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濒危名贵药用霍山石斛类原球茎
液体培养生产活性多糖的研究

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为贯彻教育部《关于实施研究生教育创新计划 加强研究生创新能力培养 进一步提高培养质量的若干意见》(教研[2005]1号)文件精神,培养研究生创新意识、创新能力,提高研究生培养质量,合肥工业大学设立了研究生科技创新基金,以支持和资助研究生的教育创新活动,为创新人才的成长创造条件。学校领导高度重视研究生教育创新,出版的《斛兵博士文丛》就是创新基金资助的项目之一。

《斛兵博士文丛》入选的博士学位论文是合肥工业大学 2008 届部分优秀的博士学位论文。为提高学位论文的出版质量,《斛兵博士文丛》以注重创新为出版原则,充分展示我校博士研究生在基础与应用研究方面的成绩。

《斛兵博士文丛》的出版,得到了相关兄弟院校和有关专家的大力支持,也得到了研究生导师和研究生的热情支持,我们谨此表示感谢,希望今后能继续得到他们的支持与帮助。

我们力求把这项工作做好,但由于我们经验不足和学识水平有限,书中难免存在不足之处,敬请读者给予批评指正。

合肥工业大学研究生学位论文出版编辑委员会

2011 年 11 月

总 序

胡锦涛总书记指出,为完成“十二五”时期经济社会发展的目标任务,在激烈的国际竞争中赢得发展的主动权,最根本的是靠科学技术,最关键的是大力提高自主创新能力。“提高自主创新能力,建设创新型国家”已明确写进了党的十七大报告。而创新型国家的建设靠人才,人才的培养靠教育。博士生教育与我国科学技术的进步与发展,与社会经济的发展有着直接而密切的联系,是国家创新体系的重要组成部分,研究生尤其是博士研究生培养质量如何,将集中体现一所高校的教育和科研水平。

博士论文的研究工作一般都能体现本领域学科发展的前沿性和某些行业多元发展的战略性,应具有一定的创新性。为鼓励广大研究生,特别是博士研究生选择具有重大意义的科技前沿课题进行研究,进一步提高研究生的创新意识、创新精神、创新能力,激励、调动我校博士研究生及其指导教师进一步重视提高博士学位论文质量和争创优秀博士学位论文的主动性和积极性,展示我校博士研究生的学术水平,学校经过精心筹划,编辑出版了《斛兵博士文丛》。

此次入选《斛兵博士文丛》的论著,均为2008年毕业并获得博士学位的优秀博士研究生学位论文。我校的优秀博士学位论文评选工作旨在逐步建立有效的质量监督和激励机制,培养和激励我校在学博士生的创新精神,构建高层次创造性人才脱颖而出的优良氛围。同时优秀博士学位论文代表着我校博士生培养的最高水平,对我校博士生教育起到了示范作用。这套丛书中的论文大体上都有以下几个显著特点:一是选题均为本学科的前沿,具有较大的挑战性;二是论文的创新性突出,或是在理论上或是在方法上有创新;三是论文的成果较为显著,大多都在国际学术刊物上发表了与该论文有关的学术论文。

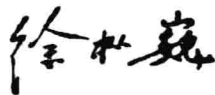
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濒危名贵药用霍山石斛类原球茎液体培养生产活性多糖的研究

《斛兵博士文丛》的出版也是我校实施研究生创新工程的一个重要举措。伴随着办学条件的不断改善、人才培养政策的日趋完善和高层次创新型人才成长的良好环境的不断构建,一定能达到多出人才、快出人才、出好人才的目标。

我衷心希望广大研究生发扬我校的优良传统,在严谨求实、开放和谐、充满生机与活力的学术环境中奋发努力、锐意进取、勇于创新,通过自己的辛勤劳动和刻苦钻研写出更好的论文,为进一步提高我校的学术水平作出更大的贡献,为把学校建设成为国内先进、国际知名的创新型高水平大学而不懈努力。

