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医学与免疫生物技术

灵芝免疫调节蛋白(Lz-8)在毕赤酵母中的表达及其免疫活性鉴定

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摘 要: 真菌免疫调节蛋白家族(Fungi immunoregulatory proteins, FIPs)各成员所具有的免疫调节和抗肿瘤活性已被广泛研究。本研究利用毕赤酵母表达系统对其成员 Lz-8 进行了重组表达。以毕赤酵母突变株 GS115 为表达宿主细胞, PCR 和 DNA 测序结果均显示 Lz-8 的 cDNA 已被成功地整合入酵母基因组。聚丙烯酰胺凝胶电泳(SDS-PAGE)、激光解析飞行时间质谱(MALDI-TOF-MS)和免疫学实验均被用于重组表达蛋白的检测。实验结果表明 Lz-8 在毕赤酵母表达系统中得到成功表达, 在 SDS-PAGE 中可观察到分子量为 14 000 D 的单一条带, MALDI-TOF-MS 的实验结果显示 rLz-8 的分子量为 12 722 D。在相关的免疫学实验中, rLz-8 可引起绵羊血红细胞凝集, 但对人血 4 种血型的红细胞并没有凝集作用, rlz-8 还可诱导巨噬细胞吞噬作用, 均与其他报道中的实验结果吻合。以上结果表明, 本实验已成功地利用毕赤酵母表达系统对 Lz-8 进行重组表达。

关键词: 灵芝, 免疫调节蛋白, 毕赤酵母, 免疫蛋白

Ganoderma lucidum immunomodulatory protein(Lz-8) expressed in *Pichia pastoris* and the identification of immunocompetence

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Abstract: Fungi immunoregulatory proteins family is effective in immunological regulation and anti-tumor. We used *Pichia pastoris* expression system for recombinant expression of Lz-8, an immunomodulatory protein isolated from fruiting body of *Ganoderma lucidum*. The Gs115 (mut^+) strains of *P. pastoris* was used as host cells. PCR and sequencing of DNA showed that Lz-8 cDNA was successfully integrated into the *P. pastoris* genome. Electrophoresis(SDS-PAGE), matrix assisted laser desorption ionisation time-of-flight mass spectrometry(MALDI-TOF-MS) and immunological techniques were used to identify recombinant Lz-8 (rLz-8). Lz-8 expressed in *Escherichia coli*, the *Pichia* system requires further optimization to obtain more active fungi immunomodulatory protein. Lz-8 was expressed in *P. pastoris* successfully, and polyacrylamide gel electrophoresis in the presence of SDS-PAGE gave a single band with an apparent Mr=14 000 D. MALDI-TOF-MS also showed that molecular weight of rLz-8 was 12 722 D. Aggregation was observed from sheep red blood cells in the presence of purified rLz-8 within the concentration range of 12.5–50 μg/mL. However, no aggregation was seen at concentration greater than 50 μg/mL for any type of human red blood cell. The

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dose at 0.5 mg/kg of rLz-8 induced macrophage cytophagocytesis, and set interferon as control at 0.5 mg/kg. These results suggested that active and stable rLz-8 was obtained in *P. pastoris* expression system.

Keywords: *Ganoderma lucidum*, immunomodulatory protein, *Pichia pastoris*, immunolomodulation

Introduction

The wild Changbaishan *Ganoderma lucidum* is a particular Chinese traditional medical and edible resource, it contains some pharmacological compositions. The Lz-8 of fungi immunoregulatory proteins family has been found to effective against immunological regulation and anti-tumor by Chinese medical study and clinical application. Lz-8 has been expressed in *E. coli* by many study groups recent years[1−8]. Our study group also expressed Lz-8 in *E. coli*, but we did not gain a mass of dissoluble and stable protein, even though we adopt some fusion-protein vectors (for example GST vector). The Lin's study group of Taiwan university has studied fungi immunoregulatory proteins family last decade, it was not until last year that they used Sf21 insect cells to express FIP-gts which had apparent homology with Lz-8, and explained that they did expression of FIP-gts in *E. coli* with same problem of our study[9].

