用于生产重组蛋白药物的抗凋亡 CHO 宿主细胞株的建立

来大志 付 玲 于长明 齐连权 翁少洁 于 婷 王海涛 陈 薇*

(北京微生物流行病研究所,北京 100071)

摘 要 哺乳动物工程细胞在大规模培养生产重组蛋白时很容易发生细胞凋亡,从而导致生产过程提前终止,造成生产成本高昂。细胞代谢产物氨已被证明可以促进细胞凋亡,而线粒体膜整合蛋白 Bcl-2 可以通过促进线粒体膜完整性而抑制细胞凋亡。本实验应用谷氨酰胺合成酶加压系统在 CHO 工程细胞中高效表达中国仓鼠 Bcl-2 蛋白,使细胞具有抗凋亡能力的同时,利用谷氨酸和氨合成谷氨酰胺而有效降低培养基中氨的含量,从而达到抑制细胞凋亡的目的。

关键词 细胞凋亡, CHO 细胞, Bcl-2, 谷氨酰胺合成酶, 氨

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哺乳动物细胞表达系统具有准确的转录后修饰功能,与原核细胞表达系统和酵母细胞表达系统相比,表达的糖基化蛋白在分子结构、理化特性和生物学功能方面最接近天然蛋白分子,是目前重组糖蛋白生产的首选体系。对于较复杂的分子如抗体,以及基因治疗用病毒载体,哺乳动物细胞是唯一的表达宿主。但哺乳动物细胞表达系统的缺点也很明显。如细胞在大规模培养时容易发生细胞凋亡[1,2],从而导致生产周期缩短而使生产成本高昂。在大规模培养中,各种轻微的不利条件即可引起细胞凋亡,如溶氧过高或过低,营养或血清缺乏,有害代谢产物积累等。仅依靠优化这些外部培养条件很难彻底解决细胞的凋亡问题。因而改造宿主细胞本身使之更易于培养,已成为当今另一研究热点[3]。细胞凋亡由各种凋亡信号诱导,并由一系列基因产物精确调控。Bcl-2 蛋白是其中的一个重要调节组分。它通过增强线粒体膜的完整性而阻止细胞色素 C 的释放,从而阻止 Caspases 蛋白酶家族的激活而抑制细胞凋亡[4]。通过过表达 *Bcl-2* 基因,已使多种工程细胞系包括 CHO 细胞、杂交瘤细胞、骨髓瘤细胞、淋巴瘤细胞以及昆虫细胞等获得了不同程度的抗凋亡能力[3]。细胞代谢产物氨对于细胞凋亡也起到明显的促进作用。细胞谷氨酰胺合成酶(GS)可以利用氨和谷氨酸合成谷氨酰胺,因而可以通过过表达 *GS* 基因,并且在无谷氨酰胺组分的培养基中培养细胞,使细胞通过氨和谷氨酸合成谷氨酰胺供给自身营养需要,而达到降低培养基中氨的目的。谷氨酰胺是必须氨基酸,*GS* 基因同时可以做为筛选扩增系统,应用硫氨甲硫氨酸(MSX,GS 的抑制剂)加压,可使表达载体在细胞中的拷贝数增加 30 倍以上[5]。本实验应用 GS 加压系统在 CHO 细胞中高表达中国仓鼠 Bcl-2 蛋白,在内源调控细胞抗凋亡能力同时,外源降低培养基中氨的含量,从而有效抑制了培养细胞的凋亡率。

1 材料与方法

1.1 中国仓鼠 Bcl-2 cDNA 的克隆

从 CHO 细胞中提取 RNA 和反转录合成 cDNA 按 Rneasy Mini Kit(Qiagen 公司产品)和 SuperScript™ Preamplification System for First Strand cDNA Synthesis (Gibco 公司产品)说明书操作。根据已发表文献[6],设计套式 PCR 引物。

外引物 正向:TGTGGGGCGGGAGTCAGG;

反向:GCATATTTTGGGGAGCAGGTC;

内引物 正向:TATCCCGGGCCACCATGGCTCAAGCTG-GGAGAAC;

反向:GCGTCTAGATTATCACTTGTGGCCCAGGTAGG。

为方便克隆进表达载体,内引物加入了 *Sma* Ⅰ

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* 通讯作者。 Tel:86-10-66948565;Fax:86-10-63815273;E-mail:cw8966@yahoo.com

