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基础研究 BASIC RESEARCH

DNA聚合酶γ的提取、纯化和鉴定

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Isolation, purification and identification of DNA polymerase gamma

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Abstract

AIM: To purify and identify the mitochondrial DNA polymerase gamma (polymerase γ, Pol γ) from HeLa cells.

METHODS: Ion exchange chromatography was used to isolate Pol γ from HeLa cells. Protein concentration was measured using the Bradford method. SDS-PAGE was performed to determine the molecular weights of the subunits of Pol γ. Following the incorporation of α-^{32}P-dTTP, the activity of Pol γ was counted using a liquid scintillation spectrometer.

RESULTS: Pol γ was purified by 150-fold to apparent homogeneity, with a 6% yield. SDS-PAGE indicated the presence of one subunit of 140 kDa, and Western blotting identified the specificity. Total activity and specific activity of Pol γ were determined to be 4.81 U and 36.17 U/mg, respectively, by chromatography.

CONCLUSION: Pol γ can be purified by ion exchange chromatography. It can then be activated and used as a target to detect the toxicity of some compounds to mitochondria *in vitro*.

Key Words: HeLa cells; DNA polymerase γ; Mitochondria; Ion exchange chromatography; Purification

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摘要

目的: 提取并纯化人宫颈癌细胞(HeLa)的线粒体DNA聚合酶γ(DNA polymerase γ, Pol γ), 鉴定其纯度和活性.

方法: 运用离子交换层析等方法提取纯化HeLa细胞的线粒体Pol γ, 并用Bradford法检测蛋白浓度. 经SDS-PAGE检测蛋白纯度和相对分子量, Western blotting验证蛋白. 用α-^{32}P-dTTP掺入法, 液体闪烁计数器进行放射性测量以确定Pol γ的活性.

结果: 成功提取并纯化HeLa细胞的线粒体Pol γ, 经SDS-PAGE鉴定, 有一个大约140 kDa的亚基单位, Western blotting证实为Pol γ. 对其进行150倍纯化, 收得率为6%, 酶的总活力为4.81 U, 比活力为36.17 U/mg.

结论: HeLa细胞的线粒体Pol γ通过离子交换层析法提取和纯化后活性较高, 可用于体外药物线粒体毒性的检测.

关键词: 人宫颈癌细胞; DNA聚合酶γ; 线粒体; 离子交换层析

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0 引言

DNA聚合酶可以进行严格的DNA复制和修复

■背景资料
DNA聚合酶γ(Pol γ)指导着线粒体DNA的正确合成, 保证线粒体很好行使其功能. 近年来新出现的核苷类抗病毒药物毒副作用主要也作用与Pol γ, 并且与许多疾病的发生(如PEO)密切相关.

■创新盘点
本文经过多次离子交换层析得到有活性的纯度较高的Pol γ, 并且将其用做体外抗病毒药物毒性的研究.

DNA损伤, 对于保持基因组的完整性是不可缺少的[1]. 在人类细胞中, 现已发现15种不同的DNA聚合酶, 然而只有4种参与DNA的复制, 余下的参与DNA的修复和特定DNA合成[2-3]. 虽然大部分酶参与核DNA的复制和修复, 然而至今, DNA聚合酶γ(DNA polymerase γ, Pol γ)仍然是线粒体内唯一的DNA聚合酶[4], 由核基因编码[5], 对于线粒体DNA(mtDNA)的复制、合成与修复起着独一无二的作用[2,6].

最近有许多抗病毒核苷类似物如治疗HIV的叠氮胸苷(AZT), 治疗乙肝的拉米呋定(lamivudine)和恩替卡韦(entecavir)等, 在治疗方面起了很大的作用, 但其毒副作用不容忽视. 这些药物的毒副作用主要作用于线粒体, 其中Pol γ对其高度敏感[7]. 很多体外研究表明这些药物可以抑制线粒体DNA聚合酶活性从而影响线粒体DNA的合成, 以至影响线粒体的功能[8-9]. 本研究旨在提取并纯化得到有活性的Pol γ, 以利于以后有关药物对于线粒体毒性的研究.