Pichia pastoris is a methylotropic yeast capable of utilizing methanol as a sole carbon source. The AOX I promoter, which regulates the AOX I gene allows for the production of the alcohol oxidase enzyme required for the oxidation of methanol and also allows for the overexpression of heterologous protein genes introduced downstream in a *Pichia* expression vector. This allows for the production of 10–100 times more recombinant protein than does the traditionally utilized *Saccharomyces cerevisiae* expression system[10]. Methanol induced *P. pastoris* cultures grown in shake-flask results in expression levels of approximately 5% of total soluble proteins. However, this level is increased to > 30% of total proteins in cell grown in fermenter cultures utilizing growth limiting rates of methanol. A key factor is the preferential use of the *P. pastoris* system for respiratory growth, which facilitates its culturing at high-cell densities relative to fermentation yeasts such as *Saccharomyces cerevisiae*[8,11,12]. The *P. pastoris* expression system allows for the recombinant protein to either be expressed intracellularly or to be secreted into the growth medium. Secretion is said to be the first step in purification of heterologous proteins produced from the *Pichia* system because it separates the recombinant protein from the bulk of the cellular proteins which are present in low concentrations in *Pichia* cells[13,14]. Hence, the recombinant protein will form a major portion of the total protein in the expression medium. The *Pichia* vectors carry the secretion signal sequence from the Saccharomyces cerevisiae α-factor prepropeptide located downstream from the AOX I promoter and upstream from the heterologous protein gene, thus facilitating secretion of the expressed protein.

The vector used in our studies is the p819 vector designed by Invitrogen Corporation (Invitrogen Corporation, Calif) and improved by Chinacdc (Chinese Center for Disease Control and Prevention). This vector contains functional sites which are designed for ease of integration of foreign protein genes, selection of transformants, purification and identification of the recombinant protein. Several investigators including Eldin *et al* [13] and Baumgartner *et al*[15] have utilized the convenience of the secretion signal and hexahistidine tag contained in the *P. pastoris* vectors to express and purify recombinant proteins in a one-step purification procedure using affinitymetal-chelating chromatography. Eldin *et al*[11] purified two single-chain antibody fragments, which were expressed at 250 mg/L and 60 mg/L, respectively. Baumgartner's group expressed and purified the kidney bean lectin phytohemagglutinin E-form, which was secreted in the expression medium to levels of 16 mg/mL. The use of both the methanol-utilizing strain *P. pastoris* GS115 (mut^+) and the slow methanol-utilizing strain *P. pastoris* KM71H (muts) to express various heterologous proteins is well documented.

The objective of this research is to investigate the suitability of the *P. pastoris* expression system as a mean of recombinant expression of Lz-8. The rationale for the use of this expression system in our research is based on the previously outlined features of the *P. pastoris* expression system as well as the widely documented success of previous works utilizing this system. Other expression systems such as the *Escherichia coli* expression system was not used in our research because Lz-8 is being investigated as a potential medicine and food preservative, *E. coli* is perceived as being a dangerous pathogen, therefore using *E. coli* would not have been appropriate for the expression of Lz-8 from a consumer acceptance perspective, and therefore using *E. coli* would not achieve our objective. However, *P. pastoris* is ideally suited as a means of producing lz-8 for human use,

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because *Pichia* cells have been previously used as a source of animal feed by the Phillips Petroleum Company, and would therefore be much more acceptable from a consumer standpoint.

1 Materials and methods

1.1 Vector selection and cloning

The EasySelect *Pichia* Expression System was used for these genetic engineering studies. All primers, vectors, and host cells were obtained from Invitrogen Corporation (Invitrogen Corporation, Calif). The vector (p819) was obtained from China CDC, Lz-8 DNA sequence and primers were synthesized by Shanghai Songon Co., Ltd. The Lz-8 cDNA was cloned into the p819 vectors and returned to our laboratory for transformation and expression experiments. The secretion signal sequence from the *Saccharomyces cerevisiae* α-factor prepropeptide can target Lz-8 to the secretory pathway, it was cloned in the vector pPIC9K and linked with Lz-8 cDNA by restrictive enzyme sites in the vector (p819).

1.2 Transformation of *P. pastoris* host strains

P. pastoris commonly used in heterologus protein expression were selected for transformation with the Lz-8 cDNA in our laboratory; *Pichia* host cells Gs115(mut$^+$), a mutant type *Pichia* strain containing the AOX I promoter which allows for rapid growth while utilizing methanol as the sole carbon source; The cultures were made competent and the transformed in our laboratory by introduction of the linearized p819 vectors into these cells using the EasySelect *Pichia* Expression System. (Invitrogen Corporation). Transformed cells were selected by growth on yeast extract peptone dextrose (YPD) agar plates containing G418 (2000 μg/mL).