和 *Xba* Ⅰ位点。PCR 扩增条件：第一轮：94℃ 40s，52℃ 50s，72℃ 50s，用外引物，共 20 循环；第二轮：94℃ 40s，56℃ 50s，72℃ 50s，用内引物，共 30 循环。其中高保真 DNA 聚合酶 Pyrobest 购自日本 TaKaRa 公司。回收扩增片段，在只有 dATP 的存在下用 *Taq* DNA 聚合酶加 A，并克隆至 pGEM-T 载体（Promega 公司产品）中，转化宿主菌 XL1-Blue（本室保存），挑取阳性克隆，并由上海生工公司完成测序。

1.2　含 GS 共扩增基因的 Bcl-2 表达载体的构建

中国仓鼠谷氨酰胺合成酶（GS）基因由本室童贻刚博士克隆，并替换真核表达载体 pCI-neo（Promega 公司产品）的 *neo* 基因，构建成新型表达载体 pCI-GS。以 *Sma* Ⅰ和 *Xba* Ⅰ从 pGEM-T 载体中切下 Bcl-2 基因，并克隆进以相应酶切割过的 pCI-GS 载体中，即为 pCI-GS-Bcl2（图 1）。

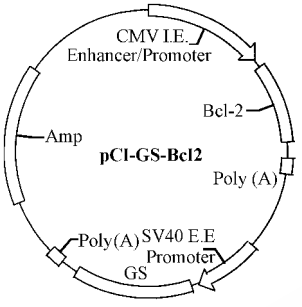

图 1　表达载体 pCI-GS-Bcl2

Fig.1　The expression vector pCI-GS-Bcl2

1.3　细胞转染及 Bcl-2 高表达株的筛选

Jkws 为本室构建的高表达人 β-干扰素的 CHO 细胞系。应用 Lipofectamine™ 2000（Gibco 公司产品）把 pCI-GS-Bcl2 转染至 Jkws 细胞中。转染后 24h，分板克隆，并更换为不含谷氨酰胺的 RPMI1640/10% FCS/25μM MSX 培养基培养。MSX（硫氨甲硫氨酸，Methionine Sulfoximine），是谷氨酰胺合成酶的抑制剂，购自 Sigma 公司。培养约 2 周，待单一细胞克隆出现后，挑取 20 个克隆，放大培养，每个细胞克隆分别转接入 4 个培养皿中，分别加入 100、200、500、1000μmol/L MSX 加压扩增。经一轮扩增，每个能耐受最高浓度的原始克隆被挑选出来，进一步进行 Western Blot 分析，筛选表达 Bcl-2 较高的细胞株：基因扩增后的细胞接入 10cm 培养皿扩大培养至完全汇片，胰酶消化下细胞，PBS 洗 1 遍，细胞重悬于 400μL 裂解缓冲液（50mmol/L Tris-HCl，pH8.0；150mmol/L NaCl；0.1% SDS；1% Triton X-100；0.5mmol/L PMSF）中冰浴 30min。10 000 × g 离心 5min，上清进行浓度为 12% 的 SDS-PAGE 电泳，而后应用 BioRad 公司的转膜系统将蛋白转至 PVDF 膜（Millipore 公司产品），5% 脱脂奶粉/PBS 封闭膜 2h，加入兔抗 Bcl-2 蛋白多抗（Santa Cruz 公司产品），反应 1h，洗涤，加入碱性磷酸酶标记的羊抗兔二抗，反应 1h，充分洗涤；应用 Western Blue® 显色液（Promega 公司产品）显色。

1.4　氨含量测定

应用英国 Randox 公司血氨检测试剂盒按说明书进行测定。

1.5　抗凋亡活性分析

转化和没有转化 Bcl-2 基因的 Jkws 细胞分别用无血清培养基培养 96h 诱导细胞凋亡，应用 Clontech 公司 ApoAlert® Annexin V 试剂盒检测细胞凋亡。

1.6　人 β-干扰素活性测定

参照文献[7]。

2　结　果

2.1　中国仓鼠 Bcl-2 cDNA 的克隆

本实验成功克隆出中国仓鼠 Bcl-2 的 cDNA，其序列已登录 GenBank（AF404339）。

2.2　Bcl-2 高表达株的筛选

应用 Western blot 分析，我们从经过一轮 MSX 加压扩增的 20 个细胞克隆中，选择出 3 个高表达 Bcl-2 蛋白的细胞株：7G，2A 和 5F。它们最高 MSX 耐受浓度分别是 200μmol/L、200μmol/L 和 500μmol/L，并且 5F 的表达量最高（图 2）。5F 细胞株经无压力连续培养 4 周，Western blot 检测 Bcl-2 的表达量没有显著变化，证明该细胞株可以稳定高表达 Bcl-2。此细胞株重命名为 Jkws-5F，供进一步分析用。

