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Studies on the acid degradation process and *in vitro* immune activity of the polysaccharide H6PC20 in *Hericium erinaceus*

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Author introduction: Tingting Li (1989.09), female, assistant professor, PhD student, and she is mainly engaged in research on active polysaccharides in natural products, and her E-mail is litt@sumhs.edu.cn.

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Abstract: In this paper, the degradation and modification of the macromolecule polysaccharide (H6PC20) extracted from *Hericium erinaceus* was carried out by acid treatment, and a set of *Hericium erinaceus* polysaccharides with proper molecular weight were obtained to improve the biological activities of H6PC20. The effects of citric acid, trifluoroacetic acid, sulfuric acid and hydrochloric acid on the degradation of polysaccharide H6PC20 were compared, and it was found that trifluoroacetic acid was most suitable for degrading the polysaccharide in *Hericium erinaceus.* With the increasing of the degradation time, the proportion of polysaccharides with large molecular weight reduced, and the *in vitro* immune activity decreased, and the DPPH removal capacity increased gradually. With the increasing of trifluoroacetic acid concentration, the molecular weights of the degraded polysaccharides decreased, and the proportion of polysaccharides with large molecular weight reduced. When the acid concentration was 0.1~0.5 M and the molecular weight was 1.57~1.97×10^6 Da, the immune activities of the degraded polysaccharides markedly enhanced and the DPPH removal rate increased gradually compared with both before the degradation of H6PC20.

1 Introduction

Hericium erinaceus, which belongs to the basidiomycota, hericiaceae and hericiaceae genus, is a famous edible and medicinal mushroom [1]. It is natured and sweet, benefitting for internal organs and digest, and it can cure neurasthenia, gastritis, gastric ulcer, etc. [2, 3]. Polysaccharide is the most important active ingredient in *H.erinaceus*, and it has the effects of strengthening immunity, antitumor, reducing glycemia, antioxidation, anti-aging and so on [4]. Because of the unique physical and chemical properties and physiological function, *H.erinaceus* has been widely used in food, medicine, health products, cosmetics and other fields in recent years. *H.erinaceus* tablets, oral liquid of *H.erinaceus*, *H.erinaceus* biscuits and other products have been produced successively, and *Hericium erinaceus* has attracted the attention of more and more researchers.

In the preliminary studies of our research group, a macromolecule β-glucan H6PC20 with *in vitro* immune activity was isolated from *H.erinaceus*. Due to the high viscosity of the polysaccharide, its

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solubility in water was low. Because of its large molecular weight, the polysaccharide was not easy to enter the body cells, and its activity was hindered. The appropriate degradation modification or chemical modification of the polysaccharide could be conducted by physical or chemical method, and polysaccharide derivatives with good solubility and high activity could be prepared, and the development and application of the polysaccharide in food, health care products and drugs would be expanded.

In this paper, the degradation modification of the macromolecule β-glucan in *H.erinaceus* was conducted through the acid hydrolysis technology, and the degradation process was optimized. The molecular weight of the polysaccharide in *H.erinaceus* before and after modification was measured by chemical analysis, spectrum analysis and modern instrument analytical technique. The correlation between structure and activity of polysaccharide was investigated combined with biological activity, and the foundation would be laid for further study of the structure-activity relationship of polysaccharide.

2. Materials and methods

2.1 Source of materials

Polysaccharide H6PC20 with large molecular weight in *H.erinaceus* was used.

2.2 Reagents

Sulphuric acid, hydrochloric acid, trifluoroacetic acid, citric acid, sodium hydroxide, anhydrous ethanol and hydrogen peroxide were all analytical pure.

2.3 Instruments

The main experimental instruments included: the centrifuge (Beckman Corp.), the rotary evaporator (BÜCHI Corp.), the freeze-drying apparatus (Thermo Savant Corp.), the Synergy HT multi-functional enzyme marking instrument (Bio-Tek Corp.).

HPSEC-MALLS-RI combined system was composed by the Waters 2695 HPLC pumps, the gel chromatography columns (TSK PWXL6000 and TSK PWXL3000), the Waters 717 plus automatic sampler, the eight-angle laser light scattering detector with helium-neon laser light source (MALLS, Wyatt Corp.) and the Waters 2414 differential detector (RI), etc.

