Extraction, Separation and Purification of Acidic Polysaccharide from *Morchella esculenta* by High Voltage Pulsed Electric Field

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Abstract: *The intracellular acidic polysaccharide from Morchella esculenta mycelium produced by submerged fermentation is extracted and separated. In the process of extracting the polysaccharide by the high voltage pulsed electric field (HVPEF), heat is not generated and the structure of the polysaccharide is not destroyed. The response surface method is used to investigate the electric field intensity, the pulse number and the liquid-to-material ratio. The optimum extraction process of the acidic polysaccharide is obtained as follows: the electric field intensity is 18 kV/cm, the pulse number is 7 and the liquid-to-material ratio is 27 mL/g. At this time, the polysaccharide yield reaches a maximum value of 56.03 μg/mL. The crude polysaccharide is separated and purified by using DEAE-52 cellulose ion exchange column and Sephadex G-100 to obtain an acidic polysaccharide F1 with high purity. The molecular weight is 1.9895×10^5 Da. Its functional and structural characteristics need to be further studied.*

Keywords: *Morchella esculenta, Intracellular polysaccharide, High voltage pulsed electric field, Extraction, Separation and purification.*

Introduction

Morchella esculenta is a rare edible and medicinal fungus, which is one of the most precious edible fungi in the world. Its fruiting bodies and mycelium contain rich nutrients, mainly including *Morchella esculenta* polysaccharides, amino acids, enzymes [2, 8] and fatty compounds. *Morchella esculenta* polysaccharide is one of the main active components of *Morchella esculenta*. Modern medical and pharmacological studies have shown that it has many pharmacological activities such as reducing blood fat, anti-tumor, antibiosis, anti-fatigue and enhancing immunity [1, 3-5, 9]. Song et al. [7] ferment *Morchella esculenta* mycelium with malt juice and soybean juice to make health-care functional beverage, which is extracted and concentrated to prepare nutritional agent suitable for patients with diabetes and hypercholesterolemia, and developed anti-cancer and anti-aging nutrient solution.

At present, *Morchella esculenta* has been tried to cultivate at home and abroad, but obtains little effect [6], and it cannot be cultivated in a large scale. The extraction technology of polysaccharide usually adopts alcohol precipitation method. However, in order to obtain higher extraction rate and extraction efficiency, many scholars have studied ultrasonic, microwave and other auxiliary extraction techniques in recent years. However, these methods will lead to the destruction of the chemical structure of polysaccharides to a certain extent, which is not conducive to the study of polysaccharides. The cold treatment method has been gradually introduced for extraction of polysaccharide, among which the advantages of high voltage pulsed electric field (HVPEF) make it the best choice for extraction of active substance. The energy

loss of HVPEF treatment caused by heating is very low [1], which is very suitable for the extraction of active polysaccharides.

HVPEF is mainly adopted to extract the intracellular polysaccharide produced by submerged fermentation of *Morchella esculenta* mycelium, and the main polysaccharides of *Morchella esculenta* are separated and purified by ion exchange resin and molecular sieve gel chromatography so as to obtain a single *Morchella esculenta* polysaccharide. In this way, the molecular structure and efficacy of *Morchella esculenta* polysaccharide can be further studied, which lays a foundation for further study of *Morchella esculenta* polysaccharide and promote the research of *Morchella esculenta* polysaccharide.

Materials and methods

Materials and equipment

Test material

Morchella esculenta: purchased from the Research Institute of Edible Fungi, Mianyang, Sichuan.

Test equipment

HVPEF: Self-made, frequency is 10-5,000 Hz.

Test methods

Activation of culture

Medium: potato – 20 g, glucose – 2 g, KH_2PO_4 – 0.3 g, $MgSO_4$ – 0.15 g, agar powder – 1.5 g, adding distilled water to 100 mL, sterilize at 121 ℃ for 30 min.

In the aseptic condition, *Morchella esculenta* is inoculated on the activation medium, and cultured in a constant temperature incubator at 25 ℃ for 2-3 d in the absence of light.