合肥工业大学校长
教授、博士生导师



二〇一一年十一月

摘要

石斛作为传统名贵中草药,其资源再生、化学成分和药理活性逐渐引起人们的关注,其中多糖是石斛属植物中主要的药用活性成分之一。本文以传统的名贵珍稀药用植物——霍山石斛为主要研究对象,在建立霍山石斛类原球茎多糖高效表达悬浮培养体系的基础上,利用生物大分子研究的最新技术和现代分离分析手段与药理实验方法,对霍山石斛类原球茎水溶性多糖进行了提取分离纯化,并对其主要活性多糖组分 HPS-1B23 的组成和一级结构进行了研究。主要研究结果如下:

(1)以霍山石斛试管苗茎段为外植体,首次诱导获得了类原球茎,并建立了类原球茎的培养体系。其中附加 NAA($7.5 \mu\text{mol L}^{-1}$)和 KIN($0.5 \mu\text{mol L}^{-1}$)组合的 MS 培养基最利于类原球茎的形成,诱导率达到 79.9%。诱导的类原球茎经长期继代培养后,从生长、植株再生、活性多糖合成、药理作用、单糖组成及基因组 DNA 多态性等方面进行品质稳定性分析,表明诱导获得的类原球茎系在品质上是稳定的。

(2)药理学研究证明了培养类原球茎具有野生霍山石斛同样的合成活性多糖的能力。野生霍山石斛总多糖能显著促进小鼠脾细胞和巨噬细胞分别产生 IFN- γ 和 TNF- α ,多糖作用浓度分别在 $800 \mu\text{g mL}^{-1}$ 和 $200 \mu\text{g mL}^{-1}$ 。RT-PCR 分析表明,总多糖是通过上调或下调 IFN- γ 和 TNF- α 的基因表达来控制各自的释放量的。经 DEAE-Cellulose 离子交换色谱层析,从类原球茎总多糖中分离出和野生霍山石斛相同的 5 个多糖组分,并表现出同样的促进小鼠脾细胞和巨噬细胞分别产生 IFN- γ 和 TNF- α 的能力,其中以水洗脱部分 HPS-1 含量最大。建立的多糖活性评价方法实现了对霍山石斛类原球茎培养体系的质量监控。

(3)研究分析了霍山石斛类原球茎液体培养过程中碳、氮代谢特征,阐

明霍山石斛类原球茎生长和多糖合成与细胞内源和外源碳、氮的关系。在整个培养过程中,没有观察到显著的细胞生长延滞期,培养 30 d 后,生物量达到最大,多糖合成与类原球茎生长表现出了非同步的关系。细胞培养启动后,在前 3 d 培养基中蔗糖浓度减少了一半,同时无葡萄糖和果糖的存在,之后随着蔗糖浓度的继续下降,培养基中逐渐检测到有葡萄糖和果糖的存在,到第 9 d 含量达到最大值。相反,类原球茎内源蔗糖随培养的启动,在培养的前 6 d,快速积累,之后快速被消耗;内源葡萄糖和果糖含量也有一定程度的增加,培养 9 d 后逐渐被消耗。可溶酸性蔗糖酶和碱性蔗糖酶随类原球茎培养的启动逐渐被激活,分别在培养的第 6 d 和第 18 d 活性达到最大值。细胞壁蔗糖酶在整个培养过程中活性很低,一直处于被抑制的状态。蔗糖合成酶在培养的第 18 d 活性达到最高,而蔗糖磷酸合成酶与细胞壁蔗糖酶相类似,活性一直保持很低的水平。培养基中 NH_4^+ 在启动培养 9 d 后几乎消耗殆尽,而类原球茎对 NO_3^- 的吸收利用才刚刚开始;胞内 NH_4^+ 在培养的前 3 d 含量急剧上升,之后又快速被消耗,直到培养的第 12 d,此时类原球茎对 NO_3^- 的利用才启动。谷氨酰胺合成酶和谷氨酸合成酶随培养的启动,活性逐渐提高,分别在培养的第 12 d 和 15 d 活性达到最大值。硝酸还原酶是细胞对 NO_3^- 利用涉及的第一个关键性酶,在类原球茎培养初期,活性表达受到了一定程度的抑制作用,培养 9 d 后,活性表达逐渐提高,到第 15 d 活性达到最大值。结果表明 soluble acid IT、alkaline IT、SS、GS、GOGAT 和 NR 在类原球茎生长的不同阶段调控碳和氮代谢,最终影响类原球茎生长和多糖合成。研究结果为培养体系的优化和提高多糖合成的提供理论指导。