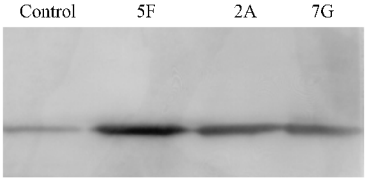

图 2　应用 Western blot 筛选 Bcl-2 蛋白的高表达细胞株

Fig.2　Screening of clones expressing high level of Bcl-2 by Western blot

2.3　Jkws-5F 细胞株具有较低的代谢产氨率

Jkws 和 Jkws-5F 细胞株分别接种 7×10^5 个细胞于 75cm² 细胞培养瓶。分别应用 RPMI1640/10% FCS 和不含谷氨酰胺的 RPMI1640/10% FCS/500μmol/L MSX 培养基连续不换液培养 10d，每隔 1d 取样测量培养基中氨浓度，结果如图 3 所示。Jkws-5F 细胞培

养基中氨浓度显著低于 Jkws 细胞培养基中氨浓度。而且随着培养时间的延长，这种差别逐渐加大。当细胞培养到第 8 ~ 10 天时，由于营养耗尽，细胞已经死亡过半，但此时 Jkws-5F 细胞培养基中氨的浓度仅约为 Jkws 细胞培养基中氨浓度的 1/2。表明 Jkws-5F 细胞具有较高的氨清除效率。

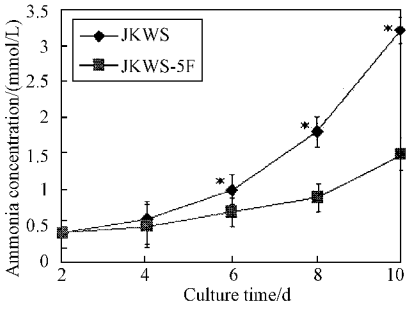

图 3 两株细胞培养基中氨含量相对于培养时间的变化

Fig.3 Ammonia concentration versus culture time in conditioned media of Jkws and Jkws-5F cell lines

The Bars represent the standard deviation of three independent measurements, and asterisks represent significant difference between the two groups tested ($P < 0.05$)

2.4 Jkws-5F 细胞株比 Jkws 具有更强的抗凋亡能力

Jkws 和 Jkws-5F 分别接种 10^5 个细胞于 25cm^2 细胞培养瓶中，分别应用 RPMI1640/10% FCS 和不含谷氨酰胺的 RPMI1640/10% FCS/500μmol/L MSX 培养基培养 24h，而后换为无血清培养基继续培养 96h 诱导细胞凋亡，同时设未诱导对照。贴壁细胞经胰酶消化及含血清的新鲜培养基洗涤 1 遍后，应用增强型绿色荧光蛋白(EGFP)标记的 Annexin V 染色。凋亡细胞由于细胞膜外翻使磷脂酰丝氨酸(PS)暴露于细胞膜外，很容易与 Annexin V 结合而被染色。经流式细胞分析，结果如图 4 所示。未经无血清诱导凋亡的 Jkws-5F 细胞，经过 96h 连续培养，仅有 19% 的细胞发生了细胞凋亡；未诱导凋亡的 Jkws 细胞的凋亡率基本相同(图中未显示)。经过无血清诱导凋亡后，两种细胞系的绝大部分细胞均发生了凋亡：Jkws-5F 的凋亡率为 54%，而 Jkws 的凋亡率为 84%。Jkws-5F 的凋亡率显著低于 Jkws 的凋亡率，说明应用本实验方案同时高表达 Bcl-2 蛋白和降低培养基中氨含量确实有利于培养细胞的存活。

2.5 在不利条件下，Jkws-5F 细胞株表达人 β-干扰素的水平显著高于 Jkws 细胞株

Jkws 和 Jkws-5F 分别接种 10^5 个细胞于 25cm^2 细胞培养瓶中，分别应用 RPMI1640/10% FCS和不含

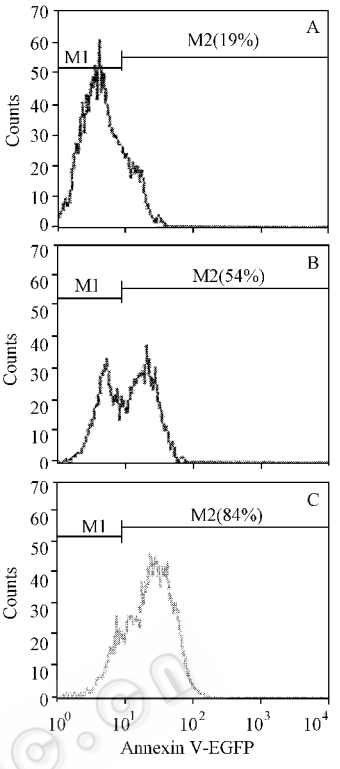

图 4 应用流式细胞术检测细胞凋亡

Fig.4 Detection of apoptosis by flow cytometry

A. Jkws-5F without inducement of apoptosis, only 19% underwent apoptosis; B. Jkws-5F with inducement of apoptosis, 54% underwent apoptosis; C. Jkws with inducement of apoptosis, 84% underwent apoptosis. Apoptosis rate was estimated by a simple gating method: EGFP strength higher than 10^1 was considered as apoptosis based on the peak form in (B)

