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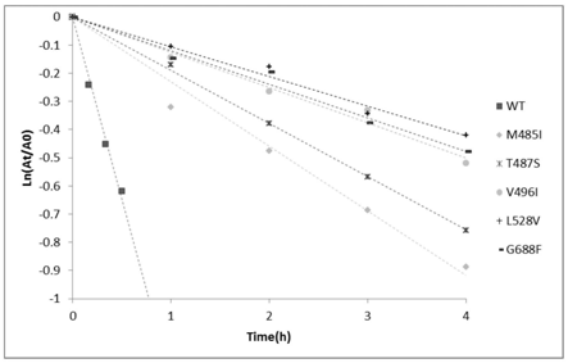
(54)发明名称

耐热的I型普鲁兰酶的突变体酶及其制备方法与应用

(57)摘要

本发明公开了耐热的I型普鲁兰酶的突变体酶，其氨基酸序列如SEQ ID No.1，或如SEQ ID No.2，或SEQ ID No.3，或SEQ ID No.4、或SEQ ID No.5所示。本发明还公开了编码突变体酶的基因，其如SEQ ID No.6，或SEQ ID No.7，或SEQ ID No.8，或SEQ ID No.9、或SEQ ID No.10。本发明还公开了突变体酶的制备方法，包括从普鲁兰酶的氨基酸序列选取突变位点，设计突变引物对；以携带普鲁兰酶基因的载体为模板，进行点突变反应，得到突变质粒；然后于转化工程菌中复制表达。本发明的突变体酶具有更优的耐热性能。



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1.耐热的I型普鲁兰酶的突变体酶,其特征在于,所述突变体酶的氨基酸序列如SEQ ID No.1所示。

2.编码权利要求1所述的突变体酶的基因,其特征在于,编码所述突变体酶的基因的核苷酸序列如SEQ ID No.6所示。

3.包含编码权利要求1所述的突变体酶的基因的工程菌。

4.包含编码权利要求1所述的突变体酶的基因的载体。

5.制备如权利要求1所述的突变体酶的方法,其特征在于,包括以下步骤:

步骤一、以携带I型普鲁兰酶基因的载体为模板,使用设计的突变引物对,利用PCR突变方法,进行点突变反应,得到带有突变体酶基因的突变质粒,其中,I型普鲁兰酶为普鲁兰酶PulF,编码普鲁兰酶PulF的基因的核苷酸序列如SEQ ID No.11所示,所述突变引物对分别为:

F:5′-CGGCGTGGGCAATGTTATCGCGACCGAACGT-3′和R:5′-GATAACATTGCCCACGCCGCTTTCATTCAGATACG-3′;

步骤二、将步骤一中得到的突变质粒转化可表达目的基因的工程菌中,随工程菌的复制表达对应的突变体酶。

6.如权利要求5所述的方法,其特征在于,步骤一中的点突变反应条件为:94℃预变性5 min;94℃解链20 s,65℃退火20 s,72℃延伸4 min,共25个循环;72℃延伸10 min;4℃保温。

7.如权利要求5所述的方法,其特征在于,步骤二中的工程菌为大肠杆菌。

8.如权利要求5所述的方法,其特征在于,步骤一中的载体为pET-22b(+)。

9.如权利要求5所述的方法,其特征在于,还包括采用Ni^{2+}亲和层析的方式对突变体酶进行分离纯化。

10.如权利要求1所述的突变体酶在制备酶解含有a-1,6糖苷键的多糖的产品中的应用。

耐热的I型普鲁兰酶的突变体酶及其制备方法与应用

技术领域

[0001] 本发明涉及基因工程和酶工程技术领域。更具体地说，本发明涉及一种耐热的I型普鲁兰酶的突变体酶及其制备方法与应用。

背景技术

[0002] 目前已经有很多普鲁兰酶被挖掘出来，甚至普鲁兰酶已经在基因层面进行了研究。但是真正能应用于工业生产中的却很少，目前商业化程度最高的是来源于Bacillus acidopullulyticus的普鲁兰酶。普鲁兰酶最主要的应用方式是用于淀粉的糖化过程，其工业应用条件为pH4.5～5.5，温度55～65℃甚至更高，持续作用时间长达48～60h(NISHA and SATYANARAYANA,2016)。大多数已报道的I型普鲁兰酶均无法适应这一工业条件，有些酶耐热性差，有些酶在pH5.5或更低的条件下酶活很低，甚至会失活。即使是商业化的B.acidopullulyticus普鲁兰酶也存在明显短板，其在60℃时的半衰期仅有34.9min，没有足够强的耐热性。

[0003] 针对以上现状，本发明以普鲁兰酶的耐热性作为首要考虑因素，拟挖掘具有较强耐热性的普鲁兰酶。耐热普鲁兰酶的微生物来源多是嗜热或极端嗜热微生物，其生长条件恶劣，培养条件苛刻，不利于取样和筛选。因此，采用基因工程技术方式得到耐热性好的普鲁兰酶是值得尝试的。

发明内容

[0004] 本发明的一个目的是解决至少上述问题，并提供至少后面将说明的优点。

[0005] 本发明还有一个目的是提供一种耐热的I型普鲁兰酶的突变体酶，以及耐热的I型普鲁兰酶的突变体酶的制备方法，得到的突变体酶具有优良的耐热性能。

[0006] 为了实现根据本发明的这些目的和其它优点，提供了一种耐热的I型普鲁兰酶的突变体酶，所述突变体酶的氨基酸序列如SEQ ID No.1所示，或如SEQ ID No.2所示，或如SEQ ID No.3所示，或如SEQ ID No.4所示、或如SEQ ID No.5所示。

[0007] 提供了一种编码所述突变体酶的基因，编码所述突变体酶的基因的核苷酸序列如SEQ ID No.6所示，或如SEQ ID No.7所示，或如SEQ ID No.8所示，或如SEQ ID No.9所示，或如SEQ ID No.10所示。

[0008] 提供一种包含编码上述突变体酶的基因的工程菌。

[0009] 提供一种包含编码上述的突变体酶的基因的载体。

[0010] 提供了一种制备所述突变体酶的方法，包括以下步骤：

[0011] 步骤一、根据NCBI数据库中公开的I型普鲁兰酶的氨基酸序列，进行同源建模，基于序列比对分析和蛋白结构分析，选取距离I型普鲁兰酶的催化位点10埃以内的氨基酸残基作为对象，通过序列比对排除掉其中非常保守的氨基酸残基，选取5个非保守氨基酸突变位点，对5个突变位点设计对应的突变引物对，所述突变引物对分别为

[0012] 5′-CGGCGTGGGCAATGTTATCGCGACCGAACGT-3′和

[0013] 5′-GATAACATTGCCCACGCCGCTTTCATTCAGATACG-3′、

[0014] 5′-CGTGGGCAATGTTATGGCGAGCGAACGTCCGAT-3′和

[0015] 5′-CTCGCCATAACATTGCCCACGCCGCTTTCATTC-3′、

[0016] 5′-CGTCCGATGCTGCGCAAATATATCATTGATACCCTG-3′和

[0017] 5′-GATATATTTGCGCAGCATCGGACGTTCGGTCGCCAT-3′、

[0018] 5′-AAAACCATGCTGGACGTGGAAAAAGAACTGC-3′和

[0019] 5′-CGTCCAGCATGGTTTTTTTATCCATCAGGC-3′、

[0020] 5′-ATTTTGCGCGCACCAAAAAATTCGATGAGAACAGC-3′和

[0021] 5′-AATTTTTTGGTGCGCGCAAAATCCTGGCCCGCAT-3′;

[0022] 步骤二、以携带I型普鲁兰酶基因的载体为模板,使用设计的突变引物对,利用PCR突变方法,分别进行点突变反应,得到带有突变体酶基因的突变质粒;

[0023] 步骤三、将步骤二中得到的突变质粒转化为可表达目的基因的工程菌中,随工程菌的复制表达对应的突变体酶。

[0024] 优选的是,步骤二中的点突变反应条件为:94℃预变性5min;94℃解链20s,65℃退火20s,72℃延伸4min,共25个循环;72℃延伸10min;4℃保温。

[0025] 优选的是,步骤二中的工程菌为大肠杆菌。

[0026] 优选的是,步骤二中的载体为pET-22b(+)。

[0027] 优选的是,还包括采用Ni^{2+}亲和层析的方式对突变体酶进行分离纯化。

[0028] 提供一种上述突变体酶在制备酶解含有a-1,6糖苷键的多糖的产品中的应用。

[0029] 本发明至少包括以下有益效果:

[0030] 第一、得到了5种耐热性能优于现有的I型普鲁兰酶PulF的突变体酶。

[0031] 第二、相比于现有的普鲁兰酶PulF,5个突变体酶在比较宽的pH范围内都具有较高的酶活,尤其是在pH5.0～5.5的范围内,突变体酶的相对酶活都大于85％。在pH4.5的条件下,5个突变体酶的相对酶活都明显高于普鲁兰酶PulF。在pH 6.0～7.0范围内,5个突变体酶的相对酶活都明显高于普鲁兰酶PulF。

[0032] 第三、5个突变体酶的相对酶活变化趋势与普鲁兰酶PulF有较大区别,但他们5个各自的趋势较为一致,当pH远离最适值时酶活缓慢下降,在pH5.0～6.5范围内相对酶活均大于50％。且不同缓冲液体系对它们的酶活影响不大,在pH6.0的磷酸盐缓冲液中的相对酶活和在pH6.0醋酸缓冲液中较为接近。因为制糖工业中普鲁兰酶反应的pH范围为4.5～5.5,所以结合这一工业应用条件分析,5个突变体酶相比于普鲁兰酶PulF来说最大的优点是:在pH5.0～5.5范围内均具有较高酶活,对糖化过程中反应液的pH环境要求不苛刻。

[0033] 第四、当普鲁兰酶PulF突变为M485I、T487S、V496I、L528V和G688F后,最适温度变为75℃。在60℃～75℃范围内,5个突变体酶的相对酶活都大于普鲁兰酶PulF在该温度下的相对酶活。

[0034] 制糖工业中普鲁兰酶反应的温度范围为55～65℃甚至是更高的温度,其中最常用的反应温度为60℃,所以结合这一工业应用条件分析,突变体酶的优势在于其在温度60℃～75℃范围内具有更高的相对酶活。

[0035] 第五、在75℃条件下,5个突变体酶的热稳定性都有所增强,它们热稳定性由强到弱的顺序为:L528V>G688F>V496I>T487S>M485I;在80℃条件下,5个突变体酶的热稳定性也