(4)建立了霍山石斛类原球茎两阶段培养高水平合成活性多糖的体系。在生长阶段,通过培养基组成的优化组合,获得适合类原球茎生长的最适碳、氮源是蔗糖和硝酸钾,浓度分别为 35 g L^{-1} 和 30 mmol L^{-1} ,最适 Ca^{2+} 、 Fe^{2+} 、 Mn^{2+} 和 Zn^{2+} 分别为 4.5 mmol L^{-1} 、 0.1 mmol L^{-1} 、 0.5 mmol L^{-1} 和 0.06 mmol L^{-1} 。在优化的生长培养基上类原球茎培养 30 d 生物量达到 693 g FW L^{-1} ,生物量增加比优化前提高了 230%。在多糖合成阶段,在培养基中补加蔗糖迅速促进多糖的合成,以在培养第 30 d 补加 50 g L^{-1} 的蔗糖类原球茎中多糖合成能力最高,在补加蔗糖继续培养 6 天多糖产率达 22 g L^{-1} ,是对照的近 10 倍。

Abstract

Dendrobium species are used as the traditional Chinese medicine herbs for centuries. Much attention has been focused on its resource regeneration, chemical components and pharmacological activities. Polysaccharides were the major active constituent from Dendrobium plants. Dendrobium huoshanense C. Z. Tang et S. J. Cheng, from which the Chinese medicine was most originally prepared, was studied in this paper. After the high expression system of polysaccharides from protocorm-like bodies (PLBs) in suspension culture was researched, water-soluble polysaccharides were extracted, isolated and purified from PLBs of D. huoshanense, with the advanced methods of isolation and purification, chemical and pharmacological instrument analysis technique of biomacromolecular. Some pharmacological action and mechanisms were investigated. The chemical properties and structural features of main polysaccharides of HPS-1B23 from PLBs of D. huoshanense (HPS) were identified. The main results were obtained as follows:

An efficient procedure was developed for PLBs formation from stem segments of D. huoshanense and subsequent subculture for a long time. The highest frequency (79.9%) of PLB formation occurred on MS medium supplemented with $7.5 \mu\text{mol L}^{-1}$ NAA and $0.5 \mu\text{mol L}^{-1}$ KIN. Growth-regulator free MS medium at half-strength was suitable for PLB proliferation. The obtained PLBs were stable in growth, plantlet regeneration, polymorphic DNA of genome and polysaccharide synthesis including content and monosaccharide ratio.

Pharmacological studies testified that PLBs have the same potential of synthesizing active polysaccharide as that of wild plants. The crude HPS from wild plants significantly stimulated interferon γ (IFN- γ) release in the supernatant of murine splenocytes and tumor necrosis factor alpha (TNF- α) release in the supernatant of peritoneal macrophages. The critical concentration of HPS was $800 \mu\text{g mL}^{-1}$ for IFN- γ and $200 \mu\text{g mL}^{-1}$ for TNF- α release. RT-PCR analysis indicated those responses were attributed to the up-regulation of the expression of their own genes in cells. Different HPS fractions showed different capability in stimulating IFN- γ and TNF- α release. By DEAE-Cellulose chromatography, five fractions were all obtained from wild plants and PLBs, in which HPS-1 eluted by distilled water was the main constituent (79%). These analytical methods of polysaccharide bioactivities could be applied to monitor the liquid culture system of PLBs.