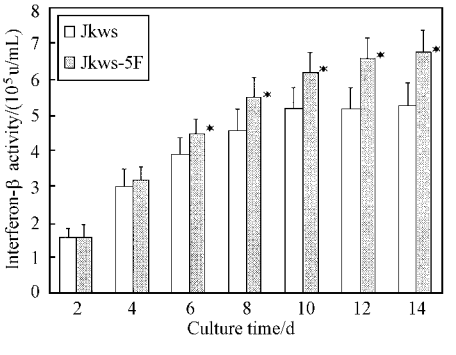

图 5 2 株细胞 β-干扰素表达水平相对于培养时间的变化

Fig.5 Interfeon-β production versus culture time of Jkws and Jkws-5F cell lines

The Bars represent the standard deviation of three independent measurements, and asterisks represent significant difference between the two groups tested ($P < 0.05$)

谷氨酰胺的 RPMI1640/10% FCS/500μM MSX 培养基连续不换液培养 2 周。随着营养的耗尽，细胞将逐渐凋亡，以利于比较两种细胞在不利条件下表达外

源蛋白能力的差别。隔日分别取样测定 β-干扰素活性。结果如图 5 所示。Jkws-5F 细胞株 β-干扰素的表达量显著高于 Jkws 细胞株,且随着培养时间的延长,这种差别日趋明显。至第 14 日,Jkws-5F 细胞株 β-干扰素的表达量比 Jkws 细胞株的表达量提高了 30%。这种现象的产生可能在于 Jkws-5F 细胞株具有更强的抗凋亡能力,在不利条件下,能够维持更高的活细胞数,所以外源蛋白的表达量也较高。

3　讨　论

哺乳动物细胞的大规模培养对于生产重组蛋白至关重要。但由于培养难度较大,国内目前上市的生物工程药物中,仅 EPO 是应用哺乳动物细胞表达系统生产的。哺乳动物细胞大规模培养的难点主要来自于细胞凋亡[3]。由于哺乳动物细胞对培养条件十分敏感,各种不利条件均可引起细胞凋亡。尤其大规模生产重组蛋白时往往需要应用无血清培养基培养,而在无血清条件下细胞更容易发生凋亡,这使得培养基的合理设计和培养条件的精确调控显得尤为重要。不同的细胞系对于不同培养条件的适应性各不相同,针对某一特定细胞系,必需设计相应的培养方案,难度很大。如果通过改造宿主细胞自身,使之'强壮'起来,能够降低对培养条件的苛求,似乎是一个很好的解决方案。国外对宿主细胞改造的研究已经进行了多年,并且取得了一些成果。如通过过表达 Vitronectin 使细胞获得在无血清条件下贴壁生长能力;通过过表达 Bcl-2 基因使细胞获得抗凋亡能力;通过敲除乳酸脱氢酶基因,降低有害代谢产物乳酸的积累而抑制细胞凋亡;以及通过可控诱导表达生长刺激因子、周期蛋白或细胞周期抑制蛋白而控制细胞周期,从而增加细胞密度及细胞活力[3]。最近有报道通过过表达细胞凋亡通路关键蛋白酶 Caspase-3 的抑制蛋白 XIAP[8] 或应用反义核酸技术[9]抑制 Caspase-3 而控制细胞凋亡。本实验尝试对生产 β-干扰素的工程细胞株 Jkws 进行了改造。该细胞株在大规模培养时,培养条件很难控制,细胞很容易发生凋亡,因而生产周期较短,使得生产成本较高。通过过表达 Bcl-2 而抑制细胞凋亡在众多哺乳动物细胞系中都有成功的报道[3]。所有报道的方案均应用人 *Bcl*-2 基因。比较 GenBank 提供的人 *Bcl*-2 基因序列和本研究克隆的中国仓鼠的 *Bcl*-2 基因序列,发现它们之间同源性超过 90%,但仍然存在一定差别。本实验直接应用中国仓鼠 *Bcl*-2 基因抑制细胞凋亡,有可能优于应用人 *Bcl*-2 基因(本实验没做比较)。谷氨酰胺合成酶(GS)加压系统很早就被用于外源基因的加压扩增。该系统由于应用氨和谷氨酸合成谷氨酰胺供细胞利用,因而可以极大地降低培养基中氨的浓度。而高浓度的氨已被证实是引起细胞凋亡的一个原因[10]。所以应用 GS 加压系统进行外源基因的扩增,有利于细胞的大规模培养。本实验应用 GS 系统对 *Bcl*-2 基因进行加压扩增,在高表达 Bcl-2 的同时,极大地降低了培养基中氨的浓度,从而对细胞凋亡起到了双重的抑制作用。