1 材料和方法

1.1 材料 HeLa S3细胞来自于本学院免疫教研室, 换液传代后, 按批冻存在-70℃冰箱待用. 层析所需预溶胀DE52填料、磷酸纤维素填料(P11)均为Whatman产品, 羟基磷灰石填料(HTP)为Bio-Rad产品, SDS-PAGE所用装置为Bio-Rad MiNi Protean-Ⅲ电泳系统, 兔抗人聚合酶γ抗体购自Neomarkers. 层析柱购自上海锦华层析设备厂, 规格有1.0×20 cm, 1.6×25 cm, 0.5×20 cm. 检测酶活性所需的活化小牛胸腺DNA购自Sigma, 反应底物dNTP为BioLabs公司产品.

1.2 方法

1.2.1 细胞培养: 常规HeLa S-3细胞培养, 收集处于对数生长期的细胞, 按照马歇克主编的《蛋白质纯化与鉴定实验指南》制备冰冻细胞－70℃保存.

1.2.2 细胞碎片制备: 若未特别说明, 所有实验均在0℃-4℃进行, 洗脱液中均含有0.5 mmol/L二硫苏糖醇(DTT). 将分批冻存的细胞从-70℃冰箱取出约20 g, 冰冻细胞悬浮在含有10 mmol/L NaCl, 1 mmol/L K_3PO_4(pH7.8), 0.5 mmol /L DTT的80 mL溶液中, 零度放置20 min后用匀浆器将细胞破碎, 1000 r/min离心10 min, 分离细胞质和细胞核, 留取上清细胞质组分. 将细胞核重悬在含有0.32 mmol/L蔗糖, 1 mmol/L $MgCl_2$, 1 mmolL K_3PO_4(pH6.8), 0.3% Triton N-101, 0.5 mmol/L DTT的120 mL溶液中, 匀浆后100 r/min离心10 min, 上清中含有细胞质碎片. 将所得上清与第一次上清混合, 20 000 *g*离心20 min后, 收集上清即为细胞质碎片(含有目的蛋白).

1.2.3 第一次DEAE除核酸: 按照说明书, 所用填料的质量根据抽出样品测得的蛋白量决定. 称取一定量的预溶胀DEAE填料, 用0.4 mol/L K_3PO_4(pH7.5)和0.5 mmol/L DTT平衡填料, 用同样的溶液平衡1.0×20 cm柱子. 将100 mL细胞质碎片上柱, 再用10倍柱子填料体积的0.4 mol/L K_3PO_4(pH7.5)和0.5 mmol/L DTT洗脱, 核酸被吸附到柱子上, 酶被洗脱. 将洗脱下来的酶溶液用蔗糖溶液浓缩过夜, 并用0.02 mol/L K_3PO_4(pH7.5)和0.5 mmol/L DTT透析平衡.

1.2.4 第二次DEAE层析: 用0.02 mol/L K_3PO_4 (pH 7.5)和0.5 mmol/L DTT平衡填料. 灌柱平衡后, 将透析后所得的溶液加入1.0×20 cm柱子, 用一倍填料体积的0.02 mol/L K_3PO_4(pH7.5)冲洗柱子, 然后用0.05-0.23 mol/L K_3PO_4(pH7.5)的浓度梯度、八倍填料的体积洗脱液洗脱柱子. 分别收集得到的洗脱液后测A_{280}值, 找出洗脱峰. 用大量峰值的K_3PO_4(pH7.5)溶液洗脱柱子, 得到的溶液即为含有Pol γ的洗脱液, 蔗糖溶液浓缩过夜, 再用0.01 mol/L K_3PO_4(pH7.5)和0.5 mmol/L DTT透析平衡.

