· 药剂与工艺 ·

A novel approach to deproteinization in isolation and purification of lentinan from *Lentinus edodes*

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Abstract **Object** Looking for a suitable approach to deproteinization in isolation and purification of lentinan from *Lentinus edodes*. **Methods** The orthogonal test was mainly used. **Results** A new approach was established for deproteinization in isolation and purification of a lentinan by orthogonal test. The results indicated that the major factor affecting the deproteinization rate (DPR) was B (2-butanol), the sub-major factor D (ethanol); while C (methanol) and E (NaCl) had little influence. The influencing order for DPR was B> D> A (chloroform) > E> C. DPR varied greatly when the amount of 2-butanol varied, going up slowly first, then going down rapidly. DPR decreased gradually as the concentration of ethanol increased. DPR went up first, and then went down, when the content of chloroform increased. And DPR altered little when the concentration of methanol increased. The optimal condition for deproteinization was $A_2B_2C_1D_1E_2$, the best composition for the deproteinizing agent was 40% of chloroform, 10% of 2-butanol, 2% of methanol, 40% of ethanol, 0.01% of NaCl. Further proven experiments showed that DPR was up to 99.98% under the best condition. The optimal technology of pretreatment of the dried *L. edodes*, such as distilling of *L. edodes*, concentrating of boiled sample, precipitating and decolorizating of rough polysaccharides, drying of rough polysaccharides, which applied to massive production for industry, was also researched out through repeated trials. **Conclusion** The novel approach to deproteinization has the superiority of simple technology, needless high-price apparatus, lower cost of processing, and easily amplifying. Thus, the technology of deproteinization is of great importance to the isolation and purification of fungi and other polysaccharides rich in proteins.

Key words *Lentinus edodes* (Berk.) Sing.; deproteinization; polysaccharides; isolation; purification

一种在多糖分离纯化过程中新的脱蛋白方法

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摘 要: 目的 寻找一种在多糖(香菇多糖)分离纯化过程中适宜的脱蛋白方法。方法 以正交试验法为主。结果 通过正交试验法找到了一种在多糖(香菇多糖)分离纯化过程中新的脱蛋白方法。5因素 3水平正交试验的结果表明,影响脱蛋白率(DPR)的主要因素是 B(正丁醇)、次主要因素是 D(乙醇),而因素 C(甲醇)和 E(氯化钠)的影响则较小;影响 DPR的主次顺序为 B> D> A(氯仿)> E> C。DPR随着正丁醇浓度的增加而变化很大,开始缓慢上升,然后迅速降低;随着乙醇浓度的增大,DPR逐渐降低;而当氯仿浓度增大时,DPR则开始上升,然后下降;当甲醇浓度增大时,DPR则变化很小。脱蛋白的最佳条件是 $A_2B_2C_1D_1E_2$,即在氯仿 40%、正丁醇 10%、甲醇 2%、乙醇 40%、氯化钠 0.01%的条件下,杂蛋白的去除率最高。验证性试验结果表明,在此条件下,杂蛋白的去除率达

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99.98%。通过反复试验,还找到了适用于工业化生产的包括香菇原料的蒸煮、煮沸样品的浓缩、粗多糖的沉淀和脱色、粗多糖的干燥等在内的原料预处理的最佳工艺。结论 这种新的脱蛋白方法具有工艺简单、无需贵重设备、加工成本低、容易放大等特点,特别适合于多糖类产品的精制或其他需要去除杂蛋白的单元操作。

