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Full Length Research Paper

Hydroxyl radical scavenging activity of peptide from sea cucumber using enzyme complex isolated from the digestive tract of sea cucumber

Sai-Kun Pan, Dong-Rui Yao, Ming-Qian Zhou and Sheng-Jun Wu*

School of Marine Science and Technology, Huaihai Institute of Technology, 59 Cangwu Road, Xinpu, 222005, China.

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In this study, an enzyme complex was derived from the digestive tract of sea cucumber. By using this enzyme complex, sea cucumber protein hydrolysis was carried out to obtain hydrolysates that have hydroxyl-radical-scavenging activity (HRSA). The hydrolytic process was monitored by HRSA and conditions for this process were optimized as follows: pH 6.5, temperature 35°C, 12 mg enzyme complex in a reaction solution (500 ml) containing 5 g sea cucumber (dry matter), and reaction time of 3 h. The protein hydrolysates (PHs) were fractionated into four ranges on the basis of molecular weight (PH_1 MW: >10 kDa; PH_2: 10 to >5 kDa; PH_3: 5 to 1 kDa; and PH_4: <1 kDa) using an ultrafiltration membrane bioreactor system. Results indicate that PHs in the PH_3 induced the highest HRSA.

Key words: Sea cucumber, enzyme complex, hydrolysis, hydroxyl-radical-scavenging activity.

INTRODUCTION

During normal physiological processes, the human body generates small amounts of free radicals of oxygen, such as the hydroxyl (OH^-), superoxide (O_2^-), nitric oxide (NO^-), and lipid peroxyl (LOO^-) radicals. The presence of too many free radicals in the human body will result in their attacking protein, lipid, DNA and other biomolecules, thereby causing damage to the cell structure, and interfering with the body's normal metabolism, all of which result in disease and acceleration of the aging process (Bagchi and Puri, 1998; Devasagayam et al., 2004). Based on a theory that considers aging a function of free radical, in recent years, the studies conducted on active oxygen and free radical research have become leading topics of modern life science research, and screening and evaluation of antioxidants that have high efficiency and low toxicity have become new trends in biology, medicine and food science.

The sea cucumber has been used traditionally as a rejuvenating food source in China and other Asian countries (Fu et al., 2005), and hydrolysates of the sea cucumber have been shown to have antioxidant activity (Chen et al., 2010; Liu et al., 2007; Mamelona et al., 2010; Wang et al., 2010; Zeng et al., 2007). However, a number of proteases have been isolated from the digestive tract of sea cucumber (Féral, 1989; Fu et al., 2005; Shimizu et al., 1994). This bring up a question whether proteases from the digestive tract of sea cucumber can hydrolyze sea cucumber protein to generate peptides that have radical-scavenging activity.

To explore this question further, there was interest to prepare peptides by hydrolysis of the protein of sea cucumber, *Stichopus japonicus*, using an enzyme complex isolated from the organism's digestive tract. In the present study, hydrolytic conditions were optimized for the application of the enzyme complex in production processes, and the hydroxyl-radical-scavenging activity (HRSA) of the product was characterized.

MATERIALS AND METHODS

Live sea cucumbers *S. japonicus* were purchased from a local

*Corresponding author. E-mail: wushengjun008@sina.com. Tel: +86-0518-8589547. Fax: +86-0518-85895427.

Abbreviation: **HRSA**, Hydroxyl-radical-scavenging activity; **PHs**, protein hydrolysates.

market (Lianyungang, Jiangsu, China). All other chemicals are of reagent grade and were used without further purification.

Preparation of enzyme complex

Thirty sea cucumbers were used for this study. The sea cucumbers were killed and dissected immediately thereafter. After removing the tissue contents, the digestive tracts were separated and rinsed with cold distilled water. These digestive tracts were then minced and homogenized in 100 mmol/L cold tris–HCl buffer (pH 6.5) and the homogenate was then centrifuged at 10,000 × *g* for 20 min. The preparation containing the enzyme complex was obtained from the supernatant by freeze-drying and was stored at –80°C.

