute of Qingdao, Qingdao, China
04/1996 – 03/1999	Master's course of Graduate School of Tokyo University of Fisheries, Tokyo, Japan
04/1999 – 03/2002	Doctor's course of Graduate School of Tokyo University of Fisheries, Tokyo, Japan
04/2002 – 07/2003	Invite Researcher of Tokyo University of Marine Science and Technology, Tokyo, Japan
08/2003 – 10/2011	Associate Professor of Ocean University of China, Qingdao, China

Research field: Fisheries rheology, Food science and engineering

Name: Zhang Zhaohui

09/1987 – 07/1991	Undergraduate course of Graduate School of Ocean University of Qingdao, Qingdao, China
09/1991 – 07/1994	Master's course of Graduate School of Ocean University of Qingdao, Qingdao, China
09/1995 – 03/2001	Faculty of Ocean University of Qingdao, Qingdao, China
04/2001 – 03/2004	Doctor's course of Graduate School of Tokyo University of Fisheries, Tokyo, Japan
04/2004 – 10/2011	Associate Professor of Ocean University of China, Qingdao, China

Research field: Food science and engineering, Fluid rheology

Tel: 86-532-82031936, Fax: 86-532-85901692, E-mail: zhangzhh@ouc.edu.cn

College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China