都高于普鲁兰酶PulF，它们的热稳定性由强到弱的顺序为：V496I>G688F>T487S>L528V>M485I。两个温度条件下，5个突变体酶热稳定性强弱的顺序是不同的。

[0036] 75℃条件下，M485I的半衰期提高的最少，比普鲁兰酶PulF提高了近4倍。80℃条件下，所有酶的半衰期都很短，其中V496I的半衰期最长，而M485I的半衰期最短。

[0037] 第六、相比于普鲁兰酶PulF而言，5个突变体酶的K_m值均有所降低，说明突变体酶与普鲁兰多糖的结合能力增强了；突变体酶M481I、V496I、L528V和G688F的催化常数K_{cat}/K_m均有所增加，说明这些突变体酶对普鲁兰多糖的催化效率提高了。

[0038] 本发明的其它优点、目标和特征将部分通过下面的说明体现，部分还将通过对本发明的研究和实践而为本领域的技术人员所理解。

附图说明

[0039] 图1为本发明的5个定点突变PCR产物核酸电泳图；

[0040] 图2为本发明的蛋白标准曲线；

[0041] 图3为本发明的纯化后的5个突变体酶的变性聚丙烯酰胺凝胶电泳图；

[0042] 图4为本发明的5个突变体酶和普鲁兰酶PulF的75℃的半衰期图；

[0043] 图5为本发明的5个突变体酶的80℃的半衰期图。

具体实施方式

[0044] 下面结合附图对本发明做进一步的详细说明，以令本领域技术人员参照说明书文字能够据以实施。

[0045] 需要说明的是，下述实施方案中所述实验方法，如无特殊说明，均为常规方法，所述试剂和材料，如无特殊说明，均可从商业途径获得。

[0046] 1、原料准备

[0047] 生物材料：如表1所示

[0048] 表1生物材料

[0049]

生物材料	用途	来源
pET-22b(+)-pulF	定点突变 PCR 模板	自己构建
E.coli DH5α	克隆宿主	购于天根科技
E.coli BL21(DE3)	表达宿主	购于天根科技

[0050] pET-22b(+)-pulF的构建方法：

[0051] 选取登录号和微生物来源为WP_011994577.1(Fervidobacterium nodosum Rt17-B1)为原始酶，为方便表述，将这个普鲁兰酶命名为PulF。

[0052] 普鲁兰酶PulF的基因序列去除信号肽编码序列后，来源于Fervidobacterium nodosum的基因片段全长为2460bp，对2460全长片段的基因进行密码子优化后，由华大基因进行合成。合成的基因序列均通过NdeI/XhoI双酶切位点连接到表达载体pET-22b(+)中，并转入感受态细胞BL21(DE3)。从添加了氨苄青霉素(终浓度100μg/mL)的LB平板中挑取阳性克隆，测序成功的重组菌保存在15％甘油管中。构建成功的重组质粒命名为pET-22b(+)-

pulF。其中，编码普鲁兰酶PulF的基因的核苷酸序列如SEQ ID No.11所示，进行密码子优化后的编码普鲁兰酶PulF的基因的核苷酸序列如SEQ ID No.12所示，普鲁兰酶PulF和密码子优化后的普鲁兰酶PulF的氨基酸序列相同，均如SEQ ID No.13所示。

[0053] 培养基：

[0054] LB液体培养基(g/L)：胰蛋白胨10，酵母浸粉5，氯化钠10，pH 7.0，121℃，灭菌15min。

[0055] LB固体培养基(g/L)：胰蛋白胨10，酵母浸粉5，氯化钠10，琼脂粉20，pH 7.0，121℃，灭菌15min。

[0056] 主要试剂：

[0057] 磷酸二氢钠、磷酸氢二钠、氯化钠、咪唑、乙二胺四乙酸(EDTA)、冰乙酸、乙酸钠、无水葡萄糖、苯酚、氢氧化钠、酒石酸钾钠、亚硫酸氢钠、3,5-二硝基水杨酸、正丁醇、无水乙醇、浓硫酸及各种金属离子的氯化盐等分析纯试剂，均购自国药集团化学试剂北京有限公司；预制胶(SurePAGE™，Bis-Tris，8%，10孔)及SDS-PAGE电泳缓冲液，购自金斯瑞生物科技股份有限公司；SDS-PAGE蛋白上样缓冲液和蛋白marker，购自大连宝生物工程公司；亲和层析柱填料(Ni Sepharose® High Performance)，购自美国通用电气医疗集团(GE)；BCA蛋白质定量试剂盒、质粒小提试剂盒、BL21(DE3)感受态和DH5α感受态，均购自天根生化科技(北京)有限公司；Fast Mutagenesis System试剂盒，购自北京全式金生物技术(TransGen Biotech)有限公司。

[0058] 菌体破碎缓冲液(Lysis buffer)：50mM磷酸盐缓冲液，100mM氯化钠，pH7.0；

[0059] 上样缓冲液(Binding buffer)：50mM磷酸盐缓冲液，500mM氯化钠，pH7.0；

[0060] 冲洗缓冲液(Washing buffer)：50mM磷酸盐缓冲液，500mM氯化钠，100mM咪唑，pH7.0；

[0061] 洗脱缓冲液(Elution buffer)：50mM磷酸盐缓冲液，500mM氯化钠，500mM咪唑，pH7.0；

[0062] 透析液A(Dialysate A)：50mM磷酸盐缓冲液，10mM乙二胺四乙酸，pH7.0；

[0063] 透析液B(Dialysate B)：50mM磷酸盐缓冲液，pH7.0。

[0064] 主要仪器：

[0065] Avanti JXN-26智能型高效离心机，美国贝克曼库尔特有限公司；超声破碎细胞仪JY92-IIN，宁波新芝生物科技股份有限公司；核酸蛋白检测仪HD-2和恒流泵HL-2，上海沪西仪器分析厂；PowerPac Basic和Mini-PROTEAN Tetra Cell电泳系统，美国Bio-Rad公司；恒温振荡培养箱，上海一恒科学仪器有限公司；凝胶成像系统，上海勤翔科学仪器有限公司；TP600型PCR仪，日本TaKaRa公司；Spark®20M多功能酶标仪，瑞士帝肯公司。

[0066] 2、实验方法

[0067] 2.1定点突变：

[0068] 以pET-22b(+)-PulF为模板，设计突变引物，使用Fast Mutagenesis System试剂盒对PulF的编码基因进行点突变。突变引物序列如表2所示，点突变反应体系如表3所示，点突变PCR的反应条件为：94℃预变性5min；94℃解链20s，65℃退火20s，72℃延伸4min，共25个循环；72℃延伸10min；4℃保温。

[0069] 表2突变引物序列

引物名称	引物序列
M485I-F	5′-CGGCGTGGGCAATGTTATCGCGACCGAACGT-3′
M485I-R	5′-GATAACATTGCCCACGCCGCTTTCATTCAGATACG-3′
T487S-F	5′-CGTGGGCAATGTTATGGCGAGCGAACGTCCGAT-3′
T487S-R	5′-CTCGCCATAACATTGCCCACGCCGCTTTCATTC-3′
V496I-F	5′-CGTCCGATGCTGCGCAAATATATCATTGATACCCTG-3′
V496I-R	5′-GATATATTTGCGCAGCATCGGACGTTCGGTCGCCAT-3′
L528V-F	5′-AAAACCATGCTGGACGTGGAAAAAGAACTGC-3′
L528V-R	5′-CGTCCAGCATGGTTTTTTTATCCATCAGGC-3′
G688F-F	5′-ATTTTGCGCGCACCAAAAAATTCGATGAGAACAGC-3′
G688F-R	5′-AATTTTTTGGTGCGCGCAAAATCCTGGCCCGCAT-3′

[0070]

[0071] 表3点突变反应体系

[0072]

试剂	体积	终浓度
pET-22b(+)-PulF	0.5 μL	5 ng
Forward Primer (10 μM)	0.5 μL	0.2 μM
Reverse Primer (10 μM)	0.5 μL	0.2 μM
2×*TransStart*® *FastPfu* Fly PCR SuperMix	12.5 μL	1×
Nuclease-free Water	to 25 μL	Not applicable

[0073]

[0074] 点突变PCR反应结束后，取5μL的PCR产物，进行1％琼脂糖凝胶电泳检测，如有明亮清晰的目的条带出现，且与预期大小一致，则说明扩增成功。在扩增成功的PCR产物中加入0.5μL的DMT酶，37℃温育1小时，以去除其中的质粒模板。

[0075] 2.2 PCR产物的转化和保存：

[0076] PCR产物经DMT酶消化后转化DMT感受态细胞进行复制和验证，转化步骤如下：

[0077] (1)取出DMT感受态细胞稍稍解冻，无菌条件下加入5μL经DMT酶消化后的PCR产物，轻弹混匀，冰浴30min；

[0078] (2)42℃水浴中热激45s，然后迅速将感受态细胞置于冰上，静置3min，此过程中动作要轻柔，不要摇动离心管；

[0079] (3)无菌条件下，向每管感受态细胞中加入500μL室温的SOC培养基(不含抗生素)，37℃，200rpm复苏1h；

[0080] (4)无菌条件下，将复苏的菌液吹打均匀，取200μL涂布于含氨苄青霉素的LB固体培养基上，37℃培养12h；

[0081] (5)挑取菌落提质粒进行测序。

[0082] 序列比对无误的质粒即为定点突变成功的质粒，然后转化E.coli BL21(DE3)感受

态细胞，用于后续表达。转化成功的重组菌E.coli BL21(DE3)冻存于甘油管中备用。

[0083] 2.3突变体酶的纯化：

[0084] 突变体酶的诱导表达：

[0085] 将构建成功的突变体重组菌从甘油冻存管中取出，在含氨苄青霉素的LB固体培养基上划线，37℃12h培养。挑取单菌落，接种于20mL LB液体培养基(Amp^+100μg/mL)中，37℃，200rpm10 h培养，形成种子液。种子液以2％接种量接种于LB液体培养基(Amp^+100μg/mL，装液量400mL/2L)，37℃，200rpm培养至OD600nm到达0.6～0.8之间。添加终浓度为1mM的IPTG，在28℃，200rpm条件下继续培养。6h后，发酵液经4℃，6000rpm离心15min，收集菌体。

[0086] 突变体酶蛋白的分离纯化：

[0087] 菌体重悬于菌体破碎缓冲液(Lysis buffer)中，在冰浴状态下150W超声破碎15min。破碎结束后于4℃，8000rpm离心20min，取上清液过0.22μm水系滤膜，收集滤液；