关键词: 香菇;脱蛋白;多糖;分离;纯化
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It's well known that 1970s is the time of gene engineering in the sector of life science, 1980s the time of protein engineering, 1990s and 21st century the time of polysaccharides engineering[1]. The great success has been achieved in the upstream of bioengineering such as having acquired many hybrid microbes. However, broad applications of them to industry and people's life are very few. The main reason affecting their application is that we have not solved the problems of downstream of bioengineering well. The problem of deproteinization is always a crucial technology in the aspects of the genetic engineering, proteins engineering, especially for polysaccharides engineering. Clinic practice indicated that higher purity of polysaccharides has greatly potential application and market. There are many methods of isolation and purification of proteins; however, the rare methods of deproteinization in isolation and purification of polysaccharides are effective. The published methods such as the improved gel electrophoresis method[2] described by Guard-Petter (1995), Submicron Magnetic Particles method[3] reported by Khng (1998), solvent-free biotransformations[4] described by D' Souza (1999), the particular emulsans method[5] invented by Gutnick (1980), Enzymatic Transformations[6] described by Millqvist-Fureby (1998), or the traditional hot phenol method[7] described by Westphal are all difficult to be used in large scale, though they are fairly efficient. In the present paper, "a novel approach to deproteinization in isolation and purification of lentinan" that can be applied to any scale commercially is reported. *L. edodes* was taken as experimental materials for the reason of the high nourishment of *L. edodes* and the largely clinic application of lentinan but rare reports about purification of lentinan around the world.

1 Materials and methods

1.1 Materials *Lentinus edodes* (Berk.) Sing. including brindle *L. edodes*, nonbrindle *L. edodes* (purchased from mushroom market of Nanyang, Henan Province). Chloroform (AR), 2-butanol (AR), methanol (AR), ethanol (Food Grade), NaCl (AR) (purchased from No. 1 Factory of Tianjin Chemical Reagent).

1.2 Methods of pretreatment

1.2.1 Grinding and decolorization of material. Dried *L. edodes* was smashed into the size of millet (d = 1 — 1.2 mm) in a pulverizer XM—7 (Xuchang Mechanic Factory). The pulverized dried *L. edodes* powder was decolorized by 95% ethanol (powder-ethanol= 1∶2).

1.2.2 Boiling and filtrating of material. The decolorized mushroom powder was boiled for 4 hours at 96℃ with the ratio of *L. edodes* to water 1.0∶12.0 first, then distilled at 96℃ for 4 hours with the ratio of *L. edodes* to water 1.0∶8.0. After each distillation, it was pressed and filtered with heat-resistant cloth, respectively.

1.2.3 Concentration of boiled sample. Put the first and second distilled liquid into a boiler, and then heated it at 94℃ until the amount of the concentrated liquid decreased to 20% of its initial volume. This process takes about 3— 5 hours.

1.2.4 Precipitation and decolorization of rough polysaccharides. Added 95% ethanol to the concentrate till the final concentration of ethanol of the precipitator is up to 70% . It should be stirred continuously while adding the ethanol. After agitation, the sample was kept still for 8— 10 hours to let rough lentinan precipitate.

1.2.5 Dry of rough polysaccharides. The precipitated rough polysaccharide was condensed under cool condition (4℃ — 10℃) and dried at 65℃ for 2— 4 hours or pressed with squeezer at 20℃ — 30℃.

1.3 Deproteinization of rough polysaccharides

1.3.1 Selection of the agents of deproteinization. The strategy of deproteinization was determined by using cheap organic solvents commercially and conveniently rather than using other methods as described in the introduction. In this experiment, chloroform (A), 2-butanol (B), methanol (C), ethanol (D), NaCl (E) were chosen from the viewpoints of commerce, application and effectiveness.

1.3.2 Technology of deproteinization. The technology of deprotenization was designed as follows.

Rough polysaccharide sample —(distilled water)→ water-soluble solution —(chloroform + 2-butanol, stirring 10–20 min)→ colloidal mixture —(methanol + ethanol + NaCl, stirring 5–10 min)→ split phase:

- water phase → polysaccharide (repeat 3—5 times from step 2)
- protein phase → separation → depletion of reagent → dry
- organic phase → recovery and reuse

1.3.3 Decision of optimal composition of deproteinization agents. The content of each agent for deproteinization was determined through orthogonal test (OT). In this research, the major factors affecting deproteinization and levels of each factor were set as Table 1. The experimental scheme was shown in Table 2.

Table 1 Factors and levels

Levels	Factors /%				
	A	B	C	D	E
1	30	5	2	40	0.005
2	40	10	4	50	0.01
3	50	20	6	60	0.02

1.3.4 Determination of the DPR. The DPR was determined at λ = 280 nm with ultraviolet spectrophotometer 756 MC (The First Analytical Apparatus Factory of Shanghai, China). The protein content was tested in rough poly-saccharides that were not deproteinized as 100% .