Determination of proteinase activity in the enzyme complex

The proteinase activity of the enzyme complex was assayed according to the method described by Ilyina et al. (2009), but with slight modifications adjusted on the basis of the amount of acid-soluble hydrolysis products, and using azocasein (5 mg/ml) as substrate in 0.05 mol/L Tris–HCl buffer (pH 6.5) with addition of Ca^{2+} ions at 35°C. One unit of activity was defined as the amount of enzyme (µg) required to increase the D_{440} value per unit per minute under experimental conditions.

Hydrolysis of sea cucumber by the enzyme complex

The body wall of dissected sea cucumber was autoclaved at 121°C for 20 min, cooled to room temperature (about 25°C), minced, and homogenized in 100 mmol/L Tris–HCl buffer (pH 6.5) to prepare a slurry with a concentration of 1% (w/v). To 500 ml of this slurry, 12 mg of the enzyme complex was added, and the mixture was incubated at 35°C for 3 h.

Separation and purification of hydrolysates

The protein hydrolysates (PHs) were centrifuged at 10,000 × *g* for 20 min and the supernatant obtained was fractionated using an ultrafiltration membrane bioreactor system (Millipore, USA) into four ranges of molecular weight as follows: PH_1: >10 kDa; PH_2: 10 to >5 kDa; PH_3: 5 to 1 kDa; PH_4: <1 kDa. The above mentioned different fractions were tested to evaluate their HRSA.

Analytical methods

The pH of the solution was recorded using a digital pH meter (PHS-3C; CD Instruments, China). Hydroxyl radicals were generated by an iron-catalyzed Fenton Haber–Weiss reaction and the hydroxyl radicals generated were rapidly made to react with nitrone spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; Rosen and Rauckman, 1984). The resultant DMPO-OH adducts could be detectable with an electron spin resonance (ESR) spectrometer. The peptide solution (20 µL) was mixed with DMPO (0.3 mol/L, 20 µL), $FeSO_4$ (10 mmol/L, 20 µL) and H_2O_2 (10 mmol/L, 20 µL) in a phosphate buffer solution (pH 7.4), and then transferred into a 100-µL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. Experimental conditions for this procedure were as follows: Magnetic field, 336.5 ± 5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 200; and sweep time, 4 min. HRSA was calculated according to the following equation:

$$HRSA = \frac{1 - H}{H_0} \times 100\%$$

Where, H and H_0 are relative peak height of radical signals with and without sample, respectively (Qian et al., 2008).

RESULTS AND DISCUSSION

Proteinase activity in the enzyme complex

The proteinase activity of the enzyme complex was found to be 14.7 U/g. This activity was determined in the presence of protein; hence, this factor became a pre-requisite for the use of the enzyme complex to hydrolyze sea cucumber protein.

Effect of time on hydrolysis of sea cucumber protein

Hydrolysis of sea cucumber protein by using the enzyme complex was carried out for 8 h. As shown in Figure 1, HRSA showed a sharp increase with time up to 2 h into the reaction, a slower increase over 2 to 3 h, reaching the maximum at 3 h, followed by a sharp decrease over 3 to 5 h, to reach a minimum after 6 h. Therefore, the optimum reaction time was thus ascertained to be 3 h. It is interesting to note that at the beginning of hydrolysis (reaction time: 0 h), the sea cucumber slurry already has some HRSA, indicating that the sea cucumber itself contains some quantities of natural materials that have HRSA.

Effect of pH, temperature and amount of the enzyme complex on hydrolysis of sea cucumber

The pH, temperature of the reaction mixture and the amount of the enzyme complex used can play pivotal roles in the hydrolysis of sea cucumber; therefore, the effect of pH, temperature, and quantity of the enzyme complex on hydrolysis of sea cucumber was investigated. The optimum hydrolytic conditions were found to be pH 6.5 (Figure 2) at a temperature of 35°C (Figure 3), with 12 mg of enzyme complex in the reaction mixture (Figure 4). These findings are in contrast with optimal conditions that were previously reported for enzymatic hydrolysis of sea cucumber protein as pH 7.1 (Chen et al., 2010) and 7.5 (Su et al., 2009) at temperatures of 43 (Chen et al., 2010) and 55°C (Su et al., 2009). These differences of reported optimal pH and temperature may be attributed to differences in substrates, enzyme sources and reaction time. It is worth noting that excessive hydrolysis of sea cucumber decreased HRSA, for example, when too much enzyme complex was added.