Expanding culture

Medium: potato – 40 g, sucrose – 12 g, peptone – 0.4 g, yeast extract – 2 g, KH_2PO_4 – 0.2 g, $MgSO_4$ – 0.2 g, adding distilled water to 400 mL, sterilize at 121 ℃ for 30 min. Stand by after natural cooling.

The activated *Morchella esculenta* is inoculated in a 500 mL triangular flask filled with 300 mL liquid medium, and cultured in a shake flask with a revolving speed of 100 r/min at 26 ℃ for 3-5 days.

Extraction by HVPEF

Freeze-dry the cultured mycelium in a lyophilizer for 24 h, pulverize the freeze-dried culture with an ultra-fine pulverizer, adding a certain amount of water, homogenizing the mycelium, treating with HVPEF, and centrifuging the supernatant.

Single factor test and response surface method are used to investigate the influence of HVPEF intensity, pulse number and liquid-to-material ratio on the crude yield of *Morchella esculenta* polysaccharide. 30 g of *Morchella esculenta* powder is used to prepare extract solution at the liquid-to-material ratio of 10, 20, 30, 40 and 50 mL/g. Under the electric field intensity of 15, 20, 25, 30, 35 and 40 kV/cm and pulse number of 2, 4, 6, 8 and 10, the single factor test is carried out. The extract solution is centrifuged at 4 000 r/min for 15 min, and the supernatant is taken to determine the polysaccharide content.

The parameters of HVPEF are calculated according to Eqs. (1)-(2).

$$C = 2 \times \frac{\pi r^2 l}{Q} \times f, \quad (1)$$

$$E = \frac{V_{pp}}{2l}, \quad (2)$$

where, C is the pulse number; Q – sample flow rate, mL/min; r – electrode radius, 0.5 mm; l – electrode length, 1.5 mm; f – number of frequencies, Hz; E – electric field intensity, kV/cm; V_{pp} – input voltage, kV.

Purification of polysaccharide

After freeze-drying, the crude *Morchella esculenta* polysaccharide is deproteinized by Savege. The polysaccharide is further purified by DEAE-cellulose ion exchange column and Sephadex G-100 chromatography.

(1) DEAE-cellulose ion exchange column chromatography

Weighing 30 mg of crude polysaccharide, adding 5 mL of distilled water to prepare polysaccharide solution, centrifuging at high speed to take the supernatant, slowly injecting into the equilibrated ion exchange chromatography column containing DEAE-52 cellulose, sequentially using 0, 0.1, 0.2 and 0.3 mol/L NaCl solution for elution, controlling flow rate of 0.4 mL/min, collecting by automatic collector, 10 min/tube.

Column chromatography is followed by treatment with 0.5 mol/L NaOH solution for 2 h and then washing to neutral with distilled water.

(2) Sephadex G-100 chromatography

Accurately weighing 15 g of Sephadex G-100. After pretreatment, the column is packed after vacuum degassing. The column size is Φ2.6×60 cm. After equilibration, 30 mg of Morchella esculenta polysaccharide of each component collected by DEAE-cellullose column chromatography is dissolved in 0.15 M NaCl solution and centrifuged at 12000 r/min for 15 min. After centrifugation, the supernatant is added to the equilibrated Sephadex G-100 column. 4 column volume and 0.15 M NaCl solution are used for elution. The flow rate is controlled between 1 mL/min and 2 mL/min. In the method, a tube of eluent is collected every 4 min, and the absorbance value of each tube is determined by anthrone-sulfuric acid colorimetry to determine the polysaccharide content. The elution tube number is taken as the abscissa and the absorbance value of each tube is taken as the ordinate to draw the elution curve of acidic polysaccharide. The eluent of each tube at absorption peak in the curve is collected. After dialysis desalting, it is processed for concentration, alcohol precipitation, freeze drying, and collection for the next step detection.

(3) Determination of molecular weight

Accurately weighing proper amount of pure polysaccharide, adding purified water to dissolve, and filtering 20 μL sample through 0.45 μm microporous filter membrane. TSK-GEL G4000 PW_{XL} (7.8×300 mm) is used for elution of sample at 45 ℃ and column pressure of 2.0 MPa with the flow rate of 0.1 mL/min to detect the difference of the sample. Standard curve (molecular weights: 2500, 21400, 41100, 84400 and 133800 Da, respectively) is made by using standard molecular weight polysaccharides of different molecular weights.