1.3 Preparation of competent cells

Pichia host cells were grown in YPD broth overnight (30^{o}C, 250 r/min) and prepared for transformation with the p819 vector containing the Lz-8 insert, according to the manufacturer's recommendations (Invitrogen Corporation). Competent cells were stored at −80^{o}C until the time of transformation.

1.4 Determination of methanol-utilizing (mut) phenotype

In order to determine the length of time required for expression of recombinant protein by the transformed *P. pastoris* host cells, it is necessary to determine the methanol utilization (mut) phenotype of the strain. The mut phenotype for the transformed *Pichia* Gs115 strain was determined by the procedure outlined in Invitrogen's Easy Select Pichia expression manual. Briefly, growth of cells on minimal media with dextrose (MD) was compared to growth on minimal media using methanol (MM) in place of dextrose. Mut$^+$ cells grow normally on both media while Muts cells grow normally on minimal media with dextrose but show negligible growth on minimal media with methanol.

1.5 Expression experiments

The isolated transformed colonies from each host strain were tested for expression of recombinant Lz-8. Expression experiments were conducted according to the methods used by Feng *et al* [14] and Yan *et al* [15], based on the procedure outlined in Invitrogen's easy select *Pichia* expression manual, with some modifications. Aliquots of expression medium were removed (timepoints 0, 6, 12, 24, 36, 48, 60, 72, 84, 96 hours) for *P. pastoris* Gs115 strain. Simultaneously, 2 colonies of *P. pastoris* GS115 blank were used as (mut$^+$) expression control. Cell pellet and supernatant were separated and frozen in liquid nitrogen and stored at −70^{o}C for analysis by polyacrylamide gel electrophoresis (SDS-PAGE) and MALDI-TOF-MS.

1.6 SDS-PAGE analysis

Time-point samples of supernatant were desalted and concentrated to approximately 5%−10% volumn by ultrafiltration and used for electrophoretic analysis and MALDI-TOF-MS. Both the control samples were also analyzed by SDS-PAGE and Malti-tof MS. Electrophoresis was performed using Bio-Rad's Mini Protean II System (Bio-Rad Laboratories, Hercules).

1.7 Polymerase chain reaction (PCR) and sequencing

PCR analysis was conducted on transformed cells of both *Pichia* strains, in order to confirm whether the Lz-8 DNA was actually integrated into the *P. pastoris* genome.

Two clones, two from each host strain [*P. pastoris* Gs115(mut$^+$)] were tested according to standard protocol for PCR analysis. DNA was extracted from cells transformed with the vector carrying the Lz-8 cDNA, using the Easy DNA Kit for genomic DNA isolation (Invitrogen Corporation). PCR products of the extracted DNA were then purified for sequencing using Qiagen's QIAquick purification kit (Qiagen, Valencia). Purified PCR fragments were sequenced (Shanghai Songon Co., Ltd.), in order to ascertain orientation of inserted cDNA into the *Pichia* host cell genome, as well as to reveal whether there were any mutations in the inserted fragment.

1.8 Agarose gel electrophoresis of PCR product

Samples of PCR product were loaded onto 1%

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agarose gel for electrophoretic separation (100 volts for 35 min). Separated bands were visualized using ultraviolet light after staining in ethidium bromide (1 μg/mL) in Trisacetate-EDTA (TAE) buffer (18 min).

1.9 MALDI-TOF-MS

MALDI-TOF-MS spectra were detected using LDI 1700 (Linear scientific Inc. USA). First, 1 mg/mL PPO I was resolved in a 0.1% solution of trifluoroacetic acid and sinapinic acid. The 1 μL sample was trickled into the sample probe target, crystallized by the laser and detected. The result was the sum of 30 times. The scanning range was from 500–200 000 D.

1.10 Hemagglutination assay

Human bloods of all four types (A, B, AB, and O) were obtained from healthy volunteers who approved the use of their blood for this study. Sheep whole blood was obtained from the People Hospital of Jilin Province was used within 1 month after being received. Six milliliter of whole blood (human or sheep) were centrifuged at 1200×g for 10 min. The plasma was removed, and two milliliter of packed red blood cells were collected from the bottom of the tubes. The cells were washed five times with 10 mL of PBS and centrifugation at 1200×g for 10 min and then suspended to 1.5% (*V*/*V*) with PBS. Suspensions of 25 μL of purified rLz-8 (50 μg/mL to 30 ng/mL final concentration) in PBS, 75 μL of 0.2% gelatin in PBS, and 25 μL of 1.5% red blood cells placed in 96-well microtiter plates (round bottom, Nunc) were shaken for 30 s at room temperature and then incubated at 37°C. The plates were examined for hemagglutination after 12 h.