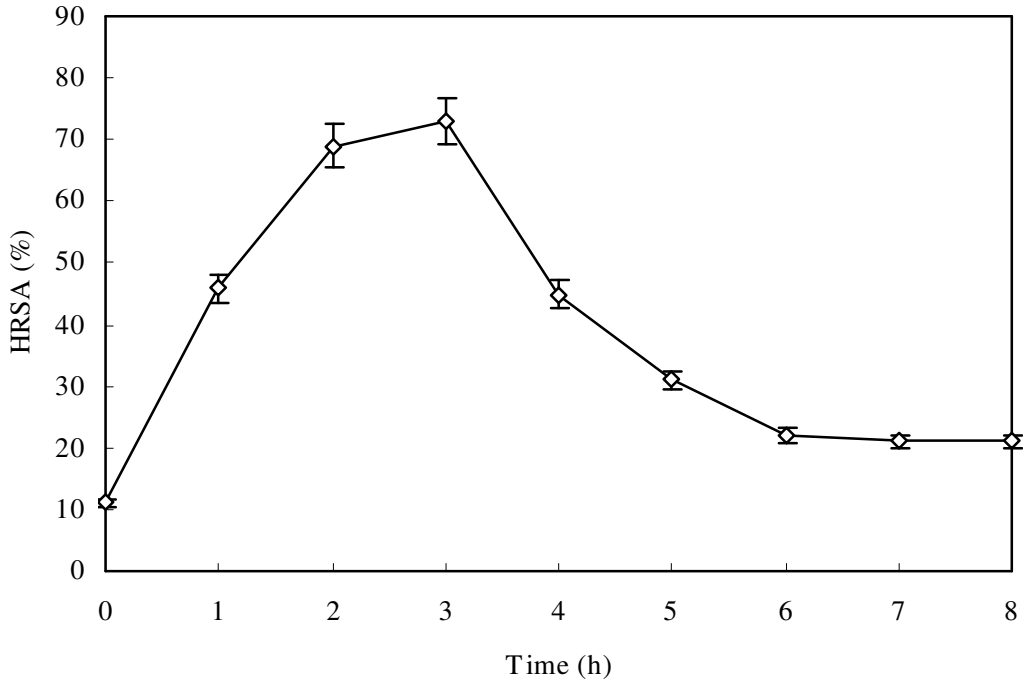

Figure 1. Effect of time on hydrolysis of protein of sea cucumber by the enzyme complex from sea cucumber. Data are shown as mean ± SD (n = 3).