Detection of polysaccharide concentration

The concentration of *Morchella esculenta* polysaccharide in solution is determined by glucose standard curve method. Accurately taking 0.1 mg/mL of the glucose standard solution of 0, 0.05, 0.10, 0.20, 0.30, 0.40, 0.60 and 0.80 mL in a colorimetric tube, and adding distilled water to 1.0 mL. Putting the colorimetric tube into cold water, adding 3.0 mL of sulfuric acid-anthrone reagent [10] along the wall, shaking it quickly, and boiling the colorimetric tube in a water bath at 100 ℃ for 10 min. Immediately putting it into flowing cool water after removal for 10 min, and then equilibrating at room temperature for 10 min. The cuvette containing the sample to be measured is put into an ultraviolet-visible spectrophotometer, and the absorbance is measured at a wavelength of 620 nm by using distilled water as a blank sample. Each group is subjected to 3 parallel tests, and the data obtained is the average value of 3 tests. The standard curve of glucose is drawn with glucose content as abscissa and absorbance as ordinate.

The elution curve of *Morchella esculenta* polysaccharide is drawn by taking the number of elution tubes of *Morchella esculenta* polysaccharide as abscissa and absorbance as ordinate, and the eluent at the elution peak of the curve is collected, concentrated, alcohol-precipitated and vacuum-dried to obtain the pure *Morchella esculenta* polysaccharide.

Result and analysis

Drawing of standard curve

The standard curve of glucose is obtained, as shown in Fig. 1.

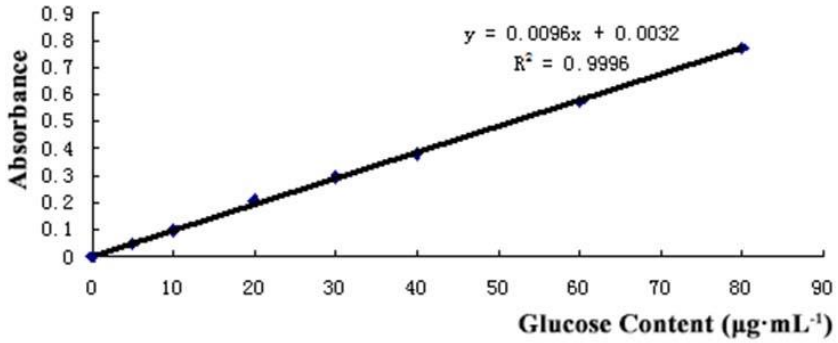

Fig. 1 Standard curve of glucose

As can be seen from Fig. 1, the linear relation of the curve is good, and the linear regression equation obtained from the data is: $y = 0.009\,6x + 0.0032$, where y is the absorbance measured at a wavelength of 620 nm; x is glucose content, μg/mL; the correlation coefficient R^2 is 0.9996.

Optimization of response surface of HVPEF extraction process

In order to study the influence of electric field intensity, pulse number, liquid-to-material ratio and their interaction on the yield of intracellular polysaccharide of *Morchella esculenta* mycelium, the central point value is selected according to the above-mentioned single factor test result (Table 1). Box-Behnken optimization test is carried out on electric field intensity, pulse number and liquid-to-material ratio. The test scheme design is shown in Table 2, and the result is shown in Table 3.

Table 1. HVPEF extraction factor horizontal coding table

Code	Factors		
	X_1	X_2	X_3
-1	15	4	20
0	20	6	30
1	25	8	40

X_1 is the electric field intensity, [kV/cm]; X_2 – pulse number; X_3 – liquid-to-material ratio, [mL/g].