1.2.5 磷酸纤维素(P11)层析: 用0.01 mol/L K_3PO_4(pH7.5)和0.5 mmol/L DTT溶胀P11填料, 灌1.6×25 cm柱平衡过夜. 将从DEAE层析获得的透析后的峰值碎片上柱, 八倍体积的K_3PO_4(pH7.5)洗脱液按浓度梯度0.1-0.28 mol/L冲洗柱子. 根据测得的洗脱成分A_{280}值, 找出洗脱峰对应的K_3PO_4(pH7.5)浓度. 大量此溶液冲洗柱子, 洗脱下来的溶液即为含有Pol γ的溶液, 蔗糖溶液浓缩后用0.05 mol/L K_3PO_4(pH7.5)和0.5 mmol/L DTT透析平衡.

1.2.6 羟基磷灰石(HTP)层析: 按照说明书用0.05 mol/L K_3PO_4(pH7.5)和0.5 mmol/L DTT平衡HTP粉剂, 灌入0.5×20 cm柱子待其平衡好后, 将P11层析后经过透析平衡后的溶液上柱, 10倍体积的K_3PO_4(pH7.5)洗脱液按浓度梯度0.05-0.40 mol/L冲洗柱子. 收集洗脱成分测其A_{280}, 用大量出现洗脱峰时的K_3PO_4(pH7.5)溶液洗脱柱子上的吸附蛋白. 收集后放在透析袋中, 用蔗糖晶体浓缩溶液一直到不能再浓缩为止.

1.2.7 线粒体Pol γ活性检测: 参照文献[10-11]建立酶活性检测反应体系: 50 μL的反应总溶

液, 其中含有45 mg/L牛血清白蛋白, 50 mmol/L Tris-HCl(pH8.5), 7.5 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 100 μg/mL活化的小牛胸腺DNA作为模板, 100 μmol/L dATP, dGTP, dCTP和α-^{32}P-dTTP 3.7×10^4 Bq, 37℃孵育30 min. 酶活性定义为: 37℃反应条件下30 min, 催化结合1 nmol脱氧核苷酸的量.

1.2.8 SDS-PAGE变性聚丙烯酰胺凝胶电泳: 制成分离胶浓度为80 mL/L, 浓缩胶为50 mL/L, 层析后抽出的1 mL样品. 分别抽出一部分将其与上样缓冲液混合, 煮沸后进行聚丙烯酰胺凝胶电泳. 其中浓缩胶所用电压为80 V, 分离胶为120 V. 电泳结束后, 考马斯亮兰染色过夜, 脱色后在凝胶成像系统下拍照.

1.2.9 Western blot检测Pol γ: 样品按照SDS-PAGE变性聚丙烯酰胺凝胶电泳结束后进行转膜, 然后用兔抗人聚合酶γ抗体进行一抗孵育, 结束后羊抗兔二抗孵育, 最后用DAB在膜上显色.

2 结果

2.1 人HeLa细胞Pol γ纯化 将破碎细胞离心所得组分上入DEAE柱除去核酸后, 收集得到的洗脱液浓缩测得体积为120 mL, 用考马斯亮兰法测得蛋白浓度为2.1 g/L. 经SDS-PAGE电泳看到一条140 kDa的亚基(图1), 并用Western blot证实其特异性(图2). 所得蛋白透析后用不同浓度梯度的K_3PO_4(pH7.5)经过第二次DEAE层析(图3A),收集峰值的洗脱液, 浓缩后约60 mL, 测得蛋白浓度为0.68 g/L. 透析后上磷酸纤维素柱子(图3A), 出现A_{280}峰值时所用的K_3PO_4(pH7.5)溶液洗脱柱子, 收集得到的洗脱液浓缩后约13 mL, 蛋白浓度为0.22 g/L. 透析后进行羟基磷灰石层析(图3A). 按照图中所示的K_3PO_4浓度梯度洗脱柱子, 收集峰值的洗脱液, 即为最后纯化所得的DNA聚合酶γ, 浓缩到最后溶液约为1.9 mL, 测得其浓度为0.07 g/L. 电泳后在140 kDa处看到一条很浅的条带, Western blotting表明此为线粒体DNA聚合酶.