[0088] 滤液上样至Ni^{2+}亲和层析柱中，层析柱已提前用上样缓冲液(Binding buffer)进行平衡。滤液全部上样完毕后，继续用上样缓冲液(Binding buffer)平衡柱子，直至流出液中检测不到蛋白；

[0089] 用冲洗缓冲液(Washing buffer)冲洗柱子中未结合的杂蛋白，直至流出液中检测不到蛋白；

[0090] 用洗脱缓冲液(Elution buffer)洗脱目的蛋白，并收集洗脱液，直至洗脱液中检测不到蛋白；

[0091] 洗脱液在透析液A中进行透析，以去除洗脱液中的盐离子和金属离子，共透析2次，每次2～3h；

[0092] 洗脱液继续在透析液B中进行透析，以去除洗脱液中的EDTA，共透析2次，每次2～3h。收集洗脱液，即为纯化后的突变体酶溶液。为方便描述，将得到的5个突变体酶的分别命名为M485I、T487S、V496I、L528V、G688F。其中，突变体酶M485I、T487S、V496I、L528V、G688F的氨基酸序列分别如SEQ ID No.1、SEQ ID No.2、SEQ ID No.3、SEQ ID No.4、SEQ ID No.5所示，编码突变体酶M485I、T487S、V496I、L528V、G688F的基因的核苷酸序列分别如SEQ ID No.6、SEQ ID No.7、SEQ ID No.8、SEQ ID No.9、SEQ ID No.10所示。

[0093] 上述过程均在4℃条件下进行。

[0094] 突变体酶的蛋白含量测定：

[0095] 使用BCA蛋白质定量试剂盒，测定纯化后突变体酶溶液的蛋白浓度，

[0096] 突变体酶的变性聚丙烯酰胺凝胶电泳(SDS-PAGE)分析：

[0097] 取5μL 5×上样缓冲液和20μL突变体酶溶液，混匀后煮沸10min。离心取上清液10μL，上样至浓度8％预制胶加样孔中，电压150V跑胶。跑胶后用考马斯亮蓝R250进行染色，染色时间不低于3h。而后脱色至背景清晰，通过凝胶成像系统进行观察。

[0098] 3、突变体酶的酶学性质测定

[0099] 酶活测定方法：

[0100] 以终浓度为0.5％(w/v)的普鲁兰多糖为底物，待测突变体酶在其最适条件下催化反应10min。反应结束后，通过3,5-二硝基水杨酸法(DNS法)测定酶反应期间释放出的还原糖的含量来间接计算酶活。具体的实验步骤为：20μL溶于最适pH缓冲液的1％(w/v)普鲁兰多糖，在最适温度条件下预热5min；将20μL经过适当稀释的酶液迅速加入预热的底物溶液

中，在最适温度下反应10min；10min后迅速加入3，5-二硝基水杨酸试剂(DNS试剂)终止反应，并将混合液的温度升至99.9℃，显色10min。显色结束后，加入100μL去离子水充分混匀，取100μL加样至96孔酶标板中，在540nm波长下测其吸光度。酶活的定义(U)：在一定反应条件下，单位时间内(1min)从普鲁兰多糖中释放出1μmol还原糖所需的普鲁兰酶的量。

[0101] 其中，最适酶反应条件：M485I、T487S、V496I、L528V和G688F都是75℃和pH5.5。

[0102] pH对酶活的影响：

[0103] 在最适温度下，以溶于不同pH缓冲液的1％(w/v)普鲁兰多糖为底物，测定酶活。pH的范围为3.5～7.5，采用两种缓冲液体系：pH3.5～6.0，0.2M醋酸盐缓冲液；pH6.0～7.5，0.2M磷酸盐缓冲液。不同pH条件下测得的酶活用相对酶活进行表示，以测得的最高酶活为100％。

[0104] 其中，酶最适反应温度：M485I、T487S、V496I、L528V和G688F都是75℃。

[0105] 温度对酶活的影响：

[0106] 以溶于最适pH缓冲液的1％(w/v)普鲁兰多糖为底物，在不同温度下(4～90℃)测定突变体酶的酶活。不同温度下测得的酶活用相对酶活进行表示，以测得的最高酶活为100％。

[0107] 其中，酶最适反应pH：M485I、T487S、V496I、L528V和G688F都是pH 5.5。

[0108] 温度对酶稳定性的影响：

[0109] 将普鲁兰酶溶液按体积比1:1加入到其最适pH缓冲液中，在不同的温度下孵育，每隔一段时间取样测酶活。以未经温育的酶液初始酶活为100％，计算残余酶活。

[0110] 酶动力学参数的确定：

[0111] 配制浓度范围为0.5～1.0mg/mL的溶于最适pH缓冲液的普鲁兰多糖系列溶液，用于测定普鲁兰酶的酶动力学参数。除普鲁兰多糖浓度不同外，在标准条件下测定该酶在系列底物浓度下的酶活，通过米氏方程(Linweaver-Burk方程)计算K_m和V_{max}值，进而计算K_{cat}和K_{cat}/K_m值。

[0112] 4、实验结果

[0113] 如图1所示，以pET-22b(+)-pulF为模板，分别使用表1中的突变引物对进行PCR反应，PCR产物通过1％琼脂糖凝胶电泳观察，图1中标号为2、3、4、5、6的条带分别为突变体酶M485I、T487S、V496I、L528V和G688F的电泳条带，从核酸电泳图可以看出，5对突变引物均扩增出了与预期大小一致的条带，虽然有多条扩增条带出现，但是不影响后续实验。

[0114] 按BCA蛋白定量试剂盒法测定纯化后突变体酶的蛋白浓度，标准曲线如图2所示，根据标准曲线的线性方程，计算突变体酶的蛋白浓度，如表4所示。

[0115] 表4突变体酶的蛋白浓度

[0116]

突变体酶	蛋白浓度（μg/mL）
M485I	2025.55±101.18
T487S	1291.09±56.05
V496I	1953.00±25.84
L528V	1403.27±61.97
G688F	2166.55±48.34

[0117] 纯化后突变体酶的变性聚丙烯酰胺凝胶电泳(SDS-PAGE)结果如图3所示。图3中标号为2、3、4、5、6的条带分别为突变体酶M485I、T487S、V496I、L528V和G688F的电泳条带，从图中可以看出，Ni^{2+}亲和层析柱对突变体酶起到了很好的分离纯化作用，5个突变体酶在凝胶中均呈现为单一条带，且根据蛋白Marker的分子量估算出的突变体酶大小与预期一致。

[0118] pH对突变体酶活的影响

[0119] 分别测定了不同pH条件下5个突变体酶的相对酶活，实验结果列于表5中。

[0120] 表5不同pH条件下突变体酶的相对酶活

[0121]

pH	WT	M485I	T481S	V496I	L528V	G688F
3.5	2.91%	2.21%	3.64%	2.42%	3.43%	2.35%
4	4.12%	3.36%	5.11%	5.25%	5.90%	3.85%
4.5	14.34%	31.18%	25.66%	35.81%	40.63%	43.76%
5	100.00%	93.29%	96.80%	95.60%	90.11%	86.08%
5.5	51.96%	100.00%	100.00%	100.00%	100.00%	100.00%
6（1）	9.52%	69.00%	80.97%	86.41%	83.30%	85.72%
6（2）	24.26%	93.80%	92.29%	89.10%	89.18%	84.93%
6.5	8.76%	62.49%	68.38%	79.29%	74.31%	73.76%
7	3.64%	9.22%	18.10%	21.43%	40.00%	22.76%
7.5	2.56%	2.87%	4.43%	3.61%	5.82%	3.39%

[0122]

[0123] 注:WT表示普鲁兰酶PulF

[0124] 从表5中可以看出，当普鲁兰酶PulF突变为M485I、T487S、V496I、L528V和G688F后，最适pH由原来的5.0变成了5.5。但是相比于普鲁兰酶PulF，5个突变体酶在比较宽的pH范围内都具有较高的酶活，尤其是在pH5.0～5.5的范围内，突变体酶的相对酶活都大于85％。在pH4.5的条件下，5个突变体酶的相对酶活都明显高于普鲁兰酶PulF。在pH6.0～7.0范围内，5个突变体酶的相对酶活都明显高于普鲁兰酶PulF。

[0125] 此外还可以看出，5个突变体酶的相对酶活变化趋势与普鲁兰酶PulF有较大区别，但他们5个各自的趋势较为一致，当pH远离最适值时酶活缓慢下降，在pH5.0～6.5范围内相对酶活均大于50％。且不同缓冲液体系对它们的酶活影响不大，在pH6.0的磷酸盐缓冲液中的相对酶活和在pH6.0醋酸缓冲液中较为接近。

[0126] 因为制糖工业中普鲁兰酶反应的pH范围为4.5～5.5，所以结合这一工业应用条件分析，5个突变体酶相比于普鲁兰酶PulF来说最大的优点是：在pH5.0～5.5范围内均具有较高酶活，对糖化过程中反应液的pH环境要求不苛刻。

[0127] 温度对突变体酶活的影响：

[0128] 分别测定了不同温度下5个突变体酶和普鲁兰酶PulF的相对酶活，实验结果列于

表6中。

[0129] 表6不同温度下突变体酶的相对酶活

[0130]

温度(℃)	WT	M485I	T481S	V496I	L528V	G688F
35	2.02%	2.84%	3.70%	2.71%	4.05%	2.53%
40	2.79%	4.70%	6.53%	5.03%	6.10%	3.57%
45	4.03%	6.56%	8.44%	6.86%	8.35%	5.67%
50	6.49%	10.53%	14.24%	11.16%	13.79%	8.86%
55	10.73%	19.14%	18.99%	15.99%	21.94%	16.25%
60	18.68%	25.18%	30.11%	25.28%	31.97%	24.63%
65	34.09%	43.93%	53.75%	47.82%	50.03%	38.55%
70	48.35%	74.59%	77.23%	75.23%	73.05%	64.18%
75	79.76%	100.00%	100.00%	100.00%	100.00%	100.00%
80	100.00%	38.19%	53.87%	62.81%	78.98%	69.70%
85	10.33%	9.28%	10.47%	11.53%	11.29%	9.96%
90	2.18%	3.22%	4.71%	3.96%	4.67%	3.54%

[0131]

[0132] 注:WT表示普鲁兰酶PulF

[0133] 从表6中可以看出,当普鲁兰酶PulF突变为M485I、T487S、V496I、L528V和G688F后,最适温度变为75℃。在60℃～75℃范围内,5个突变体酶的相对酶活都大于普鲁兰酶PulF在该温度下的相对酶活。