Table 2. Box-Behnken test scheme and result

Run order	X_1	X_2	X_3	MEP, [µg/mL]
1	-1	1	0	50.21
2	0	1	1	38.15
3	0	0	0	55.62
4	1	1	0	39.15
5	0	-1	-1	39.27
6	-1	0	-1	50.07
7	1	-1	0	35.98
8	0	0	0	53.08
9	1	0	-1	37.23
10	0	1	-1	46.96
11	0	0	0	54.92
12	0	-1	1	35.51
13	-1	0	1	37.95
14	0	0	0	52.66
15	1	0	1	36.07
16	0	0	0	50.13
17	-1	-1	0	36.04

Table 3. Variance analysis

Source	Sum of squares	DF	Mean square	*F*-value	*p*-value Prob > *F*	
Model	932.2642	9	103.5849	27.53076	0.0001	significant
X_1	83.4632	1	83.4632	22.18282	0.0022	**
X_2	95.70361	1	95.70361	25.43607	0.0015	**
X_3	83.52781	1	83.52781	22.19999	0.0022	**
X_1X_2	30.25	1	30.25	8.039835	0.0252	*
X_1X_3	30.0304	1	30.0304	7.98147	0.0256	*
X_2X_3	6.375625	1	6.375625	1.694512	0.2342	
X_1^2	166.5724	1	166.5724	44.27157	0.0003	**
X_2^2	186.046	1	186.046	49.44725	0.0002	**
X_3^2	186.8866	1	186.8866	49.67067	0.0002	**
Residual	26.33761	7	3.762515			
Lack of fit	7.825525	3	2.608508	0.563634	0.6673	not significant
Pure error	18.51208	4	4.62802			
Cor total	958.6018	16				
Degree of freedom (DF): * $p < 0.05$; ** $p < 0.01$						

The quadratic regression equation of polysaccharide yield Y fitted by Design-Expert software is as follows:

$$Y = 53.28 - 3.23X_1 + 3.46X_2 - 3.23X_3 - 2.75X_1X_2 + \\ + 2.74X_1X_3 - 1.26X_2X_3 - 6.29X_1^2 - 6.65\ X_2^2 - 6.66X_3^2$$

From the variance analysis in Table 3, the *F* value (27.53) and *p* value (0.0001) of the model indicate that the influence of the dependent variable on the polysaccharide yield is significant ($p < 0.05$). *p* value of misfit item is 0.6673 > 0.05, which indicates that the misfit item is not significant and the regression is significant. The determination coefficient of the model is $R^2 = 0.9725$, which indicates that the model fits well. The model can be used to optimize the extraction process of polysaccharides from *Morchella esculenta* mycelium. The electric field intensity, the pulse number and the liquid-to-material ratio have significant influence on polysaccharide yield. In the quadratic term, the influence of X_1^2, X_2^2, X_3^2 on polysaccharide yield is significant and the interaction term X_1X_2 and X_1X_3 have significant influence on polysaccharide yield.

As can be seen from Fig. 2, the optimum electric field intensity is 17.90 kV/cm, the pulse number is 6.76, and the liquid-to-material ratio is 26.35 mL/g, and the polysaccharide yield can reach 55.21 μg/mL under this condition. In practice, the electric field intensity is 18 kV/cm, the pulse number is 7, the liquid-to-material ratio is 27 mL/g are taken for convenience, and the polysaccharide yield is 56.03 μg/mL, which is close to the predicted value.

Purification of polysaccharide

DEAE column chromatography

After the crude *Morchella esculenta* polysaccharide extracted by HVPEF is freeze-dried, its protein is removed by Savege reagent, and two components E1 (10-13 tubes, pure water elution) and E2 (26-28 tubes, 0.1 mol/L NaCl elution) are obtained by DEAE-52 cellulose ion exchange column chromatography, as shown in Fig. 3. E2 is the main acid polysaccharide of *Morchella esculenta*.

Sephadex G-100 chromatography

The acidic Morchella esculenta polysaccharide E2 separated from DEAE-cellullose is fractionated by Sephadex G-100 column chromatography. Two absorption peaks of E2 are obtained by gel chromatography (shown in Fig. 4), labeled as E2-A (5-10 tubes, yield 66.3%) and E2-B (44 tube, yield 13.5%). The yield of E2-B is very small, which is unfavorable for preparation and E2-A is further analyzed.