1.11 Macrophage cytophagocytesis

RLz-8 was used for experimental groups in three levels (0.05 mg/kg, 0.1 mg/kg, 0.2 mg/kg; ICR mouse, 18–22 g). IFN-γ (7×104 IU/kg) was used for positive control. Daily dosage of mice was peritoneal injection for 7 days(7 times), injected 5% chicken red blood cell suspension(0.5 mL) to abdominal cavities of mice, after 30 min, injected PBS (2 mL), drew off 0.5 mL and dropped it on microscope slide, 37°C incubated 30 min and wash with PBS, observed and recorded the results in the microscope.

2 Results and discussion

2.1 Expression experiments results

*Eco*R I/*Asu* II double digest the expression vector (p819-Lz-8), agarose gel electrophoresis results showed DNA band at appx 620 bp, which correspond with length of Lz-8 cDNA. Sequencing result accorded with the cDNA sequence of α-factor-Lz-8 fusion protein (Fig. 1).

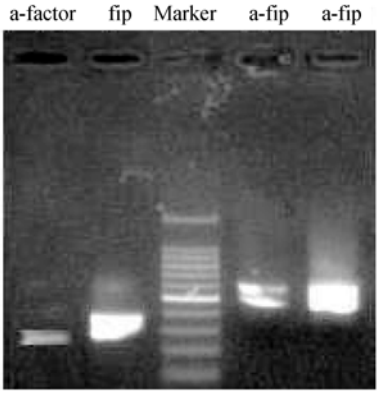

Fig. 1 Result of α-factor-fip (Lz-8).

The methanol utilization (mut) phenotype of the *Pichia* host strain *P. pastoris* Gs115 transformed with Lz-8 cDNA was confirmed to be mut$^+$ phenotype as shown in Fig. 2.

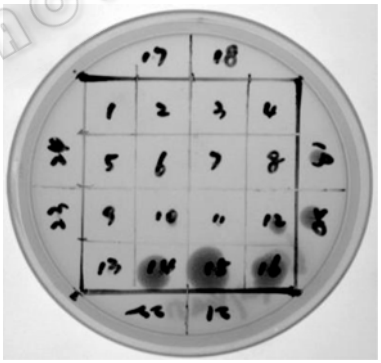

Fig. 2 Methanol utilization (mut) phenotype of the *Pichia* (2 mg/mL G418).

Initial expression experiments with transformed *P. pastoris* cells showed that SDS polyacrylamide gel was showing rLz-8 band at approximately 14 kD (Fig. 3), The results of MALDI-TOF-MS showed the molecular weight of rLz-8 was approximately 12 722.00 D (Fig. 4), which correspondwith theoretical Mr. 12 509.95 D (http://expasy.org/cgi-bin/pi_tool).

The purification methods refer to our another study paper which has been published[16]. The quantity of expressing rLz-8 in *P. pastoris* GS115 could reach 20 mg/L, which still was comparatively low quantity, but we made technical improvements in the large scale fermentation (2000 L) of *P. pastoris*, quantity has increased to 800 mg/L.

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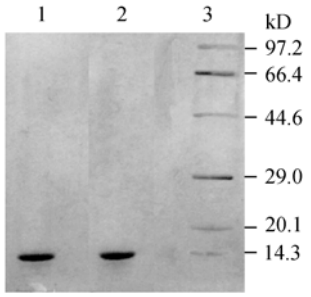

Fig. 3 Result of SDS-PAGE of rLz-8. 1: rLz-8; 2: rLz-8; 3: protein molecular marker.

