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·工具酶的功能表征·

高小朋 博士，延安大学副教授，主要从事代谢工程与合成生物学等相关研究工作。目前，主持国家自然科学基金项目1项、陕西省科技协同创新计划项目1项，已主持完成陕西省科技厅科技统筹创新工程计划项目1项、陕西省教育厅科技计划项目3项；在《微生物学通报》《中国生物工程杂志》等核心期刊发表学术论文30余篇；先后荣获陕西省生态科学技术奖二等奖、高等学校科学技术三等奖、延安市科学技术二等奖、延安市青年科技奖等奖励。

糖苷酶耐热性改造策略与应用

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摘 要: 糖苷酶作为绿色温和的生物催化剂，能够水解包含糖苷、寡糖、多糖等在内的各种含糖化合物的糖苷键生成具有高生理和药理活性的衍生物，在食品、医药等工业领域应用广泛。而工业应用的糖苷酶经常需在高温条件下进行催化，以提高反应效率并减少污染，但大多数糖苷酶属于中温酶，在实际生产条件下的活性较低且损失较快，因此，提高糖苷酶在高温下的稳定性非常重要。本文综述了近年来利用定向进化、理性设计和半理性设计等策略改造糖苷酶耐热性的研究进展与应用，比较了不同策略之间的优势与不足，并对未来糖苷酶耐热性的改造方向进行了展望。

关键词: 糖苷酶，热稳定性，催化活性，定向进化，理性设计

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Strategies for engineering the thermo-stability of glycosidase

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Abstract: Glycosidases are widely used in food and pharmaceutical industries due to its ability to hydrolyze the glycosidic bonds of various sugar-containing compounds including glycosides, oligosaccharides and polysaccharides to generate derivatives with important physiological and pharmacological activity. While glycosidases often need to be used under high temperature to improve reaction efficiency and reduce contamination, most glycosidases are mesophilic enzymes with low activity under industrial production conditions. It is therefore critical to improve the thermo-stability of glycosidases. This review summarizes the recent advances achieved in engineering the thermo-stability of glycosidases using strategies such as directed evolution, rational design and semi-rational design. We also compared the pros and cons of various techniques and discussed the future prospects in this area.

Keywords: glycosidase, thermo-stability, catalytic activity, directed evolution, rational design

糖苷酶，又称糖苷水解酶，是可以对各种糖苷键进行水解的一类酶 (EC 3.2.1.X)，广泛存在于动物、植物和微生物细胞中[1-3]，参与生物体内相关糖的代谢，生成一系列具有高附加值的糖及其衍生物[4]。糖苷酶催化水解糖苷键的过程一般需要通过两个催化氨基酸 (通常为谷氨酸或天冬氨酸) 进行催化：其中一个是亲核试剂，另一个是酸/碱质子对。根据糖苷化合物中异头碳是否发生构象改变可将糖苷酶的催化机制分为两种：保留型机制 (图 1A) 和反转型机制 (图 1B)[5]。由于糖苷酶可催化并生成具有更高附加值的产物，因而广泛应用于食品[6]、医药[7]领域，除此之外，糖苷酶在能源[8]、纸浆[9]等领域的重要性也备受关注。工业领域应用的糖苷酶一般需在高温条件下进行催化，因此，市场对耐高温的高性能糖苷酶的需求逐年增加。

糖苷酶作为生物催化剂，在催化效率、底物特异性、环境友好等方面具有明显优势，但在实际应用中，很多天然的中温糖苷酶面对生产过程中较高的温度时，其催化活性和稳定性明显降低、甚至会丧失活性，严重影响催化效率，无法满足工业生产的长效需求。例如在纸浆漂白、酿酒和能源转换等工业生产中都需要维持高温生产来降低生物质粘度、提高反应体系均一性、加速反应过程并降低污染风险[10]，而大多数糖苷酶的最适温度在 40 ℃左右，难以有效发挥作用，所以获得在高温环境下具有良好催化活性的糖苷酶就显得尤为必要[11]。目前，具有耐热性能的糖苷酶的获得主要有两个途径：直接从不同耐热微生物体内挖掘耐热糖苷酶，如从嗜热链球菌 TC11 中挖掘出的 α 葡萄糖苷酶、从嗜热脂肪土芽孢杆菌中挖掘的木聚糖酶等[12-13]；通过蛋白质改造技术提高中温糖苷酶的专一性和耐热性。

蛋白质改造技术是一种结合了分子生物学、蛋白质晶体学、生物化学、计算机科学等的多学科技术，可用于大多数酶分子的改造，从而满足工业化生产需求[14]。分子生物学的快速发展推动了蛋白质改造技术的升级，同时，随着对蛋白质