Determination of molecular weight

The purity and the molecular weight of E2-A are analyzed. It can be seen from Table 4 that two components F1 and F2 are contained after separated by Sephadex chromatography and the yield of F2 is small. Therefore, F1 is the main component of E2-A, accounting for 77.694%, and its molecular weight is 1.9895×10^5 Da. Fig. 5(a) is an E2-A gel dialysis chromatogram. TSK G-3000 gel chromatography is used to make chromatography to collect E2-A component and a symmetrical single absorption peak is obtained with high purity, as shown in Fig. 5(b).

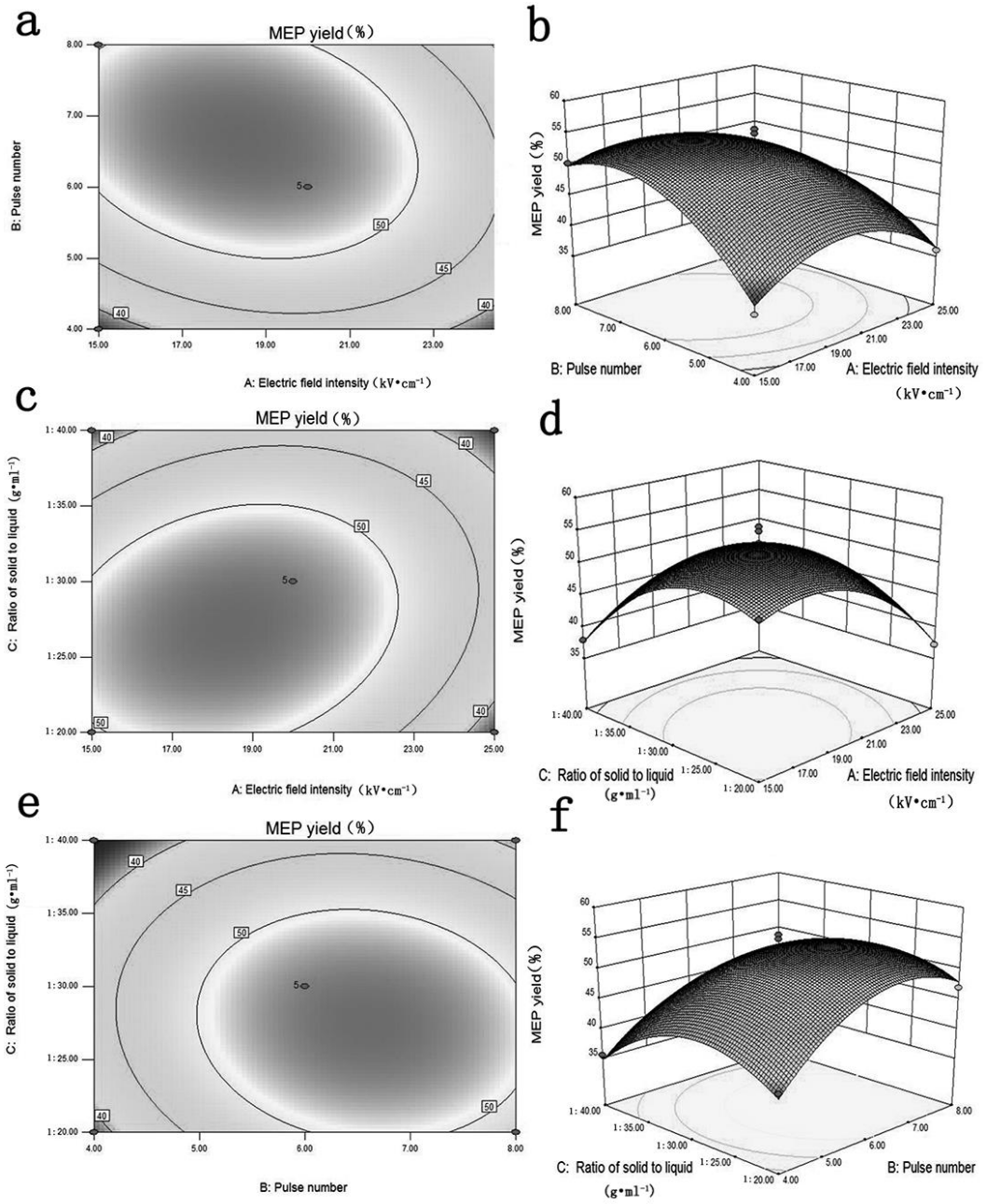

Fig. 2 3D effect graphs (a, c, e) and contour plots (b, d, f)