2.2 人HeLa细胞Pol γ鉴定 在Pol γ的逐步层析纯化中对每一步浓缩后的蛋白进行电泳(图1)鉴定其纯度, 发现其杂蛋白条带逐渐减少, 其目的蛋白的一个亚基(140 kDa)条带颜色逐步变浅, 证明其蛋白得到了有效的纯化. 进行Western blot印迹(图2)特异性结合鉴定其为Pol γ. 在纯化过程中每次层析后测得其总蛋白、总活力、比活力、纯化倍数及蛋白收得率, 结果显示酶的活力虽然逐渐降低, 但是其比活力、纯化倍数却

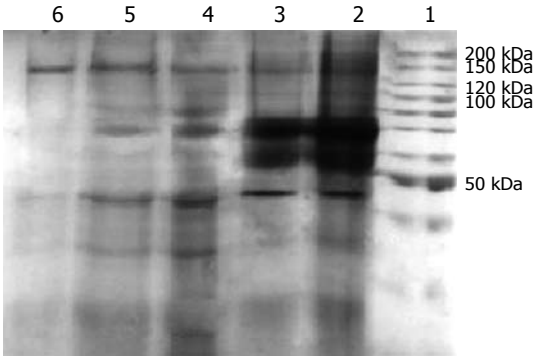

图 1 SDS-PAGE电泳图. 1: 蛋白marker; 2: 破碎细胞后所得溶液; 3: 第1次DEAE层析所得收集液; 4: 第2次DEAE层析纯化后所得蛋白组分; 5: P11层析纯化后所得蛋白组分; 6: 羟基磷灰石层析后所得纯化蛋白.

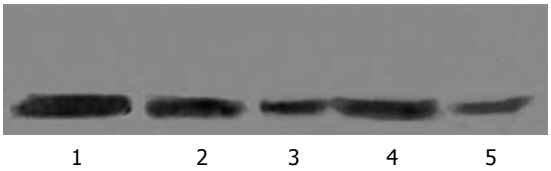

图 2 Western blot印迹. 1: 破碎细胞后所得溶液; 2: 第1次DEAE层析所得收集液; 3: 第2次DEAE层析纯化后所得蛋白组分; 4: P11层析纯化后所得蛋白组分; 5: 羟基磷灰石层析后所得纯化蛋白.

表 1 离子交换层析Hela细胞DNA聚合酶γ

步骤	总蛋白 (mg)	总活力 (Ukat)	比活力 (Ukat/mg)	纯化倍数	收得率(%)
破碎细胞后所得	330	78	0.24	/	/
第1次DEAE层析	252	103	0.40	2	132
第2次DEAE层析	40.8	67	1.64	7	86
P11层析	2.86	54	18.88	78	69
HTP层析	0.133	4.81	36.17	150	6

逐渐增高, 获得率逐渐降低, 表明目的蛋白得到了逐步的纯化, 杂蛋白量渐少(表1, 图1). 纯化完后蛋白的纯化倍数为150, 达到了较高的纯化, 说明此方法可用来提纯纯度较高的Pol γ.

3 讨论

Pol γ位于线粒体基质[1-2], 是唯一存在于线粒体中的DNA聚合酶. 虽然只占线粒体蛋白的0.008%, 但是对于维持mtDNA连续性和正确性起着重要作用, 保证线粒体正常行使功能[13-14]. 在此, 我们从人HeLa细胞中提取了Pol γ, 并用多步离子交换层析纯化. 虽然收得率较低, 但是得到了较高纯化倍数并具有活性的DNA聚合酶, 经SDS-PAGE电泳表明含有一个明确的亚基(140 kDa). 完整的Pol γ应包含有两个亚基, 一个较大

■应用要点
Pol γ可以用于药物毒性的体外研究, 也可研究其对mtDNA复制和修复的相关机制.