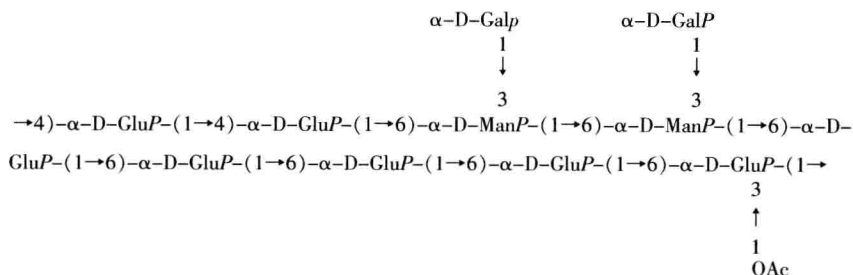
The carbon and nitrogen metabolism was analysed and the relationship between PLB growth, polysaccharide synthesis and carbon, nitrogen metabolism was elucidated in liquid culture of PLBs. No significant lag phase of PLB growth was found and the maximum biomass was obtained on day 30 of culture. The profiles of PLB growth and polysaccharide synthesis was not in synchronization. Sucrose concentration was halved as PLB growth proceeded while no change in glucose and fructose levels in the medium was found in the first 3 days followed by a gradual increase until day 9 of culture. Conversely, sucrose in PLBs accumulated dramatically in the first 6 days of culture followed a rapid decrease. At the same time, glucose and fructose content of PLBs was also increased and declined after 9 days of culture. Soluble acid invertase (soluble acid IT) and alkaline invertase (alkaline IT) were activated after inoculum, which reached the highest value on day 6 and day 18, respectively whereas cell-wall-bound invertase (cell-wall-bound IT) seemed to be repressed throughout of culture. The maximum value of sucrose synthase (SuSy) activity was observed on day 18 while sucrose phosphate synthase (SPS) stayed low and constant from inoculum to

the end of the culture. Ammonium concentration in medium decreased rapidly and it was hardly detected after culturing for 9 days while the rapid use of nitrate was onset. Corresponded to medium, ammonium in PLBs showed a sharp increase in the first 3 days of culture followed a rapid decrease until day 12 where nitrate depletion was onset. Both the peak of glutamine synthase (GS) and glutamate synthase (GOGAT) activity was observed on day 12 and 15, respectively. Nitrate reductase (NR) was repressed in the early culture stage and activated from day 9 to day 15 of culture. These results suggested that soluble acid IT, alkaline IT, SS, GS, GOGAT and NR controlled carbon and nitrogen metabolism in different growth stage of PLB of *D. huoshanense*, which regulated PLB growth and polysaccharide synthesis ultimately, which could supply the guidance for regulating PLB growth and polysaccharide synthesis in high performance.

The two-stage culture system for active polysaccharide synthesis in high performance was established. In PLB growth stage, sucrose and KNO_3 as the carbon source and nitrogen source are suitable for PLB growth and the critical concentration were 35 g L^{-1} and 30 mmol L^{-1} , respectively. The optimal concentration of Ca^{2+} , Fe^{2+} , Mn^{2+} and Zn^{2+} for PLB growth was 4.5 mmol L^{-1} , 0.1 mmol L^{-1} , 0.5 mmol L^{-1} and 0.06 mmol L^{-1} , respectively. In this medium, 693 g FW L^{-1} PLBs were harvested after 30 days of culture and biomass increase was improved 230% as compared with that of 1/2 MS. In polysaccharide synthesis stage, polysaccharide synthesis was significantly improved by the feeding sucrose. The maximum polysaccharide production (22 g L^{-1}) was seen in the case of 50 g L^{-1} sucrose feeding at day 30 of culture, which was about 9-fold higher than that of regulation before.