[0134] 制糖工业中普鲁兰酶反应的温度范围为55～65℃甚至是更高的温度,其中最常用的反应温度为60℃,所以结合这一工业应用条件分析,突变体酶的优势在于其在温度60℃～75℃范围内具有更高的相对酶活。但是突变体酶M485I、T487S、V496I、L528V和G688F的最适温度相比于普鲁兰酶PulF降了5℃(80℃→75℃),这可能意味着这5个突变体酶的热稳定性有所减弱。

[0135] 为了进一步探究突变体酶的热稳定性如何变化,测定了5个突变体酶在75℃和80℃条件下的半衰期。

[0136] 温度对突变体酶稳定性的影响

[0137] 测定了突变体酶M485I、T487S、V496I、L528V和G688F在75℃和80℃条件孵育不同时间后的残余酶活,并将残余酶活取自然对数,作其随孵育时间的变化曲线,如图4和图5。并计算每个突变体酶在两个温度下的半衰期,结果列于表7中。

[0138] 从图4和图5中可以明显看出,在75℃条件下,5个突变体酶的热稳定性都有所增强,它们热稳定性由强到弱的顺序为:L528V>G688F>V496I>T487S>M485I;在80℃条件下,5个突变体酶的热稳定性也都高于普鲁兰酶PulF,它们的热稳定性由强到弱的顺序为:V496I>G688F>T487S>L528V>M485I。两个温度条件下,5个突变体酶热稳定性强弱的顺序是不同的。

[0139] 表7不同温度下普鲁兰酶PulF和突变体酶的半衰期

[0140]

蛋白编号	75℃, $t_{1/2}$（h）	80℃, $t_{1/2}$（min）
WT	0.61	<1.00
V481C	8.42	2.24
M485I	3.02	1.83
T487S	3.67	3.04
V496I	5.55	6.05
L528V	6.55	2.38
G688F	5.81	4.56

[0141]

[0142] 注:WT表示普鲁兰酶PulF

[0143] 75℃条件下,M485I的半衰期提高的最少,但也比普鲁兰酶PulF提高了近4倍。80℃条件下,所有酶的半衰期都很短,其中V496I的半衰期最长,而M485I的半衰期最短。

[0144] 突变体酶的酶动力参数:

[0145] 突变体酶M485I、T487S、V496I、L528V、G688F和普鲁兰酶PulF的酶动力学参数列于表8中。

[0146] 表8酶动力学参数比较

[0147]

Variants	K_m (mg/mL)	V_{max} (μmol/mg/min)	K_{cat} (s^{-1})	K_{cat}/K_m (mL/mg/s)	specific activity (U/mg)
WT	3.91±0.11	33.11±1.28	52.05±2.01	13.31±0.51	25.91±0.73
M485I	1.63±0.10	24.00±1.23	37.73±1.94	23.09±1.19	20.52±0.16
T487S	3.06±0.32	15.68±1.17	24.65±1.84	8.05±0.60	10.88±0.38
V496I	1.42±0.10	21.67±0.54	34.07±0.85	23.93±0.60	19.63±0.71
L528V	1.10±0.10	12.90±0.66	20.27±1.04	18.48±0.94	12.22±0.45
G688F	1.71±0.15	24.18±1.02	38.06±1.61	22.30±0.94	22.20±0.50

[0148] 注:WT表示普鲁兰酶PulF

[0149] 从表8中可以看出,相比于普鲁兰酶PulF而言,5个突变体酶的K_m值均有所降低,说明突变体酶与普鲁兰多糖的结合能力增强了;突变体酶M481I、V496I、L528V和G688F的催化常数K_{cat}/K_m均有所增加,说明这些突变体酶对普鲁兰多糖的催化效率提高了。尽管本发明的实施方案已公开如上,但其并不仅仅限于说明书和实施方式中所列运用,它完全可以被适用于各种适合本发明的领域,对于熟悉本领域的人员而言,可容易地实现另外的修改,因此在不背离权利要求及等同范围所限定的一般概念下,本发明并不限于特定的细节和这里示出与描述的图例。

序列表

SEQUENCE LISTING

<110> 中国农业科学院农产品加工研究所

<120> 耐热的I型普鲁兰酶的突变体酶及其制备方法与应用

<130> 1

<160> 1

<170> PatentIn version 3.5

<210> 1

<211> 820

<212> PRT

<213> 人工测序

[0150] <400> 1

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Asp Gly Trp Asn Leu Trp Ile Trp Trp Val Glu Pro Ile Ser Lys Asp
20 25 30

Gly Ala Ala Tyr Gln Phe Thr Glu Lys Asp Asp Phe Gly Val Val Ala
35 40 45

Arg Val Lys Phe Asp Glu Thr Leu Thr Lys Val Gly Ile Ile Val Arg
50 55 60

Leu Asn Glu Trp Lys Glu Lys Asp Val Ala Met Asp Arg Phe Ile Ser
65 70 75 80

Ile Lys Asp Gly Lys Ala Glu Val Trp Leu Leu Gln Gly Ile Glu Gln
85 90 95

Ile Tyr Thr Thr Lys Pro Asp Thr Ser Pro Arg Val Leu Phe Ala Gln

100 105 110

Ala Arg Ala Gln Asp Val Ile Glu Ala Tyr Leu Thr Gly Gln Val Asp

115 120 125

Thr Thr Lys Val Ser Ala Lys Val Thr Val Asp Gly Val Glu Arg Lys

130 135 140

Val Ala Lys Val Glu Lys Ala Asn Pro Thr Asp Ile Ser Lys Thr Asn

145 150 155 160

His Val Lys Ile Thr Leu Ala Glu Pro Ile Lys Leu Asp Glu Val Asn

165 170 175

Lys Asp Val Gln Val Glu Ile Glu Gly Tyr Lys Pro Ala Arg Val Ile

180 185 190

[0151] Met Met Glu Ile Leu Asp Lys Ile Tyr Tyr Asp Gly Pro Leu Gly Phe

195 200 205

Glu Tyr Ser Pro Thr Lys Thr Thr Ile Arg Val Trp Ser Pro Val Ser

210 215 220

Lys Thr Val Asp Leu Leu Leu Tyr Lys Asn Trp Asp Asp Lys Glu Pro

225 230 235 240

Thr Lys Val Val Pro Met Lys Tyr Ile Gly Asn Gly Ala Trp Glu Ala

245 250 255

Val Leu Asp Gly Asp Trp Glu Gly Trp Phe Tyr Arg Ile Arg Tyr Phe

260 265 270

Ser Tyr Gly Glu Tyr Arg Glu Gly Val Asp Tyr Phe Ser Lys Ala Val

275 280 285

Thr Lys Asn Ser Ala Lys Ser Ala Ile Ile Asp Leu Lys Lys Thr Asn

290 295 300

Pro Ser Asp Trp Asp Lys Asp Val Arg Pro Thr Met Lys Ala Leu Glu

305 310 315 320

Asp Ala Ile Ile Tyr Glu Ile His Ile Ala Asp Met Thr Gly Leu Asp

325 330 335

Asn Ser Asn Val Lys Asn Lys Ala Thr Tyr Leu Gly Leu Thr Glu Lys

340 345 350

Gly Thr Arg Gly Pro Asn Gly Val Thr Thr Gly Leu Asp His Leu Val

355 360 365

Glu Leu Gly Val Thr His Val His Ile Leu Pro Met Phe Asp Phe Tyr

370 375 380

Thr Gly Asp Glu Ser Glu Arg Asp Phe Glu Lys Ser Tyr Asn Trp Gly

[0152] 385 390 395 400

Tyr Asp Pro Tyr Leu Phe Thr Val Pro Glu Gly Arg Tyr Ser Thr Asn

405 410 415

Pro Ile Asp Pro His Val Arg Ile Asn Glu Val Lys Gln Met Val Lys

420 425 430

Ala Leu His Glu Asn Gly Ile Arg Val Ile Leu Asp Met Val Phe Pro

435 440 445

His Thr Phe Gly Ile Gly Val Leu Ser Pro Phe Asp Thr Ala Val Pro

450 455 460

Tyr Tyr Phe Tyr Arg Ile Asp Lys Thr Gly Ala Tyr Leu Asn Glu Ser

465 470 475 480

Gly Val Gly Asn Val Ile Ala Thr Glu Arg Pro Met Leu Arg Lys Tyr

485 490 495

Val Ile Asp Thr Leu Lys Trp Trp Val Leu Glu Tyr His Val Asp Gly

500 505 510

Phe Arg Phe Asp Gln Met Gly Leu Met Asp Lys Lys Thr Met Leu Asp

515 520 525

Leu Glu Lys Glu Leu His Ala Ile Asp Pro Thr Ile Leu Leu Tyr Gly

530 535 540

Glu Pro Trp Gly Gly Trp Gly Ala Pro Ile Arg Phe Gly Lys Ser Asp

545 550 555 560

Val Gly Gly Thr His Ile Ala Ala Phe Asn Asp Glu Phe Arg Asp Ala

565 570 575

Met Arg Gly Ser Val Phe Asn Ala Thr Val Lys Gly Phe Leu Met Gly

580 585 590

[0153] Ala Leu Ala Lys Glu Thr Ala Ile Lys Arg Gly Val Val Gly Ser Ile

595 600 605

Glu Tyr Asp Asp Val Ile Arg Ser Phe Ala Lys Asp Pro Glu Glu Thr

610 615 620

Ile Asn Tyr Val Ala Cys His Asp Asn His Thr Leu Trp Asp Lys Asn

625 630 635 640

Tyr Leu Ala Ala Gln Ala Asp Thr Asn Ile Lys Trp Thr Glu Glu Met

645 650 655

Leu Lys Asn Ala Gln Lys Leu Ala Gly Ala Ile Leu Leu Thr Ser Gln

660 665 670

Gly Ile Pro Phe Leu His Ala Gly Gln Asp Phe Ala Arg Thr Lys Lys

675 680 685

Gly Asp Glu Asn Ser Tyr Asn Ser Pro Ile Ser Ile Asn Gly Leu Asp

690 695 700

Tyr Ala Arg Lys Ala Glu Phe Ile Asp Val Phe Asn Tyr Tyr Lys Gly

705 710 715 720

Leu Ile Glu Ile Arg Lys Ala His Pro Ala Phe Arg Gln Arg Thr Ala

725 730 735

Asp Asp Ile Lys Lys Lys Ile Thr Phe Leu Pro Thr Thr Arg Lys Met

740 745 750

Val Ala Phe Thr Ile Lys Asp Glu Asn Asp Ser Trp Lys Glu Ile Leu

755 760 765

Val Ile Tyr Asn Gly Asp Thr Lys Asp Gln Asp Phe Thr Leu Pro Glu

770 775 780

Gly Thr Trp Asn Val Val Val Asp Gln Gln Asn Ala Gly Thr Lys Val

[0154] 785 790 795 800

Leu Tyr Gln Val Ser Gly Lys Ile Thr Val Lys Ser Ile Ser Ala Met

805 810 815

Val Met Tyr Lys

820

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Asp Gly Trp Asn Leu Trp Ile Trp Trp Val Glu Pro Ile Ser Lys Asp