of the influence of electric field intensity, pulse number and liquid-to-material ratio
on *Morchella esculenta* polysaccharide yield

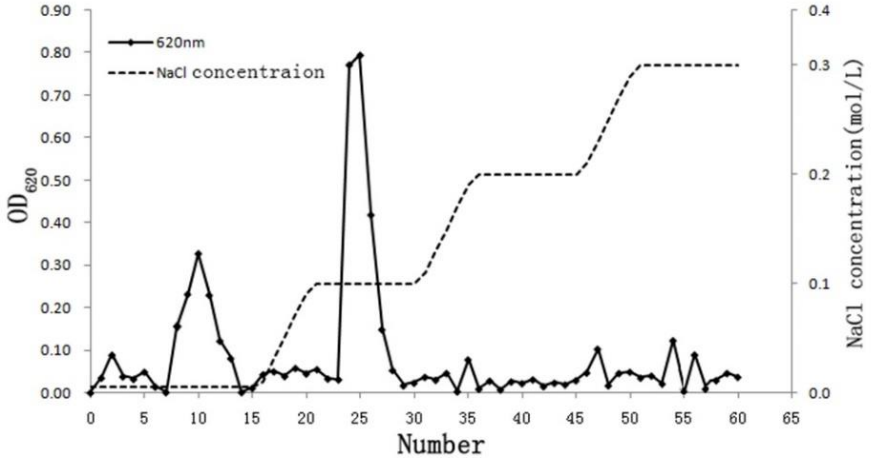

Fig. 3 DEAE-52 column chromatography map

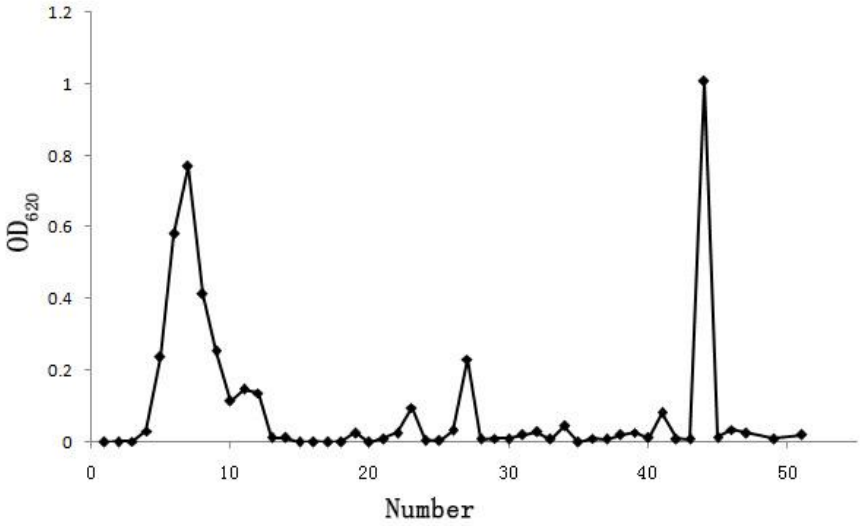

Fig. 4 Separation of *Morchella esculenta* acidic polysaccharide by Sephadex G-100 column chromatography

Table 4. Yields and relevant molecular parameters of *Morchella esculenta* acidic polysaccharide in GPC analysis

Fraction	**GPC parameter**				
	Retention time, [min]	**Yeild, [%]**	M_n**, [Da]**	M_w**, [Da]**	M_w/M_n
F1	13.656	77.694	198950	222344	1.11758
F2	20.218	22.306	413	428	1.03632

M_n – number-average molecular weight;
M_w – weight-average molecular weight; M_w/M_n – polydispersity.