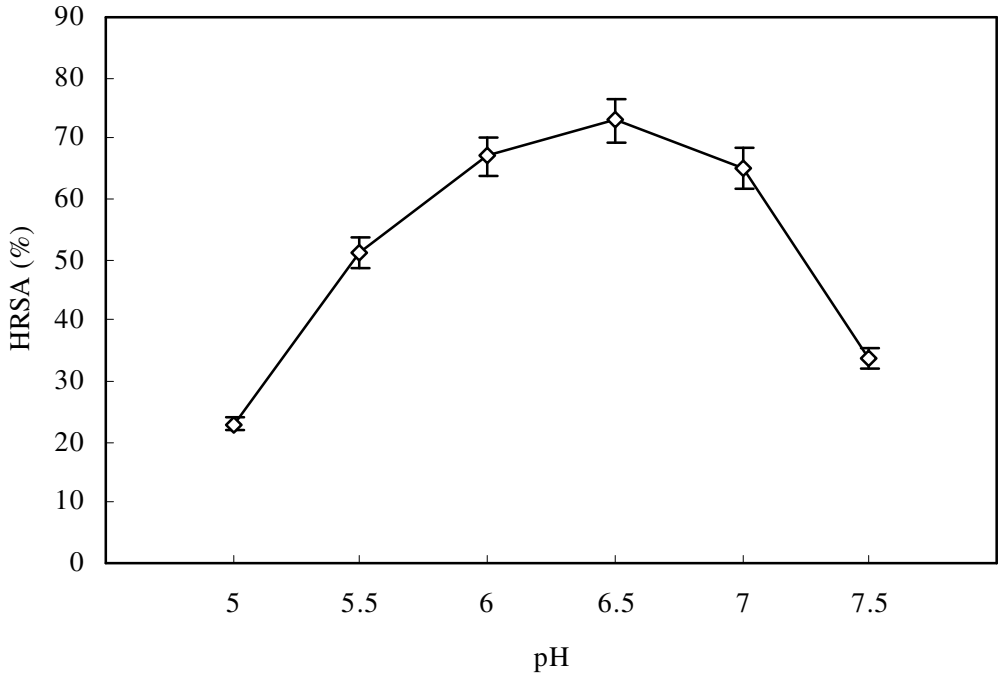

Figure 2. Effect of pH on hydrolysis of protein of sea cucumber by the enzyme complex from sea cucumber. Data are shown as mean ± SD (n = 3).

HRSA of the fractioned hydrolysates

The fractioned hydrolysates showed variable HRSA (data not shown) and the fractions can be classified on the basis of HRSA as: $PH_3 > PH_2 > PH_4 > PH_1$, indicating that peptides with too high or too low molecular weight had