2.4 Type selection of acid

A certain amount of polysaccharide H6PC20 was dissolved in water to the concentration of 1 g/L. After stirring for 2 h at 60 ˚C, the solution was cooled to room temperature. 10 mL treated H6PC20 solution was put into 20 mL plug seal centrifuge tube, and then the pre-prepared citric acid, hydrochloric acid, sulfuric acid and trifluoroacetic acid solutions with same concentration were added respectively to make the acid concentration reach 0.5 mol/L. The plug seal centrifuge tubes were placed in water bath pot at 100 ˚C, and the tubes were sealed. After hydrolysis, the acid polysaccharide solutions were immediately put in ice bath to cool the solutions, and then the solutions were neutralized using 1 M NaOH solution, and then dialysis (dialysis bags with 3500 Da), centrifugation and freeze drying were conducted to obtain acid hydrolysis products.

2.5 Degrade polysaccharide by trifluoroacetic acid

A certain amount of *H.erinaceus* polysaccharide H6PC20 was weighed and put into plug seal centrifuge tubes respectively. Then add trifluoroacetic acid according to table1 and warm up at 90 ˚C. After reaction for a period of time, the solutions were cooled and then centrifuged to remove precipitations. The liquid supernatants were dialyzed with running water to remove trifluoroacetic acid. Finally, use freeze drying method to obtain the degraded *H. erinaceus* polysaccharides

Table 1. The degradation experiments of *H. erinaceus* polysaccharide with single factor levels

factors	水平
the TFA concentrations /（mol/L）	0.1、0.3、0.5、0.7、1
reaction time /min	30、50、70、90、110

2.6 The molecular weight distribution of polysaccharide analyzed by HPSEC-MALLS-RI

2.6.1 Preparation and treatment of samples. 2 mg samples were weighed and dissolved in 1 mL flow phase. After centrifugation at 12000×g for 10 minutes, the liquid supernatants were taken and filtrated with 0.25 μm aqueous microporous membrane before analyzed by HPSEC-MALLS-RI.

2.6.2 Chromatographic analysis conditions. The analytical columns adopted TSK PWXL3000 and TSK PWXL3000 gel chromatographic columns connected in series. The mobile phase was solutions containing 0.05 mol/L NaH_2PO_4 and 0.15 mol/L $NaNO_3$ (pH=7, with 0.02% sodium azide), and the flow rate was 0.5 mL/min, and the temperature of chromatographic columns-used column oven was kept at 35 ˚C. The light source wavelength of the laser detector was 623.8 nm. The refractive index increment (dn/dc) of the polysaccharide in solutions was adopted at 0.146 mL/g for calculation [5, 6].

2.6.3 Data processing. The Astra data analysis software (version 6.1.1, Wyatt Technology, Santa Barbara, CA) was used to collect and analyse the light scattering data and calculate the molecular weight.

2.7 The removal effect of acid-degraded polysaccharide on DPPH free radical

2.5 mg degraded polysaccharide samples were taken and dissolved in 1 mL ultra-pure water to obtain the water solution with a concentration of 2.5 mg /mL, and the anti-oxidative activity was determined according to the method in the literature [7]. 1 mL prepared polysaccharide solution was taken, and 3 mL ready-prepared DPPH ethanol solution was added, and the obtained solution was acted as reaction group, which was denoted as A_1. Similarly, 1 mL 50% ethanol solution was added in the tube, and then 3 mL DPPH ethanol solution was added, and the obtained solution was acted as blank control group, which was denoted as A_0. 1 mL sample was taken and 3 mL anhydrous ethanol was added, and the obtained solution was denoted as A_2. After shaking well, all the samples were placed at dark place for 30 min at room temperature, and the absorbance of solutions at the wavelength of 517 nm were determined, respectively. The removal rate of DPPH was calculated according to the following formula:

The removal rate of DPPH by polysaccharide %=100% × $(1-(A_1-A_2)/A_0)$

Where: A_0 was the absorbance of solutions composed by 1 mL anhydrous ethanol and 3 mL DPPH solution at 517 nm; A_1 was the absorbance of solutions composed by 1 mL sample solution and 3 mL DPPH solution at 517 nm; A_2 was the absorbance of solutions composed by 1 mL sample solution and 3 mL anhydrous ethanol at 517 nm.