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Gly Ala Ala Tyr Gln Phe Thr Glu Lys Asp Asp Phe Gly Val Val Ala

35 40 45

Arg Val Lys Phe Asp Glu Thr Leu Thr Lys Val Gly Ile Ile Val Arg

50 55 60

Leu Asn Glu Trp Lys Glu Lys Asp Val Ala Met Asp Arg Phe Ile Ser

65 70 75 80

Ile Lys Asp Gly Lys Ala Glu Val Trp Leu Leu Gln Gly Ile Glu Gln

85 90 95

Ile Tyr Thr Thr Lys Pro Asp Thr Ser Pro Arg Val Leu Phe Ala Gln

100 105 110

Ala Arg Ala Gln Asp Val Ile Glu Ala Tyr Leu Thr Gly Gln Val Asp

[0155] 115 120 125

Thr Thr Lys Val Ser Ala Lys Val Thr Val Asp Gly Val Glu Arg Lys

130 135 140

Val Ala Lys Val Glu Lys Ala Asn Pro Thr Asp Ile Ser Lys Thr Asn

145 150 155 160

His Val Lys Ile Thr Leu Ala Glu Pro Ile Lys Leu Asp Glu Val Asn

165 170 175

Lys Asp Val Gln Val Glu Ile Glu Gly Tyr Lys Pro Ala Arg Val Ile

180 185 190

Met Met Glu Ile Leu Asp Lys Ile Tyr Tyr Asp Gly Pro Leu Gly Phe

195 200 205

Glu Tyr Ser Pro Thr Lys Thr Thr Ile Arg Val Trp Ser Pro Val Ser

210 215 220

Lys Thr Val Asp Leu Leu Leu Tyr Lys Asn Trp Asp Asp Lys Glu Pro

225 230 235 240

Thr Lys Val Val Pro Met Lys Tyr Ile Gly Asn Gly Ala Trp Glu Ala

245 250 255

Val Leu Asp Gly Asp Trp Glu Gly Trp Phe Tyr Arg Ile Arg Tyr Phe

260 265 270

Ser Tyr Gly Glu Tyr Arg Glu Gly Val Asp Tyr Phe Ser Lys Ala Val

275 280 285

Thr Lys Asn Ser Ala Lys Ser Ala Ile Ile Asp Leu Lys Lys Thr Asn

290 295 300

Pro Ser Asp Trp Asp Lys Asp Val Arg Pro Thr Met Lys Ala Leu Glu

305 310 315 320

[0156] Asp Ala Ile Ile Tyr Glu Ile His Ile Ala Asp Met Thr Gly Leu Asp

325 330 335

Asn Ser Asn Val Lys Asn Lys Ala Thr Tyr Leu Gly Leu Thr Glu Lys

340 345 350

Gly Thr Arg Gly Pro Asn Gly Val Thr Thr Gly Leu Asp His Leu Val

355 360 365

Glu Leu Gly Val Thr His Val His Ile Leu Pro Met Phe Asp Phe Tyr

370 375 380

Thr Gly Asp Glu Ser Glu Arg Asp Phe Glu Lys Ser Tyr Asn Trp Gly

385 390 395 400

Tyr Asp Pro Tyr Leu Phe Thr Val Pro Glu Gly Arg Tyr Ser Thr Asn

405 410 415

Pro Ile Asp Pro His Val Arg Ile Asn Glu Val Lys Gln Met Val Lys

420 425 430

Ala Leu His Glu Asn Gly Ile Arg Val Ile Leu Asp Met Val Phe Pro

435 440 445

His Thr Phe Gly Ile Gly Val Leu Ser Pro Phe Asp Thr Ala Val Pro

450 455 460

Tyr Tyr Phe Tyr Arg Ile Asp Lys Thr Gly Ala Tyr Leu Asn Glu Ser

465 470 475 480

Gly Val Gly Asn Val Met Ala Ser Glu Arg Pro Met Leu Arg Lys Tyr

485 490 495

Val Ile Asp Thr Leu Lys Trp Trp Val Leu Glu Tyr His Val Asp Gly

500 505 510

Phe Arg Phe Asp Gln Met Gly Leu Met Asp Lys Lys Thr Met Leu Asp

[0157] 515 520 525

Leu Glu Lys Glu Leu His Ala Ile Asp Pro Thr Ile Leu Leu Tyr Gly

530 535 540

Glu Pro Trp Gly Gly Trp Gly Ala Pro Ile Arg Phe Gly Lys Ser Asp

545 550 555 560

Val Gly Gly Thr His Ile Ala Ala Phe Asn Asp Glu Phe Arg Asp Ala

565 570 575

Met Arg Gly Ser Val Phe Asn Ala Thr Val Lys Gly Phe Leu Met Gly

580 585 590

Ala Leu Ala Lys Glu Thr Ala Ile Lys Arg Gly Val Val Gly Ser Ile

595 600 605

Glu Tyr Asp Asp Val Ile Arg Ser Phe Ala Lys Asp Pro Glu Glu Thr

610 615 620

Ile Asn Tyr Val Ala Cys His Asp Asn His Thr Leu Trp Asp Lys Asn

625 630 635 640

Tyr Leu Ala Ala Gln Ala Asp Thr Asn Ile Lys Trp Thr Glu Glu Met

645 650 655

Leu Lys Asn Ala Gln Lys Leu Ala Gly Ala Ile Leu Leu Thr Ser Gln

660 665 670

Gly Ile Pro Phe Leu His Ala Gly Gln Asp Phe Ala Arg Thr Lys Lys

675 680 685

Gly Asp Glu Asn Ser Tyr Asn Ser Pro Ile Ser Ile Asn Gly Leu Asp

690 695 700

Tyr Ala Arg Lys Ala Glu Phe Ile Asp Val Phe Asn Tyr Tyr Lys Gly

705 710 715 720

[0158] Leu Ile Glu Ile Arg Lys Ala His Pro Ala Phe Arg Gln Arg Thr Ala

725 730 735

Asp Asp Ile Lys Lys Lys Ile Thr Phe Leu Pro Thr Thr Arg Lys Met

740 745 750

Val Ala Phe Thr Ile Lys Asp Glu Asn Asp Ser Trp Lys Glu Ile Leu

755 760 765

Val Ile Tyr Asn Gly Asp Thr Lys Asp Gln Asp Phe Thr Leu Pro Glu

770 775 780

Gly Thr Trp Asn Val Val Val Asp Gln Gln Asn Ala Gly Thr Lys Val

785 790 795 800

Leu Tyr Gln Val Ser Gly Lys Ile Thr Val Lys Ser Ile Ser Ala Met

805 810 815

Val Met Tyr Lys

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Met Ala Thr Glu Leu Val Ile His Tyr His Arg Trp Asp Gly Asn Tyr

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Asp Gly Trp Asn Leu Trp Ile Trp Trp Val Glu Pro Ile Ser Lys Asp

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Gly Ala Ala Tyr Gln Phe Thr Glu Lys Asp Asp Phe Gly Val Val Ala