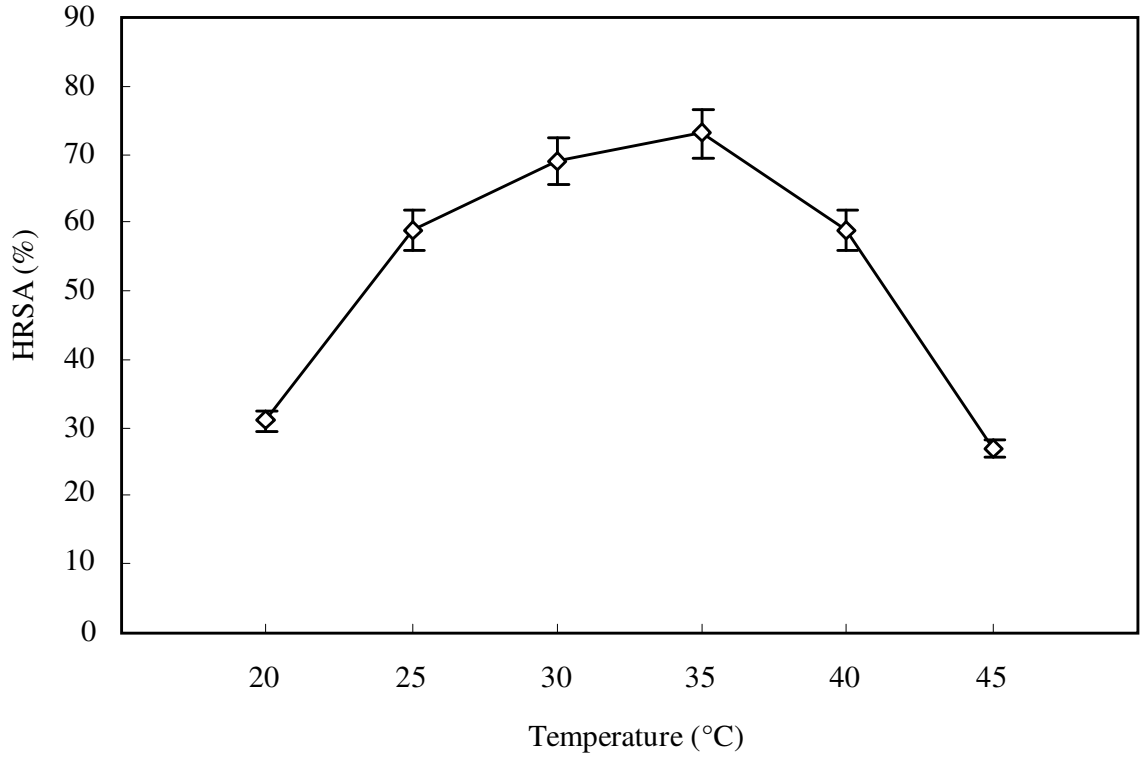

Figure 3. Effect of temperature on hydrolysis of protein of sea cucumber by the enzyme complex from sea cucumber. Data are shown as mean ± SD (n = 3).

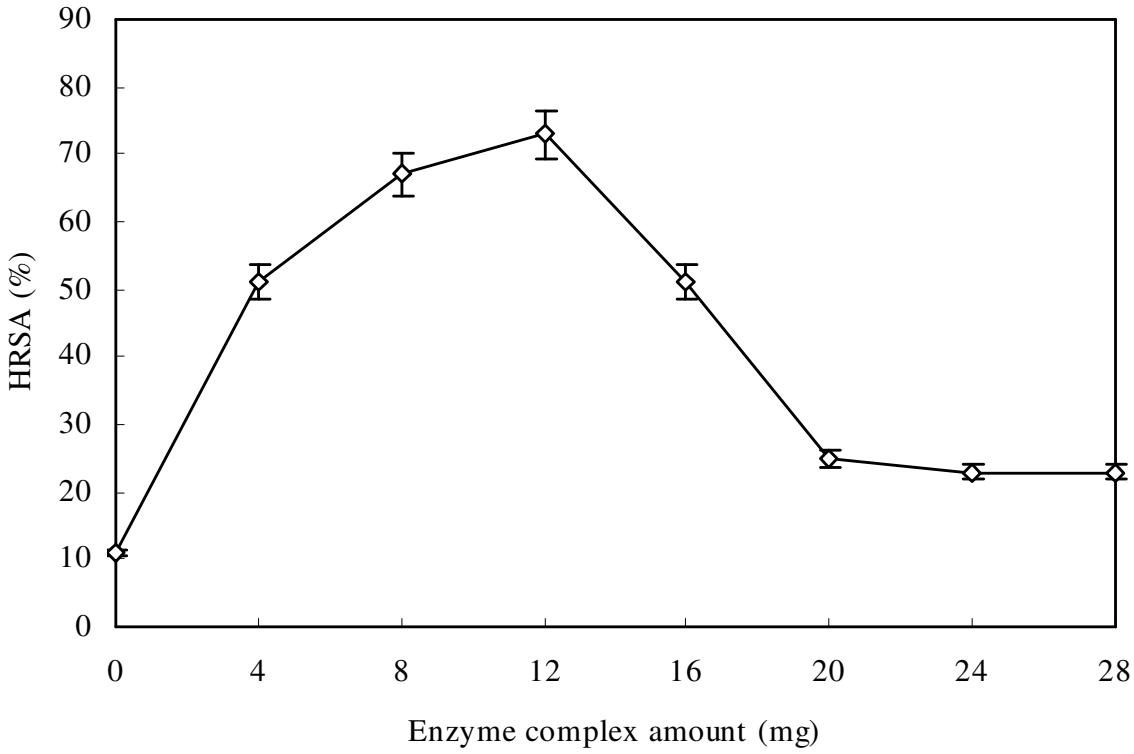

Figure 4. Effect of enzyme amount on hydrolysis of protein of sea cucumber by the enzyme complex from sea cucumber. Data are shown as mean ± SD (n = 3).