According to the above formula for calculating the removal rate of DPPH, the higher the calculated value, the higher the removal rate of DPPH and the stronger the anti-oxidative capacity.

2.8 In vitro immune activity of H.erinaceus polysaccharides with different molecular weight

5 mg polysaccharide components with different molecular weight were weighed respectively, and were dissolved in 1 mL PBS. After centrifugation at 15000×g for 30 minutes, the liquid supernatants were taken and transferred to another centrifuge tube. Then, a certain amount of solution was diluted into the concentration of 200 μg/mL and 50 μg/mL, respectively. With the 1μg/mL LPS as the positive control and PBS as the negative control, the production of NO released by macrophage under the stimulation of *H. erinaceus* polysaccharide samples was measured [8].

3. Results and analysis

3.1 Type selection of acid

Because of the weak acidity of citric acid, H6PC20 could only be degraded to polysaccharides with the molecular weight of 1.227×10^6 Da. The polysaccharide-degradation effect of citric acid was not obvious, and the subsequent treatment was complicated and the dialysis time was long. After degrading polysaccharide by sulfuric acid and hydrochloric acid, many degraded components were obtained and hard to separated from each other for their adjacent molecular weight. Trifluoroacetic acid could degrade H6PC20 to polysaccharides with the molecular weight of 7.016×10^5 Da, and the composition was single, so the polysaccharide-degradation effect of trifluoroacetic acid was optimized according to the degradation effect. The H6PC20-degradation effect of citric acid, trifluoroacetic acid, hydrochloric acid and sulfuric acid were compared in figure 1a, 1b, 1c and 1d.

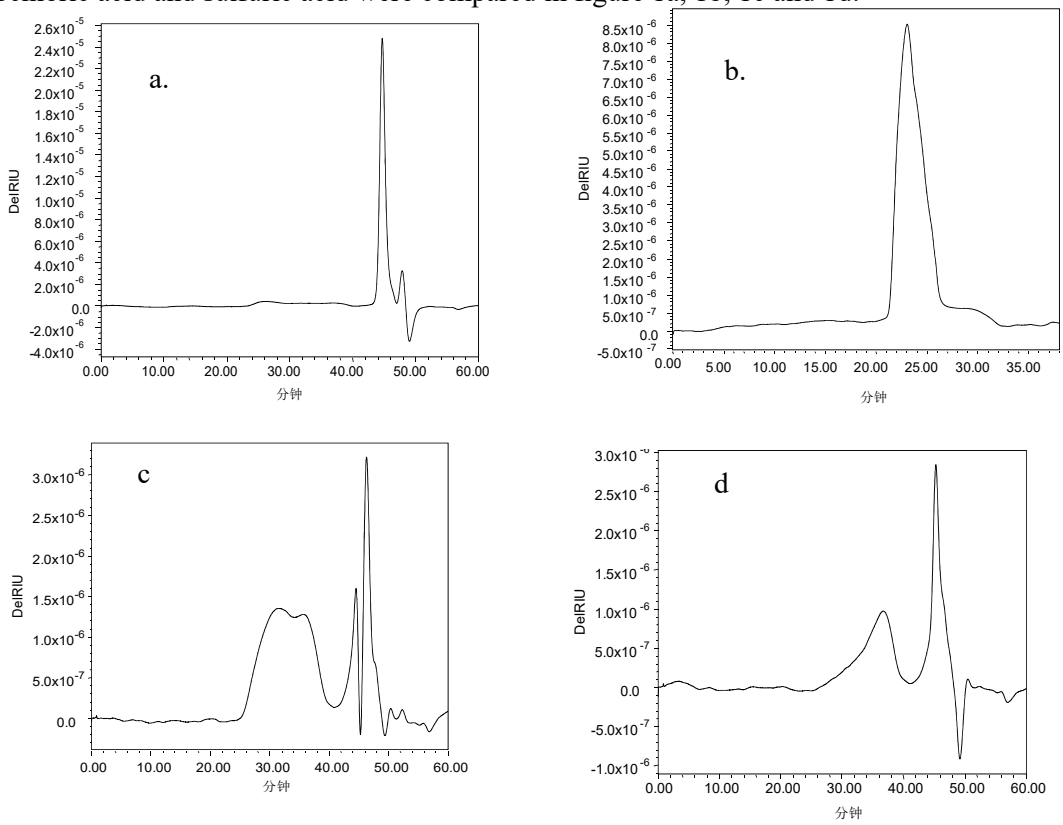

Figure 1. (a) The liquid phase diagram of H6PC20 degraded by citric acid; (b) The liquid phase diagram of H6PC20 degraded by trifluoroacetic acid; (c) The liquid phase diagram of H6PC20 degraded by citric acid; (d) The liquid phase diagram of H6PC20 degraded by sulfuric acid.