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Construction of an Anti-apoptosis CHO Cell Line for Biopharmaceutical Production

LAI Da-Zhi FU Ling YU Chang-Ming QI Lian-Quan WENG Shao-Jie
YU Ting WANG Hai-Tao CHEN Wei*
(*Beijing Institute of Microbiology and Epidemiology*, *Beijing* 100071, *China*)

Abstract Mammalian cells are prone to apoptosis when cultured in large scale for production of biopharmaceuticals. And this will reduce production duration and result in high cost of production. Apoptosis is triggered by various factors, and delicately regulated by a set of genes. Bcl-2, a component integrated in mitochondria membrane, is an important member of these genes. By maintaining the integrity of mitochondria membrane, Bcl-2 keeps cytochrome C from releasing into cytoplasm, and thus blocks the activation of caspases, and subsequent onset of apoptosis. Over-expression of Bcl-2 has proven to be useful in blocking apoptosis in various cell lines, including CHO, hybridoma, myeloma, lymphoma and insect cells. Ammonia, a metabolite of cultured cells, however, showed apparent pro-apoptosis activity. In living cells, ammonia can be utilized by glutamine synthetase (GS) to synthesize glutamine, and thus lower the concentration of ammonia in medium, and its negative effects. Glutamine is essential to living cells. If not added into medium, glutamine can only be synthesized by GS, which makes GS a qualified selection marker. This marker can be used for gene amplification by adding into medium increased concentration of MSX, an inhibitor of GS. In this study, we over-expressed Bcl-2 using GS amplification in a recombinant CHO cell line stably expressing human interferon-β. The modified cell line, with higher expression of Bcl-2 and lower production of ammonia, exhibited good anti-apoptosis quality and higher interferon-β production in continuous culture.

Key words apoptosis, CHO cells, Bcl-2, glutamine synthetase, ammonia

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* Corresponding author. Tel: 86-10-66948565; Fax: 86-10-63815273; E-mail: cw789661@yahoo.com

Purification and Properties of *Bacillus subtilis* SA-22 Endo-1,4-β-D-mannanase

YU Hong-Ying[1] SUN Yuan-Ming[2*] WANG Wei-Jun[3] YANG Yue-Sheng[3] YANG You-Hui[2]
([1] *Faculty of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou* 510090;
[2] *College of Food Science*; [3] *College of Life Science, South China Agriculture University, Guangzhou* 510642, *China*)

Abstract β-mannanase (EC 3.2.1.78) from *Bacillus subtilis* SA-22 was purified successively by ammonium sulfate precipitation, hydroxyapatite chromatography, Sephadex G-75 gel filtration and DEAE-52 anion-exchange chromatography. Through these steps, the enzyme was concentrated 30.75-fold with a recovery rate of 23.43%, with a specific activity of 34780.56 u/mg. Molecular weight of the enzyme was determined to be 38kD by SDS-PAGE and 34kD by gel filtration. The results revealed that the optimal pH value for the enzyme was 6.5 and the optimal temperature was 70℃. The enzyme is stable between pH 5 to 10. The enzyme remained most of its activity after a treatment of 4h at 50℃, but lost 25% of activity at 60℃ for 4h, lost 50% of activity at 70℃ for 3h. The enzyme activity was strongly inhibited by Hg^{2+}. The Michaelis constants (K_m) were measured as 11.30 mg/mL for locust bean gum and 4.76 mg/mL for konjac powder, while V_{max} for these two polysaccharides were 188.68 ($\mu mol \cdot mL^{-1} \cdot min^{-1}$) and 114.94 ($\mu mol \cdot mL^{-1} \cdot min^{-1}$), respectively.