35 40 45

[0159] Arg Val Lys Phe Asp Glu Thr Leu Thr Lys Val Gly Ile Ile Val Arg

50 55 60

Leu Asn Glu Trp Lys Glu Lys Asp Val Ala Met Asp Arg Phe Ile Ser

65 70 75 80

Ile Lys Asp Gly Lys Ala Glu Val Trp Leu Leu Gln Gly Ile Glu Gln

85 90 95

Ile Tyr Thr Thr Lys Pro Asp Thr Ser Pro Arg Val Leu Phe Ala Gln

100 105 110

Ala Arg Ala Gln Asp Val Ile Glu Ala Tyr Leu Thr Gly Gln Val Asp

115 120 125

Thr Thr Lys Val Ser Ala Lys Val Thr Val Asp Gly Val Glu Arg Lys

130 135 140

Val Ala Lys Val Glu Lys Ala Asn Pro Thr Asp Ile Ser Lys Thr Asn

145 150 155 160

His Val Lys Ile Thr Leu Ala Glu Pro Ile Lys Leu Asp Glu Val Asn

165 170 175

Lys Asp Val Gln Val Glu Ile Glu Gly Tyr Lys Pro Ala Arg Val Ile

180 185 190

Met Met Glu Ile Leu Asp Lys Ile Tyr Tyr Asp Gly Pro Leu Gly Phe

195 200 205

Glu Tyr Ser Pro Thr Lys Thr Thr Ile Arg Val Trp Ser Pro Val Ser

210 215 220

Lys Thr Val Asp Leu Leu Leu Tyr Lys Asn Trp Asp Asp Lys Glu Pro

225 230 235 240

Thr Lys Val Val Pro Met Lys Tyr Ile Gly Asn Gly Ala Trp Glu Ala

[0160] 245 250 255

Val Leu Asp Gly Asp Trp Glu Gly Trp Phe Tyr Arg Ile Arg Tyr Phe

260 265 270

Ser Tyr Gly Glu Tyr Arg Glu Gly Val Asp Tyr Phe Ser Lys Ala Val

275 280 285

Thr Lys Asn Ser Ala Lys Ser Ala Ile Ile Asp Leu Lys Lys Thr Asn

290 295 300

Pro Ser Asp Trp Asp Lys Asp Val Arg Pro Thr Met Lys Ala Leu Glu

305 310 315 320

Asp Ala Ile Ile Tyr Glu Ile His Ile Ala Asp Met Thr Gly Leu Asp

325 330 335

Asn Ser Asn Val Lys Asn Lys Ala Thr Tyr Leu Gly Leu Thr Glu Lys

340 345 350

Gly Thr Arg Gly Pro Asn Gly Val Thr Thr Gly Leu Asp His Leu Val

355 360 365

Glu Leu Gly Val Thr His Val His Ile Leu Pro Met Phe Asp Phe Tyr

370 375 380

Thr Gly Asp Glu Ser Glu Arg Asp Phe Glu Lys Ser Tyr Asn Trp Gly

385 390 395 400

Tyr Asp Pro Tyr Leu Phe Thr Val Pro Glu Gly Arg Tyr Ser Thr Asn

405 410 415

Pro Ile Asp Pro His Val Arg Ile Asn Glu Val Lys Gln Met Val Lys

420 425 430

Ala Leu His Glu Asn Gly Ile Arg Val Ile Leu Asp Met Val Phe Pro

435 440 445

[0161] His Thr Phe Gly Ile Gly Val Leu Ser Pro Phe Asp Thr Ala Val Pro

450 455 460

Tyr Tyr Phe Tyr Arg Ile Asp Lys Thr Gly Ala Tyr Leu Asn Glu Ser

465 470 475 480

Gly Val Gly Asn Val Met Ala Thr Glu Arg Pro Met Leu Arg Lys Tyr

485 490 495

Ile Ile Asp Thr Leu Lys Trp Trp Val Leu Glu Tyr His Val Asp Gly

500 505 510

Phe Arg Phe Asp Gln Met Gly Leu Met Asp Lys Lys Thr Met Leu Asp

515 520 525

Leu Glu Lys Glu Leu His Ala Ile Asp Pro Thr Ile Leu Leu Tyr Gly

530 535 540

Glu Pro Trp Gly Gly Trp Gly Ala Pro Ile Arg Phe Gly Lys Ser Asp

545 550 555 560

Val Gly Gly Thr His Ile Ala Ala Phe Asn Asp Glu Phe Arg Asp Ala

565 570 575

Met Arg Gly Ser Val Phe Asn Ala Thr Val Lys Gly Phe Leu Met Gly

580 585 590

Ala Leu Ala Lys Glu Thr Ala Ile Lys Arg Gly Val Val Gly Ser Ile

595 600 605

Glu Tyr Asp Asp Val Ile Arg Ser Phe Ala Lys Asp Pro Glu Glu Thr

610 615 620

Ile Asn Tyr Val Ala Cys His Asp Asn His Thr Leu Trp Asp Lys Asn

625 630 635 640

Tyr Leu Ala Ala Gln Ala Asp Thr Asn Ile Lys Trp Thr Glu Glu Met

[0162] 645 650 655

Leu Lys Asn Ala Gln Lys Leu Ala Gly Ala Ile Leu Leu Thr Ser Gln

660 665 670

Gly Ile Pro Phe Leu His Ala Gly Gln Asp Phe Ala Arg Thr Lys Lys

675 680 685

Gly Asp Glu Asn Ser Tyr Asn Ser Pro Ile Ser Ile Asn Gly Leu Asp

690 695 700

Tyr Ala Arg Lys Ala Glu Phe Ile Asp Val Phe Asn Tyr Tyr Lys Gly

705 710 715 720

Leu Ile Glu Ile Arg Lys Ala His Pro Ala Phe Arg Gln Arg Thr Ala

725 730 735

Asp Asp Ile Lys Lys Lys Ile Thr Phe Leu Pro Thr Thr Arg Lys Met

740 745 750

Val Ala Phe Thr Ile Lys Asp Glu Asn Asp Ser Trp Lys Glu Ile Leu

755 760 765

Val Ile Tyr Asn Gly Asp Thr Lys Asp Gln Asp Phe Thr Leu Pro Glu

770 775 780

Gly Thr Trp Asn Val Val Val Asp Gln Gln Asn Ala Gly Thr Lys Val

785 790 795 800

Leu Tyr Gln Val Ser Gly Lys Ile Thr Val Lys Ser Ile Ser Ala Met

805 810 815

Val Met Tyr Lys

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Asp Gly Trp Asn Leu Trp Ile Trp Trp Val Glu Pro Ile Ser Lys Asp

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Gly Ala Ala Tyr Gln Phe Thr Glu Lys Asp Asp Phe Gly Val Val Ala

35 40 45

Arg Val Lys Phe Asp Glu Thr Leu Thr Lys Val Gly Ile Ile Val Arg

50 55 60

Leu Asn Glu Trp Lys Glu Lys Asp Val Ala Met Asp Arg Phe Ile Ser

65 70 75 80

Ile Lys Asp Gly Lys Ala Glu Val Trp Leu Leu Gln Gly Ile Glu Gln

85 90 95

Ile Tyr Thr Thr Lys Pro Asp Thr Ser Pro Arg Val Leu Phe Ala Gln

100 105 110

Ala Arg Ala Gln Asp Val Ile Glu Ala Tyr Leu Thr Gly Gln Val Asp

115 120 125

Thr Thr Lys Val Ser Ala Lys Val Thr Val Asp Gly Val Glu Arg Lys

130 135 140

Val Ala Lys Val Glu Lys Ala Asn Pro Thr Asp Ile Ser Lys Thr Asn

145 150 155 160

His Val Lys Ile Thr Leu Ala Glu Pro Ile Lys Leu Asp Glu Val Asn

165 170 175

[0164] Lys Asp Val Gln Val Glu Ile Glu Gly Tyr Lys Pro Ala Arg Val Ile

180 185 190

Met Met Glu Ile Leu Asp Lys Ile Tyr Tyr Asp Gly Pro Leu Gly Phe

195 200 205

Glu Tyr Ser Pro Thr Lys Thr Thr Ile Arg Val Trp Ser Pro Val Ser

210 215 220

Lys Thr Val Asp Leu Leu Leu Tyr Lys Asn Trp Asp Asp Lys Glu Pro

225 230 235 240

Thr Lys Val Val Pro Met Lys Tyr Ile Gly Asn Gly Ala Trp Glu Ala

245 250 255

Val Leu Asp Gly Asp Trp Glu Gly Trp Phe Tyr Arg Ile Arg Tyr Phe

260 265 270

Ser Tyr Gly Glu Tyr Arg Glu Gly Val Asp Tyr Phe Ser Lys Ala Val

275 280 285

Thr Lys Asn Ser Ala Lys Ser Ala Ile Ile Asp Leu Lys Lys Thr Asn

290 295 300

Pro Ser Asp Trp Asp Lys Asp Val Arg Pro Thr Met Lys Ala Leu Glu

305 310 315 320

Asp Ala Ile Ile Tyr Glu Ile His Ile Ala Asp Met Thr Gly Leu Asp

325 330 335

Asn Ser Asn Val Lys Asn Lys Ala Thr Tyr Leu Gly Leu Thr Glu Lys

340 345 350

Gly Thr Arg Gly Pro Asn Gly Val Thr Thr Gly Leu Asp His Leu Val

355 360 365

Glu Leu Gly Val Thr His Val His Ile Leu Pro Met Phe Asp Phe Tyr

[0165] 370 375 380

Thr Gly Asp Glu Ser Glu Arg Asp Phe Glu Lys Ser Tyr Asn Trp Gly

385 390 395 400

Tyr Asp Pro Tyr Leu Phe Thr Val Pro Glu Gly Arg Tyr Ser Thr Asn

405 410 415

Pro Ile Asp Pro His Val Arg Ile Asn Glu Val Lys Gln Met Val Lys

420 425 430

Ala Leu His Glu Asn Gly Ile Arg Val Ile Leu Asp Met Val Phe Pro

435 440 445

His Thr Phe Gly Ile Gly Val Leu Ser Pro Phe Asp Thr Ala Val Pro

450 455 460

Tyr Tyr Phe Tyr Arg Ile Asp Lys Thr Gly Ala Tyr Leu Asn Glu Ser

465 470 475 480

Gly Val Gly Asn Val Met Ala Thr Glu Arg Pro Met Leu Arg Lys Tyr

485 490 495

Val Ile Asp Thr Leu Lys Trp Trp Val Leu Glu Tyr His Val Asp Gly

500 505 510

Phe Arg Phe Asp Gln Met Gly Leu Met Asp Lys Lys Thr Met Leu Asp

515 520 525

Val Glu Lys Glu Leu His Ala Ile Asp Pro Thr Ile Leu Leu Tyr Gly

530 535 540

Glu Pro Trp Gly Gly Trp Gly Ala Pro Ile Arg Phe Gly Lys Ser Asp

545 550 555 560

Val Gly Gly Thr His Ile Ala Ala Phe Asn Asp Glu Phe Arg Asp Ala

565 570 575

[0166] Met Arg Gly Ser Val Phe Asn Ala Thr Val Lys Gly Phe Leu Met Gly

580 585 590

Ala Leu Ala Lys Glu Thr Ala Ile Lys Arg Gly Val Val Gly Ser Ile

595 600 605

Glu Tyr Asp Asp Val Ile Arg Ser Phe Ala Lys Asp Pro Glu Glu Thr

610 615 620

Ile Asn Tyr Val Ala Cys His Asp Asn His Thr Leu Trp Asp Lys Asn

625 630 635 640

Tyr Leu Ala Ala Gln Ala Asp Thr Asn Ile Lys Trp Thr Glu Glu Met

645 650 655

Leu Lys Asn Ala Gln Lys Leu Ala Gly Ala Ile Leu Leu Thr Ser Gln

660 665 670

Gly Ile Pro Phe Leu His Ala Gly Gln Asp Phe Ala Arg Thr Lys Lys

675 680 685

Gly Asp Glu Asn Ser Tyr Asn Ser Pro Ile Ser Ile Asn Gly Leu Asp

690 695 700

Tyr Ala Arg Lys Ala Glu Phe Ile Asp Val Phe Asn Tyr Tyr Lys Gly

705 710 715 720

Leu Ile Glu Ile Arg Lys Ala His Pro Ala Phe Arg Gln Arg Thr Ala

725 730 735

Asp Asp Ile Lys Lys Lys Ile Thr Phe Leu Pro Thr Thr Arg Lys Met

740 745 750

Val Ala Phe Thr Ile Lys Asp Glu Asn Asp Ser Trp Lys Glu Ile Leu

755 760 765

Val Ile Tyr Asn Gly Asp Thr Lys Asp Gln Asp Phe Thr Leu Pro Glu

[0167]

770 775 780

Gly Thr Trp Asn Val Val Val Asp Gln Gln Asn Ala Gly Thr Lys Val

785 790 795 800

Leu Tyr Gln Val Ser Gly Lys Ile Thr Val Lys Ser Ile Ser Ala Met

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Val Met Tyr Lys

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Asp Gly Trp Asn Leu Trp Ile Trp Trp Val Glu Pro Ile Ser Lys Asp

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Gly Ala Ala Tyr Gln Phe Thr Glu Lys Asp Asp Phe Gly Val Val Ala