2 Results and discussion

2.1 Pretreatment of the dried *L. edodes*. It is very important to break the dried *L. edodes* to pieces with the size of millet (d = 1— 1.2 mm). If it wasn't shattered with a kibbler or it wasn't small enough, too much non-starch polysaccharides will remain in the material and won't be extracted effectively. If it is ground too much, it will be difficult to press and filter the sample. Thus, in order to extract polysaccharides from *L. edodes* completely, the material must be shattered properly.

2.2 Distillation of *L. edodes*. For both fresh and dried materials, the operation of distillation is the first and key step during the whole process of lentinan preparation. The suitable boiling condition decides whether or not enough non-starch polysaccharides of *L. edodes* can be extracted. It is crucial to choose the optimal distilling temperature and proportion of the material to water. The experimental results indicated that the optimum distilling temperature is 96 ℃, the proportion of the material to water is 1∶20, the proper period of boiling is 4— 6 hours. If the steaming tem-perature is beyond 100 ℃, it will not only increase the cost of operation, but also lead to the lentinan degradation. If the temperature is too low, the liquid can't be boiled, and the effect of steaming is lessened. To decrease the remaining polysaccharides thoroughly in the material, steaming it again and it should be carried out after the first treatment at the same condition.

2.3 Concentration of boiled sample. The concentrating is another important step to save the consumption of precipitate agent. There are many ways of concentrating the sample to choose from. In this experiment, we culled the boiling way in the light of lessening the investment of production equipment. The practice showed that the boiled liquid volume was decreased to 20% — 25% of its initiated volume at 94 ℃ for 3— 5 hours. It is still fairly effective.

2.4 Precipitation and decolorization of rough polysaccharides. The concentrated sample is thick and dark. In order to get pure preparation, decolorization has to be conducted. Much pigment in rough polysaccharides can be dissolved in ethanol. Some flocculent white sediment appeared instantly along with adding ethanol into the concentrate. The more ethanol was added, the more flocculent white sticky sediment appearred. For the high effi-

ciency of sedimentation and decolorization, ethanol was added till the final concentration of ethanol reached to 65% — 70%. The solution should be stirred with putting ethanol in. The sample was kept still for 8— 10 hours to get more flocculent. This step was repeated to deplete the pigment more. Rough polysaccharides 98.5% have been obtained and pigment (95%) has been eliminated finally.

2.5 Drying of rough polysaccharides. The best method of drying bio-products is lyophilization or freeze-drying. In order to keep the continuity of whole operation process and reduce the cost, the way of pressing or squeezing was chosen. By this way, the whole period of drying can be decreased within 30 minutes.

2.6 Optimum composition of deproteinization agent. The results of orthogonal test for DPR with five factors, three levels are shown in Table 2.

The values of the maximum difference R in Table 2 indicated that the major factor affecting DPR is B (2-butanol), the sub-major factor is D (ethanol); the minor factor is C (methanol), and the sub-minor factor is E (NaCl). The influencing order for DPR is B> D> A> E> C.

It can be seen that the DPR varied greatly when the amount of 2-butanol varied, going up slowly first, then going down rapidly from the size of numerical value and its changing tendency of K_1, K_2, and K_3, of each factor in Table 2. The DPR decreased gradually as the concentration of ethanol increased. The DPR went up first, and then went down, when the concentration of chloroform scaled up. The DPR altered little when the concentration of methanol increased.

The optimal combination for deproteinization is $A_2B_2C_1D_1E_2$ according to the maximum value among K_1, K_2, and K_3, of each factor in Table 2. That is, the best composition for the deproteinization agent is 40% of chloroform, 10% of 2-butanol, 2% of methanol, 40% of ethanol, and 0.01% of NaCl. Further proved experiments indicated that the DPR was up to 99.98% under the best condition. The obvious advantage for this approach is that all agents used are easily removed from the products compared with other methods.