Key words β-mannanase, purification, inhibition

β-mannanase (β-1,4-D-mannan mannanohydrolases, EC 3.2.1.78) catalyzes the hydrolysis of β-1,4-mannosidic linkages in various β-mannans, including mannan, galactomannan, glucomannan and galactoglucomannan. It has been reported that mannooligosaccharides can support the growth of *Bifidobacterium* sp. and *Lactobacillus* sp., these bacteria are responsible in maintaining the normal flora of intestines in human[1]. Mannanase is also widely used in foodstuff manufacture, the paper-making industry and oil-well[2,3] as a gel breaker. The properties of mannanase vary significantly, especially in their thermal stability. In the previous paper[4], we reported that under optimized fermentation conditions *Bacillus subtilis* SA-22 produced β-mannanase with activity as high as 168.15 u/mL. The enzyme, also responsible in hydrolyzing konjac to oligosaccharides, may increase the chance of applying *Bacillus* in industrial applications. In this paper, we report results on the purification and properties of endo-1,4-β-D-mannanase.

1 Materials and Methods

1.1 Materials

Konjac powder was obtained from Xie Li Co. Ltd., locust bean gum from Sigma Chemical Co., hydroxyapatite from Bio-Rad, DEAE-cellulose 52 from Whatman, Sephadex G-75 from Pharmacia Biotech, and protein marker from Shanghai Boao Biotech. All other reagents were of analytical grade.

1.2 Methods

1.2.1 Microorganism and cultivation: A loopful of cells were taken from the stock and precultured at 25℃ in a shaker (170 r/min) for 28h. β-mannanase was yielded under optimized conditions by inoculating 2% of the overnight pre-culture in 30 mL medium containing 20 g/L konjac gum, 5 g/L yeast extract, 5 g/L $NaNO_3$, 5 g/L K_2HPO_4 and 0.2 g/L $MgSO_4$ in a 250 mL flask and incubated overnight at 25℃ (pH 6.5). After 24h, the bacteria were removed by centrifugation (10 000 r/min, 4℃) for 10 min and the supernatant was used as a crude enzyme preparation.

1.2.2 Enzyme assay: The reaction mixture containing 0.5% locust bean gum, 50 mmol/L phosphate buffer (pH 6.5), and diluted enzyme solution was incubated at 70℃ for 10min. The amount of reduced sugars formed during the reaction was determined by the Miller's method[5]. One

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* Corresponding author. Tel: 86-20-85283448; E-mail: ymsun@scau.edu.cn

unit of β-mannanase activity was defined as the amount of enzyme that liberated 1 μmol equivalent D-mannose reduced sugars per minute.

1.2.3 Protein assay: The contents of the protein was measured by the Bradford's method[6], with bovine serum albumin as the standard.

1.2.4 SDS-polyacrylamide gel electrophoresis: SDS-PAGE was performed on a vertical slab gel of 12.5% polyacrylamide described by Zhang[7]. Protein was stained with Coomassie brilliant blue R-250.

1.2.5 Measurements of the effects of various factors: The mannanase activity was measured under different pH conditions. In addition, three buffers of acetate-sodium acetate (pH 4.0 ~ 5.0), di-sodium phosphate sodium phosphate monobasic (pH 6.0 ~ 8.0) and glycine-sodium hydroxide (pH 9.0 ~ 10.6) at 50 mmol/L were used for measuring the effect of pH on β-mannanase stability at 28℃.

Temperature was varied from 10℃ to 80℃ to detect the effect of temperature on the activity of the enzyme. The enzyme was incubated at various temperatures for different time duration, cooled, and the residual activity was measured for evaluating the thermal stability.

For measurement on the sensitivity of the enzyme to various ions, the enzyme was incubated in separate solutions, each containing an ion at a concentration of 1 mmol/L in 50 mmol/L phosphate buffer (pH 6.5). The mixtures were kept at 30℃ for 2h and the residual activity was measured by the standard method.

1.2.6 Determination of K_m and V_m: Different concentrations of konjac and locust bean gum were used as the substrates to measure the β-mannanase activities under standard conditions. K_m and V_m were determined by the Linewear-Burk's method.

2 Results and Analysis

2.1 Purification and separation of mannanase

The crude enzyme preparation was added with ammonium sulfate to 80% saturation and stored for 12h at 4℃, then subjected to centrifugation at 9000 r/min for 10 min. The precipitation was collected and dissolved in a small volume of 50 mmol/L phosphate buffer (pH 8.0), followed by an overnight dialysis against the same buffer at 4℃. The dialyzed enzyme solution was then applied onto a hydroxyapatite column (3.0cm × 15cm) that had been equilibrated with the same buffer. It was found that β-mannanase did not bind to the column in chromatography analysis. The active elution fractions were collected and concentrated further with PEG6000, and then subjected to Sephadex G-75 columns (2cm × 60cm) that had been equilibrated with 5 mmol/L Tris-HCl buffer (pH 8.0). Further purification of the eluted fractions was achieved by using DEAE-52 columns (3cm × 15cm), which had been equilibrated with 5 mmol/L Tris-HCl buffer (pH 8.0). The column was subsequently washed with the same buffer to remove any unbound proteins and the bound proteins were eluted with a linear gradient of sodium chloride (0.025 ~ 0.5 mol/L in Tris buffer, pH 8.0, 300 mL). The elution was fractionated and the active forms were collected. The purification results is summarized in Table 1.