■名词解释
DEAE: 一种阴离子交换纤维素, 装柱后可以使交换剂的外水空间增大, 结构更牢固, 并且电荷分布更均匀, pH变化范围宽, 可以根据需要选择不同的种类吸附不同电荷大小的蛋白质或者核酸, 稳定性较好.

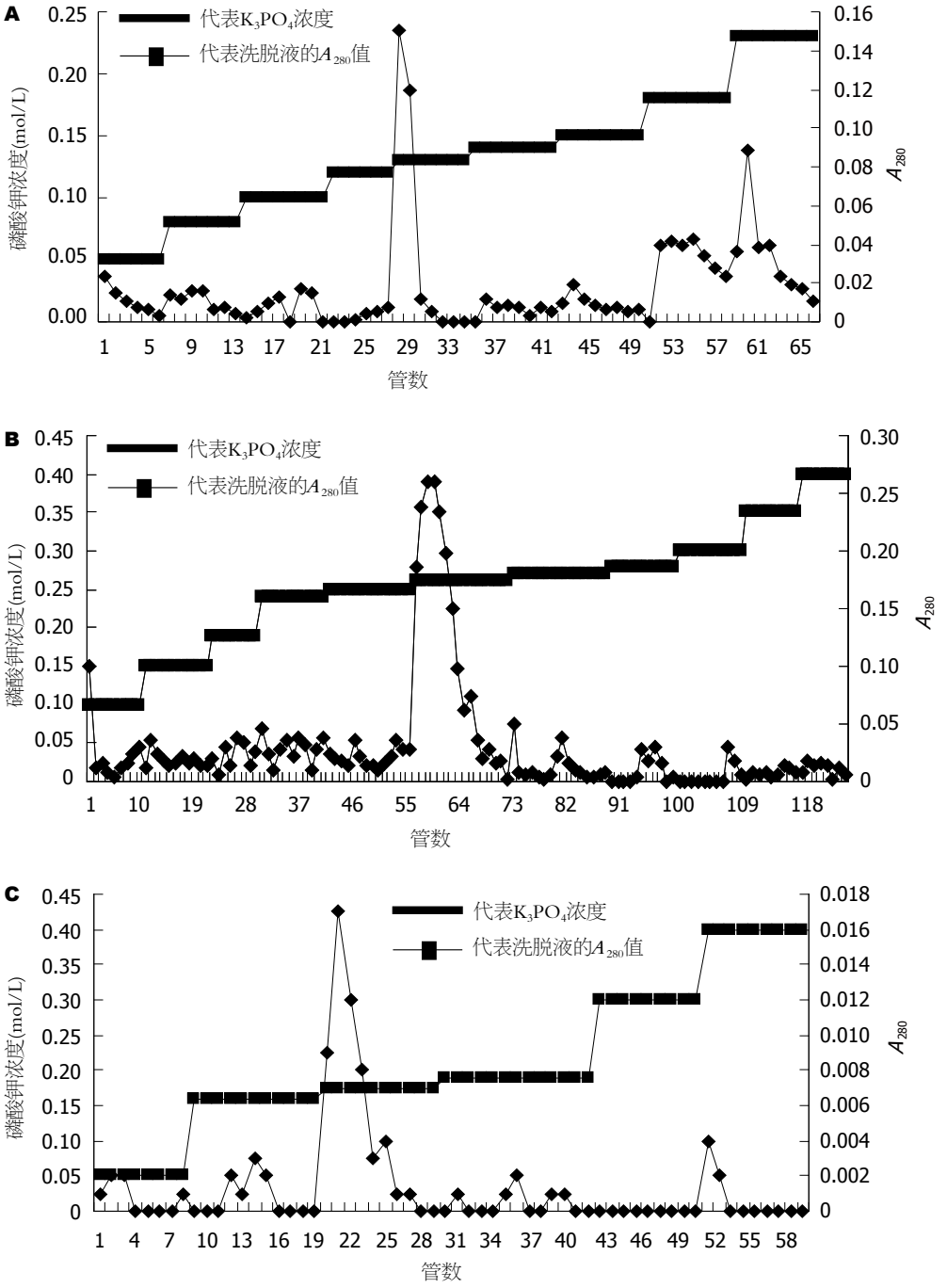

图 3 离子交换层析法. A: 第二次DEAE层析; B: 磷酸纤维素层析; C: 羟基磷灰石层析.