One homogenous fraction of HPS-1B23 was obtained after purification of HPS and its property and structure were further elucidated. The water-soluble polysaccharide of *D. huoshanense* was fractionated by DEAE-Cellulose anion-exchange and gel filtration chromatography of Sephacryl S-200,

Sephadex G-75 and Sephadex G-100 in turn, giving one homogenous fraction of HPS-1B23 with molecular weight of 2.2×10^4 Da and optical rotation of $[\alpha]_D^{20} +130.7$. HPS-1B23 was white powder and its microstructure was characterized as a honeycomb by SEM. HPS-1B23 from D. huoshanense was composed of glucose, mannose and galactose in molar proportions 31:10:8 by analysis of complete acid hydrolysates. The ultraviolet and infrared spectra analysis revealed that the HPS-1B23 was sugar without proteins. By using techniques of smith degradation, methylation analysis, partial acid hydrolysis and NMR spectrometry, the repeating unit of HPS-1B23 was characterized as:



Keywords: *Dendrobium huoshanense*; polysaccharide; protocorm-like bodies (PLBs); suspension culture; extraction isolation and purification; structural analysis; biological activity

英文缩写词表

缩写词	英文全名	中文名
ABA	Abscissic acid	脱落酸
BA	Benzyladenine	苄基嘌呤
B5	Gamborg Medium	B5 培养基
ConA	Concanavalina A	刀豆素 A
2,4-D	2,4-dichlorophenoxyacetic acid	2,4-二氯苯氧乙酸
DEAE-Cellulose	Dithyl-amino-ethyl cellulose	二乙胺基乙基纤维素
DEPC	Diethyl pyrocarbonate	焦碳酸二乙酯
EI	Electron impact	电子轰击离子源
ESS	Exogenous soluble sugar	外源可溶性糖
F6P	Fructose-6-phosphate	果糖-6-磷酸
FTIR	Fourier transform infrared spectrum	傅立叶变换红外光谱
Gal	Galactose	半乳糖
GC	Gas chromatography	气相色谱
GC-MS	Gas chromatography-mass spectrum	气质联用
Glu	Glucose	葡萄糖
GOGAT	Glutamate synthase	谷氨酸合成酶
GPC	Gel permeation chromatography	凝胶渗透色谱
GS	Glutamine synthase	谷氨酰胺合成酶
GUC	Galactose uronic acid	半乳糖醛酸
HPGPC	High performance gel permeation chromatography	高效凝胶渗透色谱
HPLC	High performance liquid chromatography	液相色谱
HPS	Polysaccharides from D. huoshanense	霍山石斛总多糖

(英文缩写词表续表)

缩写词	英文全名	中文名
IFN- γ	interferon- γ	干扰素- γ
IR	infrared spectrum	红外光谱
ISS	Intracellular soluble sugar	胞内可溶性糖
IT	Invertase	蔗糖酶
KIN	Kinetin	激动素
LPS	Lipopolysaccharide	脂多糖
Man	Mannose	甘露糖
MS	Murashige and Skoog Medium	MS 培养基
N6	Zhuzhiqing Medium	N6 培养基
NAA	1-naphthaleneacetic	萘乙酸
NMR	Nuclear magnetic resonance spectrum	核磁共振波谱
NR	Nitrate reductase	硝酸还原酶
PLBs	Protocorm-like bodies	类原球茎
RAPD	Random amplified polymorphic DNA	随机扩增多态性 DNA
RT-PCR	Reverse transcription polymerase chain reaction	反转录聚合酶链式反应
SPS	Sucrose phosphate synthase	蔗糖磷酸合成酶
SS	Sucrose synthase	蔗糖合成酶
TFA	Trifluoroacetic acid	三氟乙酸
TIC	Total ion chromatography	总离子流色谱
TNF- α	tumor necrosis factor alpha	肿瘤坏死因子- α
UDPG	UDP-glucose	尿苷二磷酸葡萄糖
UV	Ultraviolet spectrum	紫外光谱

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