Table 1 Purification of β-mannanase from *Bacillus subtilis* SA-22

Purification step	Protein/mg	Total units (× 10^3 u)	Specific activity/(u/mg)	Purification (fold)	Yield/%
Culture filtrate supernatant	47.25	53.440	1131.01	1.0	100
Hydroxyapatite	8.00	43.233	5404.13	4.78	80.90
Sephadex G-75	0.85	13.718	16138.82	14.27	25.67
DEAE-52	0.36	12.521	34780.56	30.75	23.43

2.2 Properties of the purified β-mannanase

2.2.1 Molecular weight of β-mananase: β-mannanase masses were determined to be 38 kD by SDS-PAGE (Fig.1) and 34 kD by gel filtration (Fig.2).

2.2.2 Effects of pH and temperature on the enzyme activity: The effects of pH and temperature on β-mannanase activity were shown in Fig.3 (A ~ D). The optimum pH obtained for the purified enzyme was between 6.0 ~ 6.5. When the incubation was carried out in various buffers at different pH values (pH 5 ~ 10) at 28℃ for 2h, the enzyme was able to retain 80% or more of its full activity. The optimum temperature obtained for the purified enzyme was 70℃. The enzyme was relatively stable at 50℃, but the activity dropped to 74.2 % after a 4h incubation at 60℃ and 51.3 % after a 3h incubation at 70℃. From the above results it can be deduced that the enzyme is somewhat thermally stable.

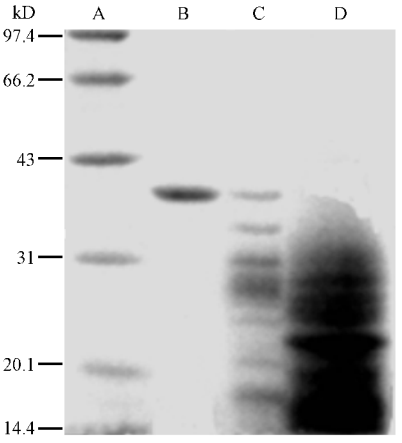

Fig.1 Molecular weight of β-mannanase determined by SDS-PAGE

A. Molecular weight of the standard proteins. 1. Phosphorylase b (97.4 kD); 2. Bovine serum albumin (66.2 kD); 3. Rabbit actin (43 kD); 4. Carbonic anhydrase (31 kD); 5. Trypsin inhibitor (20.1 kD); 6. Lysozyme (14.4 kD)

B. Purified by DEAE-52

C. Purified by Sephadex G-75

D. Crude enzyme

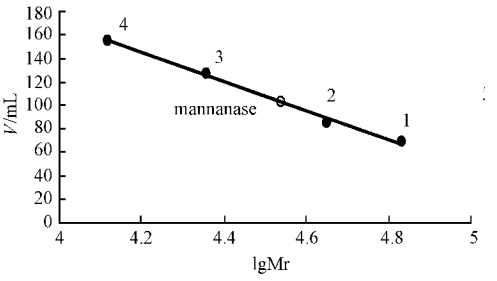

Fig.2 Molecular weight of β-mannanase determined by Sephadex G-75

1. Bovine serum albumin (67 kD); 2. Egg albumin (45 kD); 3. Trypsin bovine pancreas (23.3 kD); 4. Cytochrom C (12.3 kD)

2.2.3 Effects of various reagents on the enzyme activity: The effects of various metal ions (in various compounds) on β-mannanase activities were shown in Table 2. The data indicates that although Hg^{2+} strongly inhibited the enzyme activity, Fe^{2+}, Mg^{2+}, Zn^{2+}, Ag^{+} and Co^{2+} had no effect. The addition of EDTA had prominent effect on protecting the enzyme from Hg^{2+}, Cu^{2+}, and Mn^{2+} inhibition.