3.2 Degrade polysaccharide by trifluoroacetic acid

3.2.1 Effect of degradation time on the molecular weight of polysaccharides. Under the conditions that the concentration of trifluoroacetic acid was 0.5 mol/L and the reaction temperature was 90 ˚C, the relative molecular mass of the obtained polysaccharide fragments after reaction for different time were shown in table 2.1 and figure 2.1. It can be seen from figure 2.1 that, with the increasing of reaction time, the molecular weight of polysaccharides reduced and dropped rapidly in 50~90 min. Then, with the further increasing of reaction time, the change of molecular weight was small, and the reduction of relative molecular mass was slow in 90~110 min. There was certain degradation effect in 90 min.

Table 2.1. The effect of degradation time on the degraded effect of polysaccharides.

Reaction time (min)	molecular weigh（Da）	area ratio
30min	1.65×10^6	1
50min	1.54×10^6	0.92
70min	1.27×10^6	0.53
90min	9.10×10^5	0.34
110min	8.80×10^5	0.26

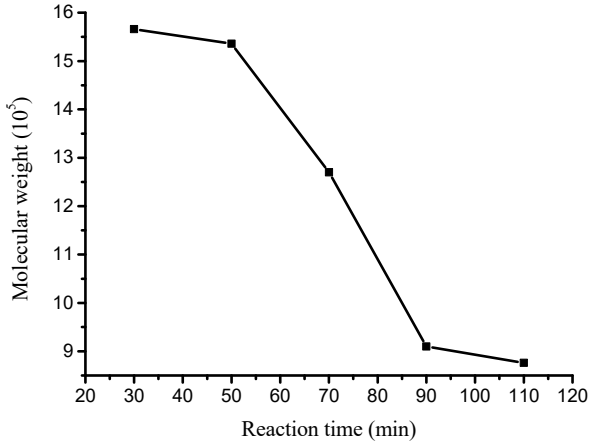

Figure 2.1. The relation between the relative molecular mass of H6PC20 and time.

3.2.2 Effect of degradation time on immune activity. Under the conditions that the acid concentration was 0.5 mol/L and the reaction temperature was 90 ˚C, the NO-releasing effect of RAW264.7 macrophage under the stimulation of degraded polysaccharide molecular fragments at different degradation time was compared in figure 2.2. The results showed that after degraded by trifluoroacetic acid for 30 min and 50 min, the obtained molecular fragments could stimulate the RAW264.7 macrophage to release more NO than the control group. It indicated that the macromolecule polysaccharide in *H.erinaceus* could be degraded into molecular fragments with high activity by trifluoroacetic acid in 50 min, and the phagocytosis of macrophage could be enhanced. However, when the reaction time increased to more than 70 min, the activity of the degraded molecular fragments reduced. According to the molecular weight distribution, it was found that the peak area of macromolecular polysaccharides was relatively large and its relative content was high in the initial stage of degradation. However, as the degradation time was prolonged, the proportion of macromolecular polysaccharide in degraded polysaccharide fragments in the later period was low, which further indicated that the immune activity of polysaccharide in *H.erinaceus* was mainly donated by the macromolecular polysaccharide component.