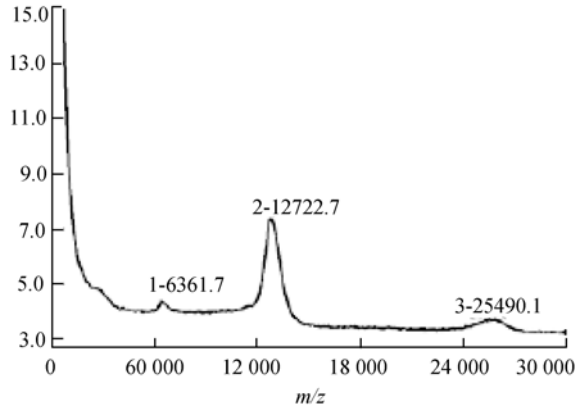

Fig. 4 MALDI-TOF-MS results.

2.2 Hemagglutination reaction results

Using human red blood cells of all four types (A, B, AB, and O), and sheep red blood cells, the hemagglutination activity of rLz-8 was examined. No aggregation was observed between any types of human red blood cells in the presence of purified rLz-8 within the concentration range of 0.05–3 μg/mL. However, with sheep red blood cells, aggregation was seen in the presence of rLz-8 at concentrations greater than 12.5 μg/mL. For a control, any of the types of human red blood cells or sheep red blood cells became aggregated in the presence of PHA. The effect of monomer and disaccharides on the hemagglutination activity of rLz-8 on sheep red blood cells was examined(Tables 1, 2).

2.3 Macrophage dytophagocytesis results

Some cells, like chicken blood red cells, can take up small particulate matter by surrounding the particle with extrusions of the cell membrane by a process termed phagocytosis. Macrophage phagocytosis of micro-organisms is important in host immunity and activated macrophages kill ingested pathogens by production of reactive oxygen and nitrogen metabolites.

Table 1 Hemagglutination activity of Lz-8 on sheep red blood cells

Groups	Blank	Lz-8 (μg/mL)						
		50.00	25.00	12.50	6.25	3.13	0.78	0.05
A	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–
O	–	–	–	–	–	–	–	–
AB	–	–	–	–	–	–	–	–
Sheep	–	+++	++	+	–	–	–	–

Table 2 Hemagglutination activity of PHA on sheep red blood cells

Group	PHA (μg/mL)						
	50	25	12.5	6.25	3.13	1.56	0.78
A	++++	++++	+++	+++	++	+	–
B	++++	++++	+++	++	++	+	–
O	++++	++++	+++	+++	++	+	+
AB	++++	++++	+++	++	++	+	–
Sheep	++++	++++	+++	+++	++	+	–

Medically important pathogens including the mycobacteria which cause tuberculosis and leprosy evade immune destruction by surviving inside the macrophage. As shown in the Fig. 5, the quantity of red blood cells which was phagocytized by macrophage was in direct proportion to the dose of rLz-8, they had a dose-dependent relationship. The percent and index of cytophagocytesis was shown as below (Fig. 5, Table 3).

Among active ingredients found in traditional Chinese medicines, certain fungal lectins have been found to possess beneficial effects in modulating the immune system and in inhibiting tumor growth. FIPs have been shown to possess stimulatory activities toward human peripheral blood leucoctyes.

Therefore, FIPs are adequate candidates for developing new therapeutic agents used in the treatment

Table 3 The percent and index results of cytophagocytesis

Group	Phagocytic percent (%)	Phagocytic index
Normal control	17.17±1.47	0.20±0.02
TFN (7×10^4 IU/kg)	36.17±3.06	1.64±0.04
rLz-8 low dose 0.05 mg/kg	39.00±2.37	1.67±0.04
rLz-8 mide dose 0.1 mg/kg	63.33±2.16	3.58±0.04
rLz-8 high dose 0.2 mg/kg	82.83±3.06	4.85±0.04

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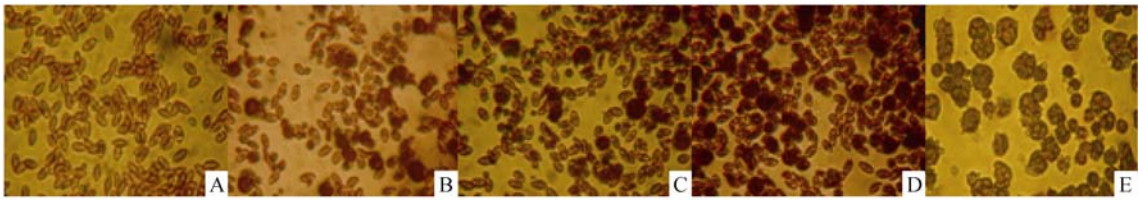