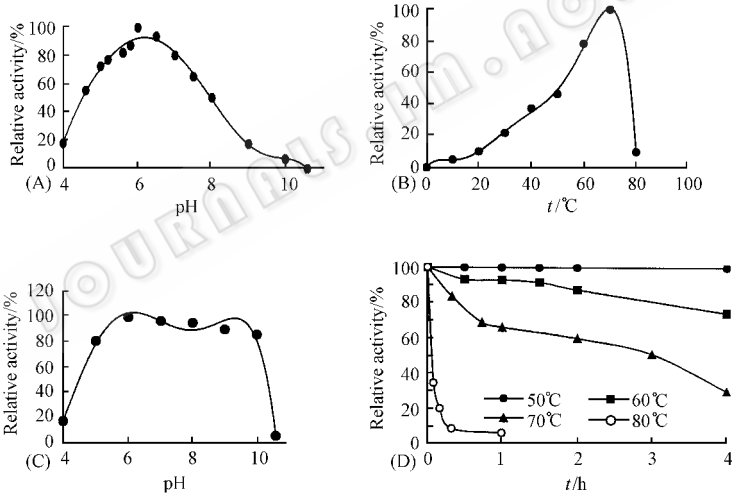

Fig.3 Effects of pH and temperature on the activity and stability of β-mannanase

(A) β-mannanase activity at various pH; (B) β-mannanase activity at various temperature; (C) β-mannanase stability at various pH; (D) Thermal stability of β-mannanase

Table 2 The effects of various reagents on the activity of *B. subtilis* SA-22

Reagents	Relative activity of the enzyme/%	Reagents	Relative activity of the enzyme/%
Control	100	$BaCl_2$	89.7
$HgCl_2$	1.3	$CuSO_4$	73.4
$HgCl_2$ + EDTA·Na_2	77.5	$CuSO_4$ + EDTA·Na_2	88.9
$AlCl_3$	87.9	$MgSO_4$	103.0
$FeSO_4$	101.1	$MnSO_4$	66.9
$FeCl_3$	87.6	$MnSO_4$ + EDTA·Na_2	79.2
$CaCl_2$	88.8	$Pb(NO_3)_2$	81.3
$CoCl_2$	116.7	$ZnSO_4$	100.8
$AgNO_3$	104.4	NaCl	89.5

2.2.4 Kinetics of β-mannanase reaction: Linewear-Burk plots of the enzyme for locust bean gum and Konjac powder are shown in Fig. 4. The values of K_m and V_{max} for β-mannanase from locust bean gum were 11.32 mg/mL and 188.68 $\mu mol \cdot mL^{-1} \cdot min^{-1}$, respectively, and for Konjac powder were 4.76 mg/mL and 114.94 $\mu mol \cdot mL^{-1} \cdot min^{-1}$, respectively.

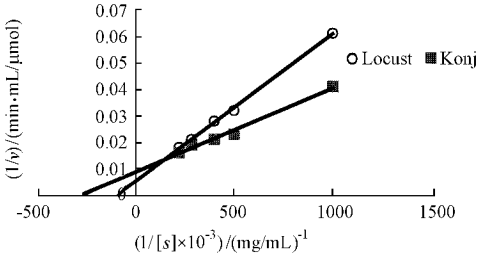

Fig.4 Linewear-Burk of β-mannanase

3 Discussion

It has been reported that β-mannanase from different sources has different molecular weight, for example, *Bacillus* sp. reported by Emi is 22 kD[8] and the multiple forms of β-mannanase from alkalophilic *Bacillus* sp. are between 34.7 ~ 58 kD[9]. From our study the molecular weight of β-mannanase purified from *Bacillus subtilis* SA-22 was between 34 ~ 38 kD. During the purification process, β-mannanase did not bind to the hydroxyapatite column under any pH conditions tested. Other proteins were removed successfully during HA chromatography, achieving a 4.78 fold concentration with a recovery rate of 80.90%, indicating HA chromatography is an effective purification process. The dialysis and PEG6000 condensation were run at 4℃, whereas all the other purification processes were performed at room temperature. This indicates that the enzyme was relatively stable.

Our study shows that Hg^{2+} strongly inhibited the enzyme activity, indicating the possibility of thiol groups on the active site. The assumption was confirmed by further experiments of amino acid modification of the enzyme (details are to be published in a separate article). The study on substrate specificity showed that konjac gum was the most suitable substrate, similar to the findings of the enzyme of *Bacillus subtilis* BM9602[10], but different from that of *Bacillus licheniformis* NK-27[3]. This could be due to the differences in the number of acetyl groups in the substrate and on the influence of galactose chain on the combination of enzyme with the substrate.

The optimal pH and temperature for *Bacillus subtilis* SA-22 were 6.5 and 70℃, respectively. The enzyme was tested stable between pH 5.0 ~ 10.0, and remained most of its activity after a treatment at 50℃ for 4h and lost 50% of the activity at 70℃ for 3h. Because the enzyme is relatively heat-resistant and can perform high efficient hydrolysis under moderate pH conditions, the enzyme may find many apparent advantages in industrial applications.

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