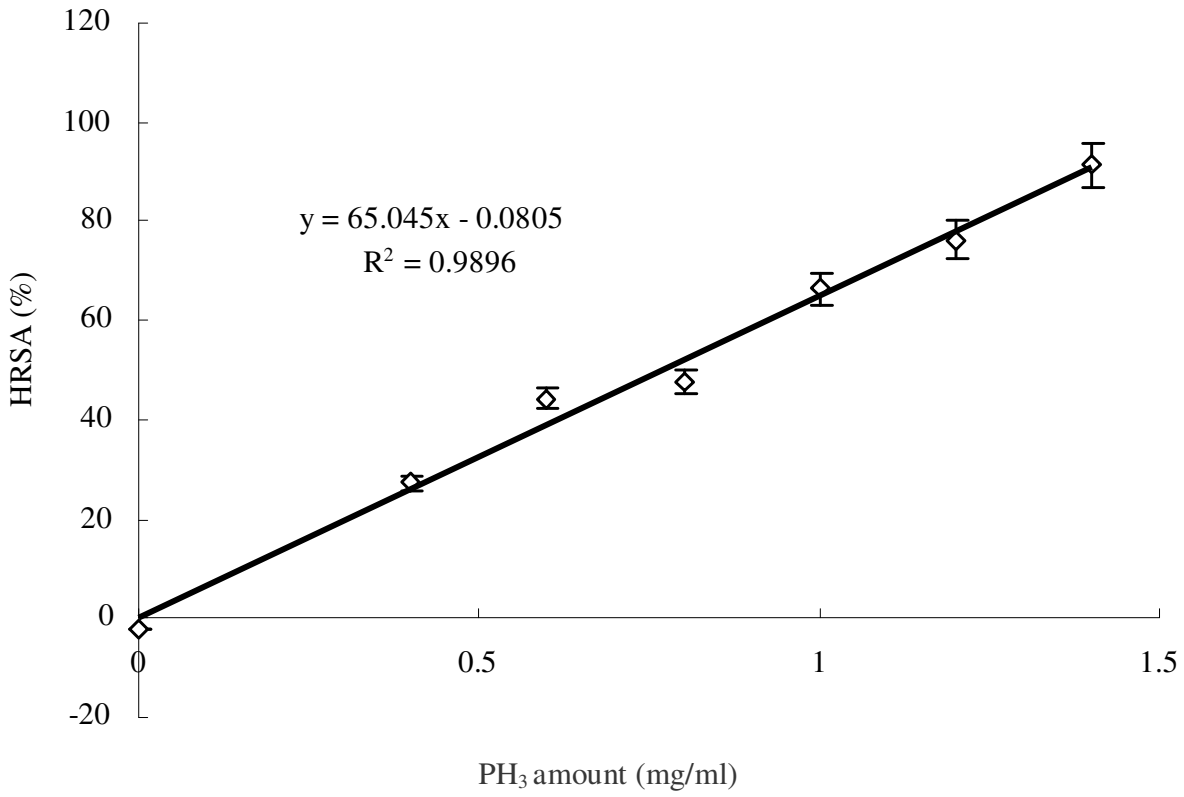

Figure 5. The hydroxyl radical scavenging activity (HRSA) of the fractioned protein hydrolysates (PH_3).

decreased HRSA. The effect of concentration of PH_3 on HRSA is shown in Figure 5. The results were subjected to best-fit linear regression and the coefficients were calculated, producing a fitted equation for predicting HRSA (Y) as follows:

$$Y = 65.045 \times x - 0.0805$$

Where, x is the PH_3 concentration. The regression coefficient was 0.9896 for this reaction. In general, a regression model having an r^2 value >0.9 is considered to indicate a very high correlation (Haaland, 1989). The HRSA of PH_3 reached 91.30% at a concentration of 1.4 mg/ml, thus indicating that PH_3 has a very high HRSA.

Conclusion

The protein of the sea cucumber can be hydrolyzed by the enzyme complex derived from the digestive tract of the sea cucumber itself to produce peptides that have HRSA. The optimum conditions for such hydrolysis were pH 6.5, temperature of 35°C, 12 mg of enzyme complex in a reaction solution (500 ml) containing 5 g sea cucumber (dry matter), and a reaction time of 3 h. Among the four fractioned hydrolysates (PH_1, PH_2, PH_3 and PH_4), PH_3 had the highest HRSA.

ACKNOWLEDGEMENT

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