35 40 45

Arg Val Lys Phe Asp Glu Thr Leu Thr Lys Val Gly Ile Ile Val Arg

50 55 60

Leu Asn Glu Trp Lys Glu Lys Asp Val Ala Met Asp Arg Phe Ile Ser

65 70 75 80

Ile Lys Asp Gly Lys Ala Glu Val Trp Leu Leu Gln Gly Ile Glu Gln

85 90 95

[0168] Ile Tyr Thr Thr Lys Pro Asp Thr Ser Pro Arg Val Leu Phe Ala Gln

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Thr Thr Lys Val Ser Ala Lys Val Thr Val Asp Gly Val Glu Arg Lys

130 135 140

Val Ala Lys Val Glu Lys Ala Asn Pro Thr Asp Ile Ser Lys Thr Asn

145 150 155 160

His Val Lys Ile Thr Leu Ala Glu Pro Ile Lys Leu Asp Glu Val Asn

165 170 175

Lys Asp Val Gln Val Glu Ile Glu Gly Tyr Lys Pro Ala Arg Val Ile

180 185 190

Met Met Glu Ile Leu Asp Lys Ile Tyr Tyr Asp Gly Pro Leu Gly Phe

195 200 205

Glu Tyr Ser Pro Thr Lys Thr Thr Ile Arg Val Trp Ser Pro Val Ser

210 215 220

Lys Thr Val Asp Leu Leu Leu Tyr Lys Asn Trp Asp Asp Lys Glu Pro

225 230 235 240

Thr Lys Val Val Pro Met Lys Tyr Ile Gly Asn Gly Ala Trp Glu Ala

245 250 255

Val Leu Asp Gly Asp Trp Glu Gly Trp Phe Tyr Arg Ile Arg Tyr Phe

260 265 270

Ser Tyr Gly Glu Tyr Arg Glu Gly Val Asp Tyr Phe Ser Lys Ala Val

275 280 285

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305 310 315 320

Asp Ala Ile Ile Tyr Glu Ile His Ile Ala Asp Met Thr Gly Leu Asp

325 330 335

Asn Ser Asn Val Lys Asn Lys Ala Thr Tyr Leu Gly Leu Thr Glu Lys

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Gly Thr Arg Gly Pro Asn Gly Val Thr Thr Gly Leu Asp His Leu Val

355 360 365

Glu Leu Gly Val Thr His Val His Ile Leu Pro Met Phe Asp Phe Tyr

370 375 380

Thr Gly Asp Glu Ser Glu Arg Asp Phe Glu Lys Ser Tyr Asn Trp Gly

385 390 395 400

Tyr Asp Pro Tyr Leu Phe Thr Val Pro Glu Gly Arg Tyr Ser Thr Asn

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Pro Ile Asp Pro His Val Arg Ile Asn Glu Val Lys Gln Met Val Lys

420 425 430

Ala Leu His Glu Asn Gly Ile Arg Val Ile Leu Asp Met Val Phe Pro

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His Thr Phe Gly Ile Gly Val Leu Ser Pro Phe Asp Thr Ala Val Pro

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Tyr Tyr Phe Tyr Arg Ile Asp Lys Thr Gly Ala Tyr Leu Asn Glu Ser

465 470 475 480

Gly Val Gly Asn Val Met Ala Thr Glu Arg Pro Met Leu Arg Lys Tyr

485 490 495

[0170] Val Ile Asp Thr Leu Lys Trp Trp Val Leu Glu Tyr His Val Asp Gly

500 505 510

Phe Arg Phe Asp Gln Met Gly Leu Met Asp Lys Lys Thr Met Leu Asp

515 520 525

Leu Glu Lys Glu Leu His Ala Ile Asp Pro Thr Ile Leu Leu Tyr Gly

530 535 540

Glu Pro Trp Gly Gly Trp Gly Ala Pro Ile Arg Phe Gly Lys Ser Asp

545 550 555 560

Val Gly Gly Thr His Ile Ala Ala Phe Asn Asp Glu Phe Arg Asp Ala

565 570 575

Met Arg Gly Ser Val Phe Asn Ala Thr Val Lys Gly Phe Leu Met Gly

580 585 590

Ala Leu Ala Lys Glu Thr Ala Ile Lys Arg Gly Val Val Gly Ser Ile

595 600 605

Glu Tyr Asp Asp Val Ile Arg Ser Phe Ala Lys Asp Pro Glu Glu Thr

610 615 620

Ile Asn Tyr Val Ala Cys His Asp Asn His Thr Leu Trp Asp Lys Asn

625 630 635 640

Tyr Leu Ala Ala Gln Ala Asp Thr Asn Ile Lys Trp Thr Glu Glu Met

645 650 655

Leu Lys Asn Ala Gln Lys Leu Ala Gly Ala Ile Leu Leu Thr Ser Gln

660 665 670

Gly Ile Pro Phe Leu His Ala Gly Gln Asp Phe Ala Arg Thr Lys Lys

675 680 685

Phe Asp Glu Asn Ser Tyr Asn Ser Pro Ile Ser Ile Asn Gly Leu Asp

[0171] 690 695 700

Tyr Ala Arg Lys Ala Glu Phe Ile Asp Val Phe Asn Tyr Tyr Lys Gly

705 710 715 720

Leu Ile Glu Ile Arg Lys Ala His Pro Ala Phe Arg Gln Arg Thr Ala

725 730 735

Asp Asp Ile Lys Lys Lys Ile Thr Phe Leu Pro Thr Thr Arg Lys Met

740 745 750

Val Ala Phe Thr Ile Lys Asp Glu Asn Asp Ser Trp Lys Glu Ile Leu

755 760 765

Val Ile Tyr Asn Gly Asp Thr Lys Asp Gln Asp Phe Thr Leu Pro Glu

770 775 780

Gly Thr Trp Asn Val Val Val Asp Gln Gln Asn Ala Gly Thr Lys Val

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805 810 815

Val Met Tyr Lys

820

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<212> DNA

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<400> 6

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aaggatgatt ttggcgtggt ggcgcgcgtg aaatttgatg aaaccctgac caaagtgggc 180

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aaaccggata ccagtccgcg tgtgctgttt gcacaagcgc gtgcgcaaga tgtgattgaa 360

gcgtatctga ccggccaagt ggataccacc aaagtgagcg cgaaagtgac cgtggatggc 420

gtggaacgca aagtggcgaa agtggaaaaa gcgaatccga ccgatattag caaaaccaac 480

catgtgaaaa ttaccctggc ggaaccgatt aaactggatg aggtgaacaa agatgtgcag 540

gtggaaattg aaggctataa accggcgcgc gtgattatga tggaaatcct ggacaagatt 600

tactatgatg gcccgctggg ctttgaatat agcccgacca aaaccaccat tcgcgtgtgg 660

agcccggtta gcaaaaccgt ggatctgctg ctgtataaga attgggatga taaggaaccg 720

accaaagtgg tgccgatgaa gtatattggc aatggcgcgt gggaagcagt tctggatggc 780

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aaaaagacca atccgagcga ttgggataaa gatgtgcgcc cgaccatgaa agcgctggaa 960

gatgcgatta tctacgaaat ccacatcgcg gatatgaccg gcctggataa tagcaatgtg 1020

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aaggatgatt ttggcgtggt ggcgcgcgtg aaatttgatg aaaccctgac caaagtgggc 180

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<210> 8

<211> 2460

<212> DNA

<213> 人工测序

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<210> 9

<211> 2460

<212> DNA

<213> 人工测序

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<210> 10

<211> 2460

<212> DNA

<213> 人工测序

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aaggatgatt ttggcgtggt ggcgcgcgtg aaatttgatg aaaccctgac caaagtgggc 180

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<211> 2460

<212> DNA

[0181] <213> 人工测序

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aaagatgatt tcggtgtcgt agcaagagtt aaattcgacg aaactttgac aaaagttgga 180

atcattgtta gacttaatga atggaaggaa aaagatgttg cgatggatag atttatatcg 240

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aaaccggata ccagtccgcg tgtgctgttt gcacaagcgc gtgcgcaaga tgtgattgaa 360

gcgtatctga ccggccaagt ggataccacc aaagtgagcg cgaaagtgac cgtggatggc 420

gtggaacgca aagtggcgaa agtggaaaaa gcgaatccga ccgatattag caaaaccaac 480

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gtggaaattg aaggctataa accggcgcgc gtgattatga tggaaatcct ggacaagatt 600

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ccggaagaga ctattaatta tgtggcgtgc catgataatc ataccctgtg ggacaaaaat 1920

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cagaaactgg cgggtgcgat tttgttaacc agccagggca ttccgttttt gcatgcgggc 2040

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aatggcctgg attatgcgcg caaagcggaa tttatcgacg tgttcaacta ttataagggc 2160

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Ile Tyr Thr Thr Lys Pro Asp Thr Ser Pro Arg Val Leu Phe Ala Gln
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Ala Arg Ala Gln Asp Val Ile Glu Ala Tyr Leu Thr Gly Gln Val Asp
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Thr Thr Lys Val Ser Ala Lys Val Thr Val Asp Gly Val Glu Arg Lys
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Val Ala Lys Val Glu Lys Ala Asn Pro Thr Asp Ile Ser Lys Thr Asn

145 150 155 160

His Val Lys Ile Thr Leu Ala Glu Pro Ile Lys Leu Asp Glu Val Asn

165 170 175

Lys Asp Val Gln Val Glu Ile Glu Gly Tyr Lys Pro Ala Arg Val Ile

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Met Met Glu Ile Leu Asp Lys Ile Tyr Tyr Asp Gly Pro Leu Gly Phe

195 200 205

Glu Tyr Ser Pro Thr Lys Thr Thr Ile Arg Val Trp Ser Pro Val Ser

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Pro Ser Asp Trp Asp Lys Asp Val Arg Pro Thr Met Lys Ala Leu Glu

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Asp Ala Ile Ile Tyr Glu Ile His Ile Ala Asp Met Thr Gly Leu Asp

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Asn Ser Asn Val Lys Asn Lys Ala Thr Tyr Leu Gly Leu Thr Glu Lys

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Gly Thr Arg Gly Pro Asn Gly Val Thr Thr Gly Leu Asp His Leu Val

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Glu Leu Gly Val Thr His Val His Ile Leu Pro Met Phe Asp Phe Tyr

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Thr Gly Asp Glu Ser Glu Arg Asp Phe Glu Lys Ser Tyr Asn Trp Gly