Table 2 DPR with five factors, three levels by OT

No.	Factors					DPR/%
	A	B	C	D	E	
1	1	1	1	1	1	80.5
2	1	1	1	1	2	82.7
3	1	1	1	1	3	81.3
4	1	2	2	2	1	88.1
5	1	2	2	2	2	92.9
6	1	2	2	2	3	89.7
7	1	3	3	3	1	50.9
8	1	3	3	3	2	55.8
9	1	3	3	3	3	53.2
10	2	1	2	3	1	70.8
11	2	1	2	3	2	74.2
12	2	1	2	3	3	73.2
13	2	2	3	1	1	98.5
14	2	2	3	1	2	99.3
15	2	2	3	1	3	99.0
16	2	3	1	2	1	63.3
17	2	3	1	2	2	66.6
18	2	3	1	2	3	64.7
19	3	1	3	2	1	75.2
20	3	1	3	2	2	79.8
21	3	1	3	2	3	76.4
22	3	2	1	3	1	83.3
23	3	2	1	3	2	88.2
24	3	2	1	3	3	84.9
25	3	3	2	1	1	67.7
26	3	3	2	1	2	70.6
27	3	3	2	1	3	68.1
K_1	675.1	694.1	695.5	747.7	678.3	
K_2	709.6	823.9	695.1	696.7	710.1	
K_3	694.2	560.9	688.1	634.5	690.5	
R	34.5	263	7.4	113.2	31.8	

As described in the introduction, higher purity of polysaccharides has greatly potential clinic application. And proteins are major residues of polysaccharides in the preparation of fungi polysaccharides as edible mushrooms are rich in proteins. It will influence the therapeutical effect of polysaccharides heavily if the residue was not removed.

Further studies need to be carried on whether the new approach for deproteinization searched out could be adapted for others materials and polysaccharides.

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交泰丸方药 4种提取方法的比较研究

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摘 要: 目的 优选交泰丸方药的提取工艺。方法 以小檗碱、肉桂酸、总生物碱、挥发油、干浸膏为指标,在药材粒度、溶剂量、煎提温度、滤过、浓缩等条件相同的前提下,对半仿生提取法(SBE法)、水提取法(WE法)、半仿生提取醇沉法(SBAE法)、水提取醇沉法(WAE法)进行比较研究。结果 5个指标综合评价 Y 值为: SBE法> WE法> SBAE法> WAE法。结论 交泰丸方药的提取法以 SBE法为佳。

关键词: 交泰丸;提取方法;小檗碱;肉桂酸;总生物碱
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Comparison of four extracting methods for Jiaotai Pill*

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Abstract **Object** To evaluate the extracting technology of Jiaotai Pill components. **Methods** Four methods—the semi-bionic extraction (SBE), the water extraction (WE), the semi-bionic extraction by precipitation with alcohol (SBAE), and the water extraction by precipitation with alcohol (WAE) were used to extract Jiaotai Pill component, with berberine, cinnamic acid, total alkaloids, volatile oil and dried extract taken as the markers and to study the four methods under the same conditions of drug granularity, solvent amount, decocting temperature, filtration, concentration, *etc*. **Results** The comprehensive values Y were SBE> WE> SBAE> WAE. **Conclusion** SBE method is better than the other three methods in the extraction of Jiaotai Pill components.

Key words Jiaotai Pill; extracting methods; berberine; cinnamic acid; total alkaloids

* Jiaotai Pill is a Chinese prescription consisted of *Rhizoma Coptidis* and *Cortex Cinnamomi*. It has the function of coordination between the heart and the kidney.

交泰丸由黄连、肉桂组成,具有交通心肾的功能。临床用于失眠、心律失常、口腔疾病等取得较好效果[1]。为进一步探讨该方药采用半仿生提取(SBE)法提取是否较目前普遍应用的提取方法为佳,本实验根据 SBE法理论[2],在优选出 SBE法提取条件[3]、药材组合方式及醇沉较佳浓度的基础上,以小檗碱、肉桂酸、总生物碱、挥发油、干浸膏量为指标,对该方药作 4种提取方法的比较研究。

1 仪器与药品

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