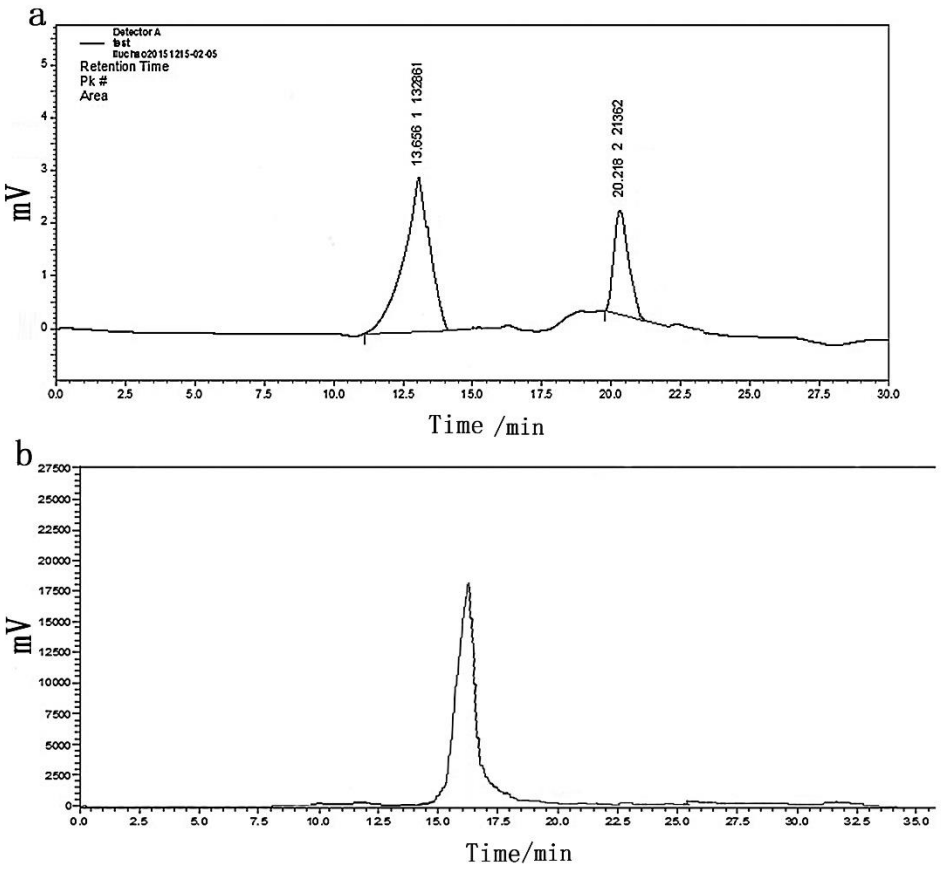

Fig. 5 GPC spectra of *Morchella esculenta* acidic polysaccharide (a) and prepared F1 (b)

Conclusions

The extraction and separation are conducted on acidic polysaccharide from *Morchella esculenta* mycelium produced by submerged fermentation. HVPEF is adopted to extract polysaccharide, during which heat is not generated and the structure of the polysaccharide is not damaged. The response surface method is used to investigate the electric field intensity, pulse number and liquid-to-material ratio. The optimum extraction process of polysaccharide is obtained as follows: the electric field intensity is 18 kV/cm, the pulse number is 7 and the liquid-to-material ratio is 27 mL/g. The polysaccharide yield reaches a maximum of 56.03 μg/mL. The crude polysaccharide is separated and purified by DEAE-52 cellulose ion exchange column and Sephadex G-100 column, and an acidic polysaccharide E1-A is obtained. An acidic polysaccharide with molecular weight of 1.9895×10^5 Da is obtained by Sephadex G-100 chromatography column, accounting for 77.694% of F1. The polysaccharide is the main component of acid polysaccharides in the mycelium of *Morchella esculenta* and is probably the main active substance. Its efficacy and chemical structure need to be further studied.

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