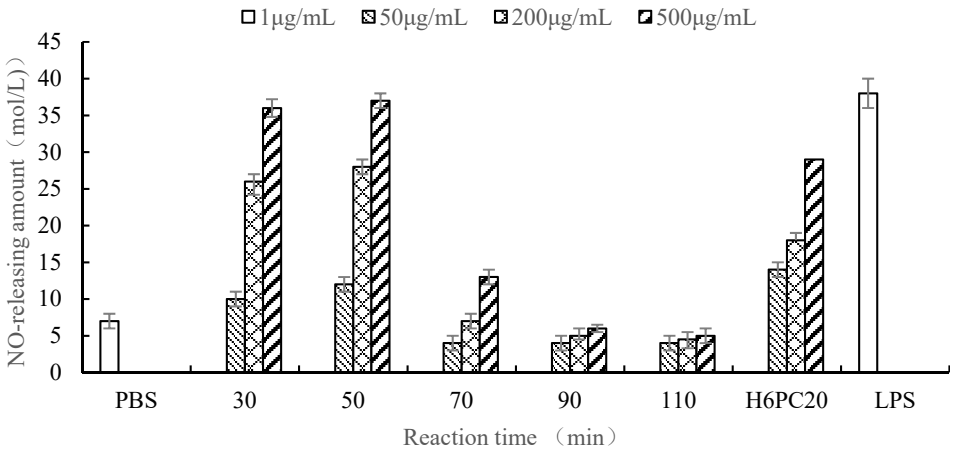

Figure 2.2. The NO-releasing amount of macrophage under the stimulation of degraded molecular fragments obtained through degrading H6PC20 by trifluoroacetic acid.

3.3 Effect of degradation time on antioxidative activity

Under the conditions that the acid concentration was 0.5 mol/L and the reaction temperature was 90 ˚C, the DPPH free radical removal rates of the degraded polysaccharide molecular fragments after degradation for different time were shown in figure 2.3. It can be seen from the figure that with the extension of trifluoroacetic acid reaction time, the DPPH removal rates of degraded polysaccharide fragments increased. When the reaction time was 70-110 min, the increasing rate of removal rates was obviously larger than that in the initial reaction stage, which indicated that the degraded molecular fragments with antioxidative activity increased with the increasing of time. This result was opposite to the *in vitro* immune activity result. It indicated that with the increasing of trifluoroacetic acid degradation time, the proportion of the degraded polysaccharides with small molecular weight or oligosaccharide increased, but the proportion of polysaccharides with large molecular weight decreased. As the polysaccharides with immune activity usually had a relatively large molecular weight ($>1\times10^5$ Da) and the polysaccharide with small molecular weight had a better antioxidative activity, the results showed that the activity of polysaccharide was significantly correlated with its molecular weight.

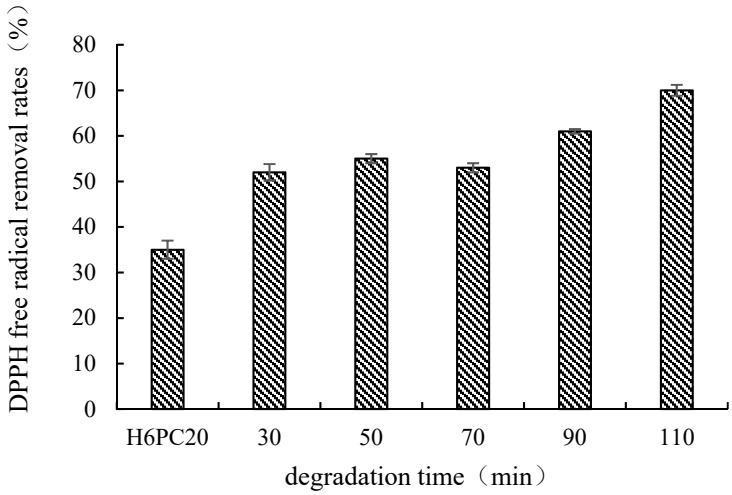

Figure 2.3. The effect of trifluoroacetic acid degradation time on the DPPH free radical removal rates.

3.4 Effect of trifluoroacetic acid concentration on the molecular weight of polysaccharides

Under the conditions that the reaction temperature was 90 ˚C and the reaction time was 50 min, the relative molecular mass of the degraded polysaccharide molecular fragments at different acid concentrations were shown in table 2.2 and figure 2.4. When the acid concentration increased to 1 mol/L, the molecular weight was degraded to 6.66×10^5 Da. The molecular weight of macromolecular polysaccharide reduced by an order of magnitude when the acid concentration was 0.7~1.0 M, and the degradation effect was good. As the main detected polysaccharide fragments had a small peak area after degraded by 1 mol/L trifluoroacetic acid, and the other degraded molecular fragments could not be detected under the given HPLC analysis condition for their low molecular weight, it was not advisable to increase the acid concentration from the point of yield rate of the degraded polysaccharide.