Fig. 5 Macrophage cytophagocytesis induced by rLz-8. (A) Normal control. (B) IFN(7×10^4 IU/kg). (C) rLz-8(dose 0.05 mg/kg).

of cancer and autoimmune diseases. Direct extraction of native FIPs from mushrooms of Lingzhi, however, is costly and time-consuming. As reported earlier, approximately 5–10 mg of purified rLz-8 was obtained from 340 g of mycelia. Moreover, the biological activities of the purified FIPs are usually dependent on the quality and quantity of Lingzhi sources. Obviously, a stable supply of active FIPs of high purity is indispensable for further evaluation of these candidate proteins for oral administration and medicinal applications. Previously, Lz-8 was produced in *E. coli* and used for protein characterization. However, the rLz-8 extracted from *E. coli* cells appeared to be unsuitable for oral administration due to the potential problem of endotoxin contamination in the protein preparation. To avoid this potential problem, active rLz-8 of high yield and purity was produced in yeast cells by the *P. pastoris* expression system in this study. The native Lz-8 as well as other FIPs has been identified as a glycoprotein, *P. pastoris* expression system makes glycosylation for recombinant Lz-8, is lack of glycosylation was functionally expressed in *E. coli*, and some data indicated that glycosylation of recombinant Lz-8 had more biology activity. Obviously, the glycosylation of Lz-8 is essential for its biological activity, in our crystal structure of rLz-8 also confirm the argument about glycosylation.

It has been reported that expression of IL-2 and interferon was induced in spleen cells of Lz-8 treated mice, implying that Lz-8 exerted its pharmacological effect by modulating the Th-1 subset of T-cells and up regulating IL-2 receptors on the surface of T cells. Hence, to assess the immunomodulatory potential of Lz-8, some study teams detected the expression of IL-2 on the lymphocyte of murine splenocytes. As expected, the rLz-8 produced in both *P. pastoris* showed elevating effects on the release of IL-2 from murine splenocytes, but lower specific immunomodulatory activity as observed for rLz-8 produced in *E. coli* cells than in that produced in *P. pastoris*. The lower specific immunomodulatory activity of rLz-8 produced in *E. coli* cells might result from improper folding of a eukaryotic protein in prokaryotic cells.

Apparently, rLz-8 produced in *P. pastoris* cells is a better source than that produced in *E. coli* cells for evaluating its potential applications in administration. We have finished analysis of crystal structure and large scale production of fermentation (100 L), which can provide basic for further researches about immunologic mechanism of Fungi immunoregulatory proteins family and recombinant protein production of Chinese medical drugs.

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综述	Reviews	内容要求新颖丰富, 观点明确, 论述恰当, 必须包含作者自己的工作内容和见解
小综述	Mini-review	能尽快反映生物学领域的最新进展的小型综述
科学进展/名家论坛	Perspective	邀请生物领域专家对最新生物技术科研成果进行评述
动物及兽医生物技术	Animal and Veterinary Biotechnology	转基因动物；动物生物反应器；动物疾病控制、免疫生物技术
海洋生物技术	Marine Biotechnology	海洋生物资源；海洋生物基因组学、蛋白质组学和代谢组学；海水养殖生物技术；藻类生物技术；海洋天然产物；海洋生物产品、生物材料和生物能源
环境生物技术	Environmental Biotechnology	微生物多样性；分子生物学方法及应用；环境过程的生物监测；污染控制新工艺；生物修复
工业生物技术	Industrial Biotechnology	工业酶和微生物；代谢工程与应用；生物催化剂与生物转化；工业生物原料、生物燃料、生物能源和生物基化学品；生物过程工程；生物工程控制及优化
农业生物技术	Agricultural Biotechnology	农业功能基因组；植物生物反应器；转基因作物；转基因产品的生物安全及转化；农业重组微生物
食品生物技术	Food Biotechnology	食品微生物及功能性食品；转基因食品；食品安全及公众健康
系统生物技术	Systems Biotechnology	系统生物学理论在工业、医药、环境、农业生物技术领域的应用
医学与免疫生物技术	Medical and Immunological Biotechnology	基因治疗和干细胞治疗 RNAi 技术及应用；生物标记与诊断；单克隆抗体；工程蛋白和疫苗
组织工程与细胞培养	Tissue Engineering and Cell Cultivation	细胞发育与生物材料；干细胞工程；细胞培养工程
生物技术与方法	Methods in Biotechnology	生物技术各个领域中的方法学

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