的催化亚基Pol γ A(140 kDa)和一个较小的调节亚基Pol γ B(55 kDa). 大亚基除了主要的聚合酶作用外, 还有3'-5'核酸外切酶的作用, 在碱基切除修复中去除5'脱氧核糖核酸(dRP). 小亚基Pol γ B有增加大亚基持续合成的能力, 在碱基切除修复中起到增强催化亚基的作用, 通过识别RNA引物激发mtDNA的合成[15-19]. 双链DNA(dsDNA)的结合也是通过Pol γ B协助Pol γ A进行定位[20]. 生物化学对于人Pol γ的鉴定表明, 在酶的纯化过程中, 由于Pol γ B的缔合常数太小可导致其丢失[21-22], 故我们电泳只看到一个大的催化亚基, 小亚基可能已丢失. 但是通过活性检测证明, 在体外他仍然有正确合成DNA的作用并具有较高的活性. 酵母菌中提取出来的聚合酶本身就只有一个大亚基, 但是仍然有高的持续合成能力[23].

最近研究显示, 许多疾病与Pol γ的功能密切相关, 如进行性眼外肌麻痹(PEO)[24-26], 弥漫性进行性脑灰质变性[27]、感觉性共济失调、神经病变、发音困难和帕金森综合征[28], 男性不育[29-31]也与其聚合酶的活性有关. 正常mtDNA由于Pol γ的存在, 其错配率很低. 但是随着年龄的

增长, 由于Pol γ的活性和功能的下降, 修复氧化损伤的能力会随着DNA聚合酶的活性及碱基切除修复的能力的下降而下降. 线粒体是进行有氧呼吸的主要场所, 导致活性氧族的堆积, 与氧化损伤的有关疾病如黄斑变性和阿尔茨海默病也由此出现[12]. 曾有研究者用有Pol γ功能缺陷的转基因小鼠模型, 但他缺乏核酸外切酶的校正功能不能保证mtDNA的正确复制, 从而增加线粒体DNA的突变率[32]和心肌病的危险性[33-34]. 另外常染色体显性或隐性疾病的发生也与线粒体DNA聚合酶的活性有关. 如PEO就是由于大脑中突变的mtDNA超过总数的50%, 而Pol γ的功能异常不能正常修复[35-37]. 近年来有许多对于抗HBV和HIV的抗病毒药物(拉米呋定、恩替卡韦和叠氮胸苷等)的研究, 其机制为竞争抑制病毒的逆转录酶或者病毒的DNA聚合酶. 而作为人线粒体中唯一的DNA聚合酶Pol γ对其敏感性很高, 毒性大[38-42]. 大量的基础和临床研究一致报道, 核苷类逆转录酶抑制剂(NRTIs)如扎西他宾、地达诺新、司他夫定对于Pol γ有很强的抑制作用[43-50]. 由此可见DNA聚合酶γ在临床疾病的发生发展及治疗中起着巨大的作用. 他可提供一个新的药理治疗的目标来推动DNA的修复, 也可用于核苷类似物药物的毒性检测及药物的筛选等. 我们提纯有活性的酶以利于体外研究, 而在纯化的过程中, 洗脱液里都加了少量的抗氧化剂DTT. 虽然酶的活性好, 并得到了很好的纯化, 但是酶的获得量仍然很少, 用此来做药物的毒性检测尚可, 但是若要用于其他研究就远远不够. 因此, 我们应寻找到更好的提取与纯化此酶的方法以得到大量有活性的酶.

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■同行评价
本文思路清晰, 实验步骤严谨, 讨论较为系统, 具有一定的学术价值和临床指导意义.

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