385 390 395 400

Tyr Asp Pro Tyr Leu Phe Thr Val Pro Glu Gly Arg Tyr Ser Thr Asn

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485 490 495

Val Ile Asp Thr Leu Lys Trp Trp Val Leu Glu Tyr His Val Asp Gly

500 505 510

Phe Arg Phe Asp Gln Met Gly Leu Met Asp Lys Lys Thr Met Leu Asp

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Leu Glu Lys Glu Leu His Ala Ile Asp Pro Thr Ile Leu Leu Tyr Gly

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Glu Pro Trp Gly Gly Trp Gly Ala Pro Ile Arg Phe Gly Lys Ser Asp

545 550 555 560

Val Gly Gly Thr His Ile Ala Ala Phe Asn Asp Glu Phe Arg Asp Ala

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Met Arg Gly Ser Val Phe Asn Ala Thr Val Lys Gly Phe Leu Met Gly

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Ala Leu Ala Lys Glu Thr Ala Ile Lys Arg Gly Val Val Gly Ser Ile

595 600 605

Glu Tyr Asp Asp Val Ile Arg Ser Phe Ala Lys Asp Pro Glu Glu Thr

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Tyr Leu Ala Ala Gln Ala Asp Thr Asn Ile Lys Trp Thr Glu Glu Met

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675 680 685

Gly Asp Glu Asn Ser Tyr Asn Ser Pro Ile Ser Ile Asn Gly Leu Asp

690 695 700

Tyr Ala Arg Lys Ala Glu Phe Ile Asp Val Phe Asn Tyr Tyr Lys Gly

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Gly Thr Trp Asn Val Val Val Asp Gln Gln Asn Ala Gly Thr Lys Val

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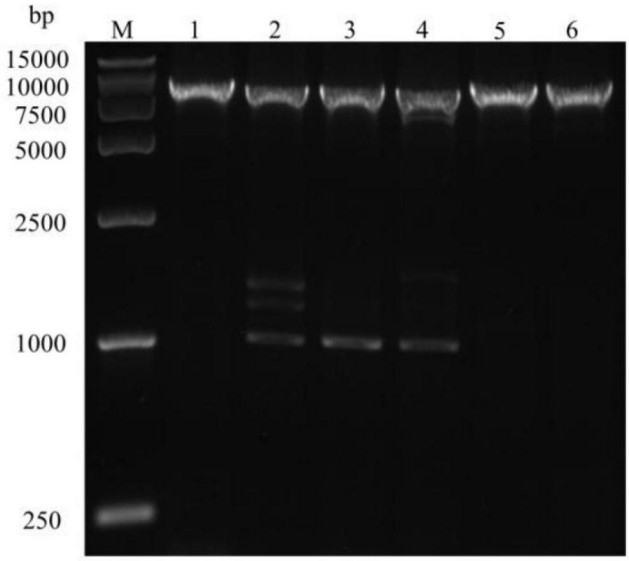
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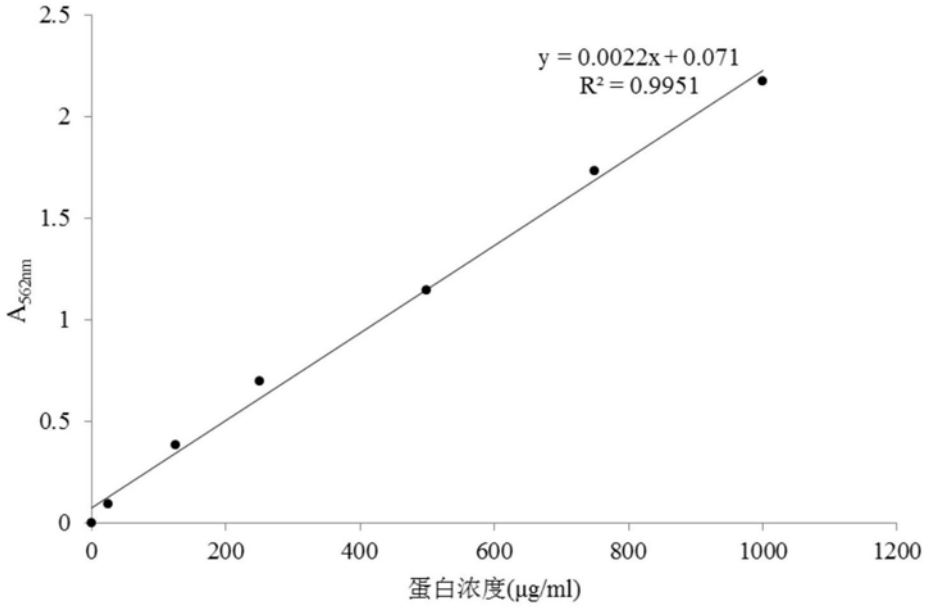
图1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0
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600
800
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1200

图2

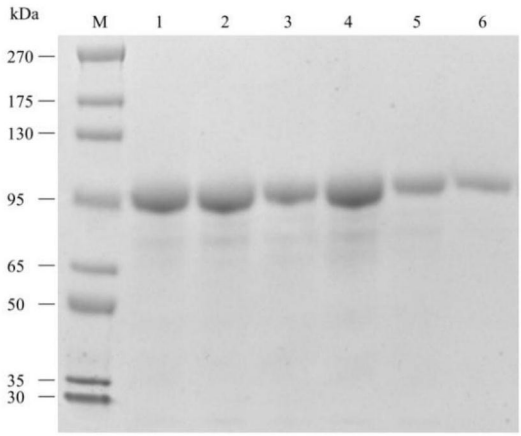
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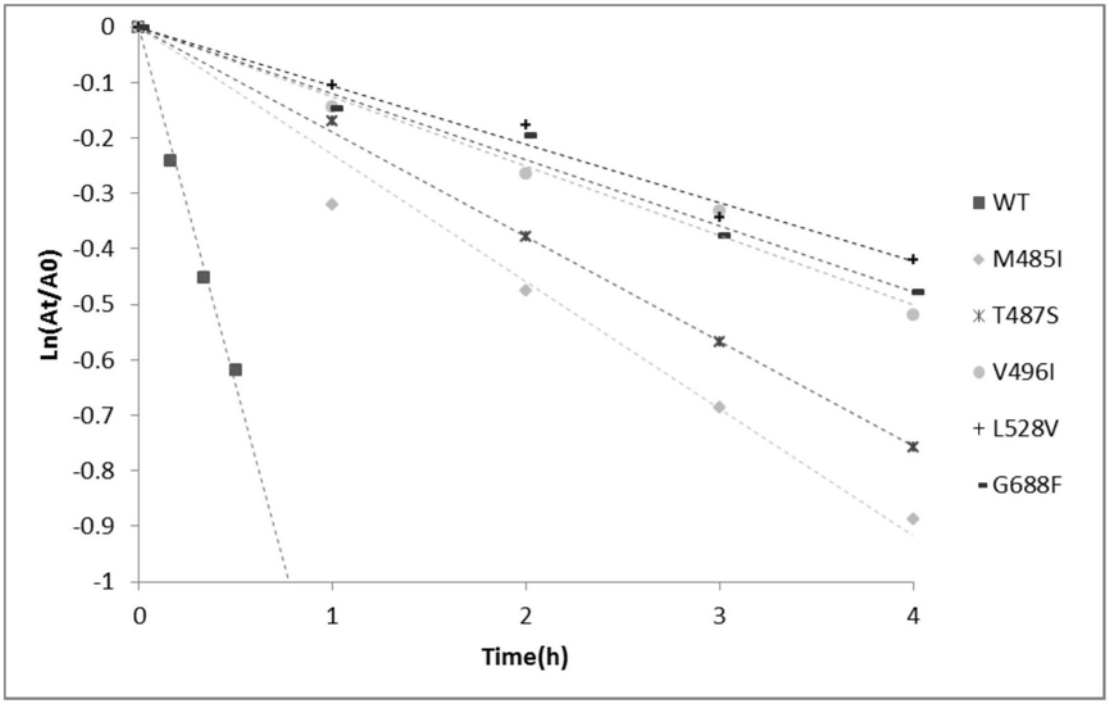
图3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-1
Ln(At/A0)
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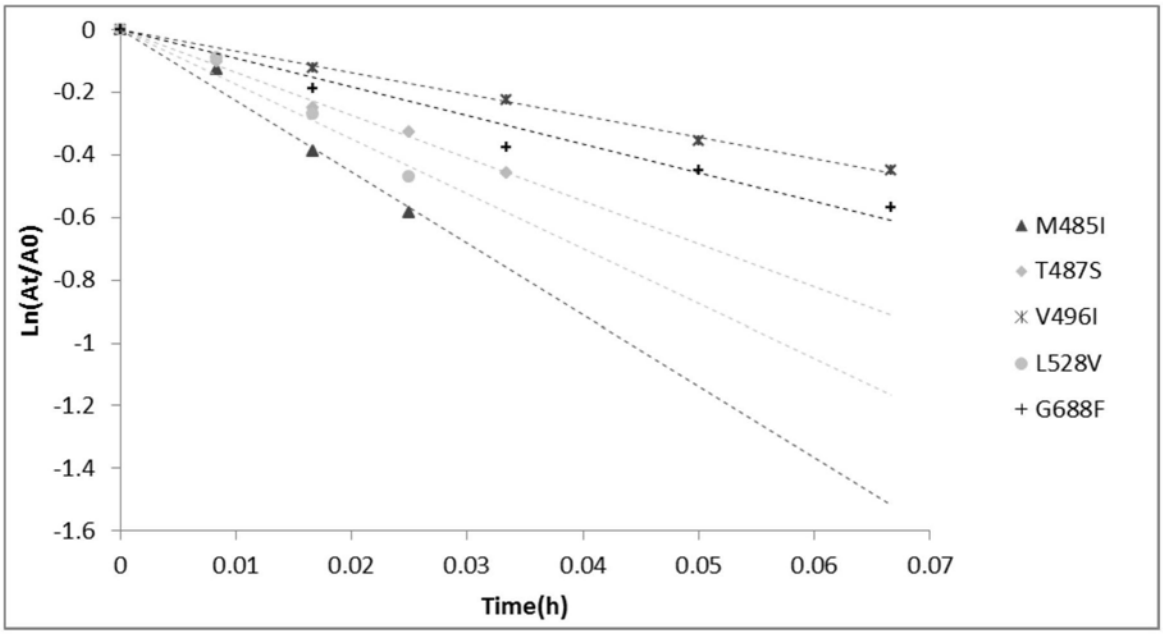
1
2
3
4
Time(h)
WT
M485I
T487S
V496I
L528V
G688F

图4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Time(h)
M485I
T487S
V496I
L528V
G688F

图5