Table 2.2. Influence of trifluoroacetic acid concentrations on the degradation effect of polysaccharide.

acid concentrations (mol/L)	molecular weight (Da)	area ratio
0.1	1.97×10^6	1
0.3	1.62×10^6	0.84
0.5	1.57×10^6	0.61
0.7	1.43×10^6	0.27
1	6.66×10^5	0.18

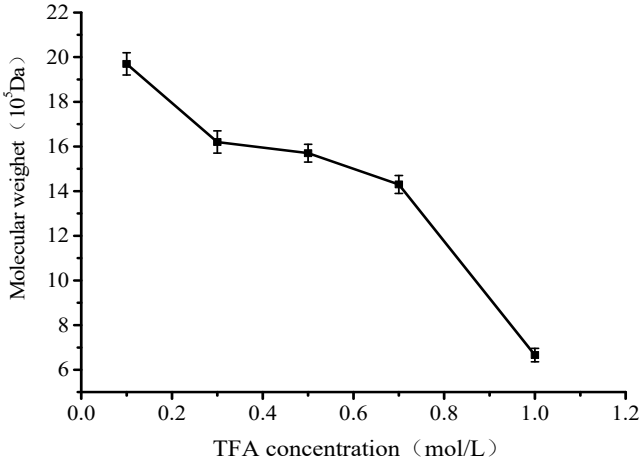

Figure 2.4. The relation between the molecular weight of H6PC20 and time.

3.5 Effect of trifluoroacetic acid concentrations on the immune activity of degraded polysaccharide molecule fragments.

The reaction temperature was set at 90 ˚C and the reaction time was 50 min. The NO amounts released by macrophage under the stimulation of degraded polysaccharide molecule fragments at different acid concentrations were shown in figure 2.5. Compared with the control group, the polysaccharide molecule fragments degraded by 0.1 M, 0.3 M and 0.5 M TFA significantly enhanced the phagocytosis of macrophage. However, with the increasing TFA concentration, the relative molecular mass of obtained polysaccharide molecule fragments decreased and the NO release amounts reduced significantly. The results showed when the concentration of TFA was 0.1 M, 0.3 M and 0.5 M, the obtained polysaccharide molecular fragments could stimulate RAW264.7 macrophages to release more NO than the control group. It indicated that 0.1~0.5 M TFA could degrade the macromolecular polysaccharide in *H.erinaceus* into molecular fragments with higher activites, and the phagocytosis of macrophage would be enhanced. However, with the increasing of acid concentration, the activities of degraded molecular fragments decreased. Combined with the molecular weight distribution, it can be found that the peak area of macromolecular polysaccharide was relatively large and its content was high when the acid concentration was relatively low. With the increasing trifluoroacetic acid concentration, the proportion of the degrade polysaccharide fragments with large molecular weight reduced. It was a further sign that the immune activity of polysaccharide in *H.erinaceus* was mainly donated by the macromolecular polysaccharide components.

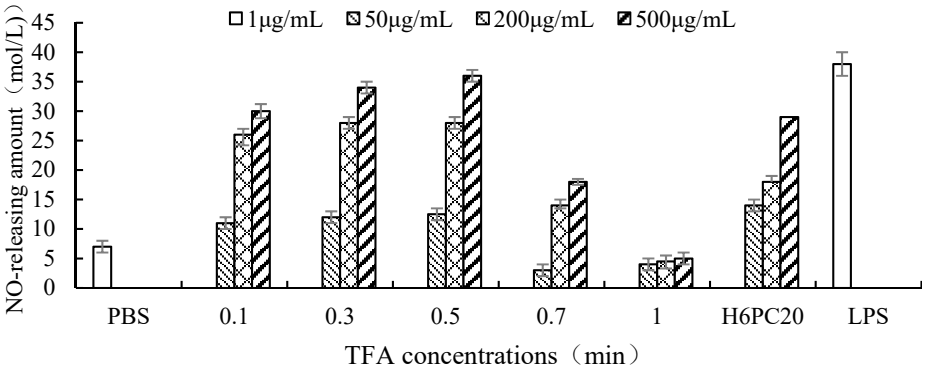

Figure 2.5. The NO amounts released by macrophage under the stimulation of molecule fragments ob tained through degrading H6PC20 by trifluoroacetic acid.

3.6 Effect of trifluoroacetic acid concentrations on the antioxidative activity of degraded polysaccharide molecule fragments

Under the conditions that the reaction temperature was 90 ˚C and the reaction time was 50 min, the DPPH removal rates of the polysaccharide molecular fragments degraded by trifluoroacetic acid with different concentrations were shown in figure 2.6. The DPPH removal rates increased with the increasing of the trifluoroacetic acid concentration, which indicated that the degraded molecular fragments with antioxidative activity increased with the increasing of acid concentration. This result was opposite to the *in vitro* immune activity results. It indicated that with the increasing of trifluoroacetic acid degradation time, the proportion of the degraded polysaccharide with small molecular weight or oligosaccharide increased, and the proportion of polysaccharide with large molecular weight decreased. As the polysaccharide with immune activity usually had a relatively large molecular weight (>1×10^5 Da), and the polysaccharide with small molecular weight had a better antioxidative activity, the results showed that the activity of polysaccharide was significantly correlated with its molecular weight.

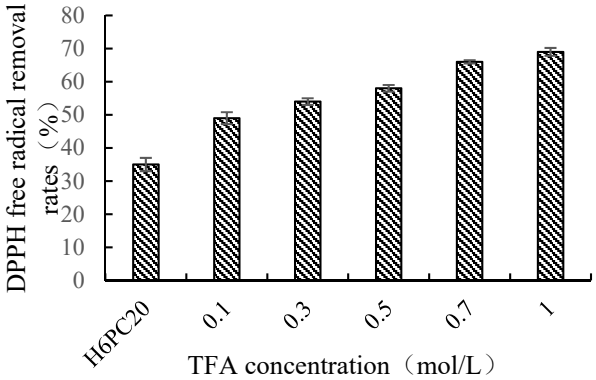

Figure 2.6. The effect of trifluoroacetic acid concentration on the DPPH removal rates of H6PC20.

4. Discussions

Citric acid, hydrochloric acid, sulfuric acid and trifluoroacetic acid (TFA) were adopted to select the type of acid. Among these acids, the acid of citric acid was very weak, and H6PC20 could only be degraded to polysaccharide with the molecular weight of 1.227×10^6 Da by citric acid. The polysaccharide-degradation effect of citric acid was not obvious, and the subsequent treatment was complicated and the dialysis time was long. After degrading polysaccharides by sulfuric acid and

hydrochloric acid, many components were obtained and hard to be separated from each other for their adjacent molecular weights. Trifluoroacetic acid could degrade H6PC20 to polysaccharide with the molecular weight of 7.016×10^5 Da, and the composition was single, so the polysaccharide-degradation effect of trifluoroacetic acid was optimized according to the degradation effects.

When studying the H6PC20-degradation effect of trifluoroacetic acid, it was found that with the increasing of degradation time, the molecular weight of degraded polysaccharides reduced, and the proportion of the polysaccharide with large molecular weight reduced, and the *in vitro* immune activity reduced, and the DPPH removal capacity increased gradually. With the increasing of trifluoroacetic acid concentration, the molecular weight of degraded polysaccharide decreased gradually, and the proportion of the polysaccharides with large molecular weight reduced. When the acid concentration was 0.1~0.5 M, and the degraded polysaccharide had a relative molecular mass in the range of $1.57\sim1.97\times10^6$ Da, and its immune activity was markedly enhanced and was stronger than that of H6PC20 before degradation, and the DPPH removal rates was gradually enhanced. It was indicated that with the increasing of degradation time and trifluoroacetic acid degradation time, the proportion of the degraded polysaccharide with small molecular weight or oligosaccharide increased, but the proportion of polysaccharide with large molecular weight decreased. As the polysaccharide with immune activity usually had a relatively large molecular weight ($>1\times10^5$ Da) and the polysaccharide with small molecular weight had a better antioxidative activity, the results showed that the activity of polysaccharide was significantly correlated with its molecular weight.

In general, polysaccharide structures have certain relationships between biological activities for edible fungi [9,10,11]. The correlation between polysaccharide molecular weights and *in vitro* biological activities were investigated, and the foundation would be laid for further application of *H.erinaceus*.

Acknowledgements

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