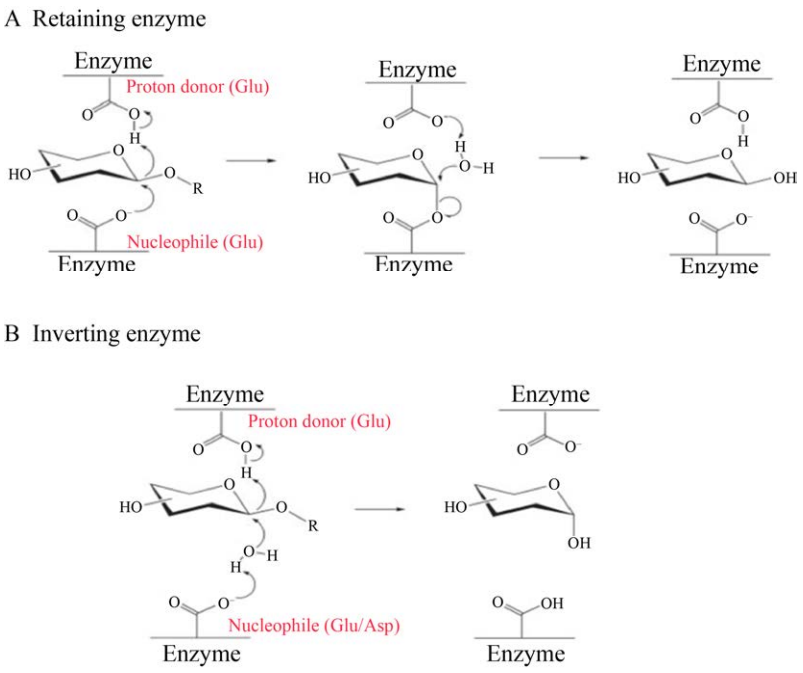

图 1　糖苷酶的催化机制[5] (A：保留型机制；B：反转型机制)
Fig. 1　Catalytic mechanism of glycosidase[5]. (A) Retention mechanism. (B) Inversion mechanism.

结构和功能认识的逐步深入，蛋白质的从头设计成为了解决蛋白质结构和功能问题的新兴方法之一[15]。总而言之，蛋白质改造技术为按需改造蛋白质结构和功能提供了可能，并为工业化应用提供了多种催化效率更高的酶源。目前，从生物角度来看，蛋白质改造技术主要以定向进化、理性设计和半理性设计 3 种策略应用居多(图 2)。本文对近年来利用这 3 种策略改造糖苷酶耐热性的研究进展与应用进行综述，比较不同策略之间的优势和不足，并对未来糖苷酶耐热性的改造方向进行展望。

1　糖苷酶的定向进化与高通量筛选方法

1993 年美国科学家 Arnold 首次提出酶定向进化的概念并应用于天然酶的改造，此后，蛋白质的定向进化得以广泛应用[16]。定向进化，不需要已知蛋白质结构和作用机制，仅通过模拟自然进化过程 (随机突变、重组和选择)，使基因发生大量变异来构建突变体库，并进一步结合高通量筛选方法筛选出具有预期特性突变酶[17]。定向进化有两个关键环节：一个是产生随机突变，创建尽可能大的随机突变体库，以 DNA 改组技术为代表的有性进化和以易错 PCR 技术为代表的无性进化是最常用的产生随机突变的方法；另一个是开发高效快捷的高通量筛选方法。

易错 PCR 法是利用 PCR 过程中出现的碱基错配，对特定基因进行随机诱变的技术，是糖苷酶定向改造研究中常用到的随机突变方法之一。目前，市面上众多的突变试剂盒均能实现定向进化突变体库的构建，是三维结构还未解析的糖苷酶分子耐热性改造的有力工具。Sürmeli 等[18]对硫化土杆菌来源的 GH51 家族 α-L-阿拉伯呋喃糖酶进行定向进化，用一轮易错 PCR，结合酶催化特异性底物对硝基苯基 α-L-阿拉伯呋喃糖苷水解产生的透明圈大小进行筛选，从 73 个突变体中筛选出 2 个透明圈最大的优良突变体，最终突变体在

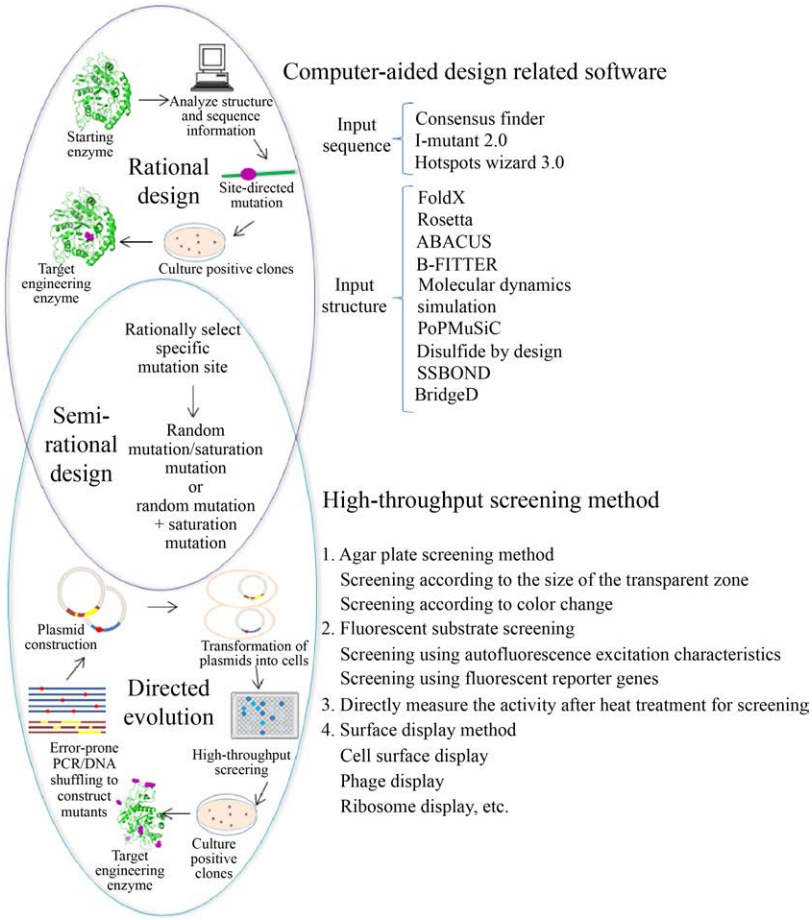

图 2　糖苷酶耐热性改造流程图

Fig. 2　Procedure for engineering the thermo-stability of glycosidase.

71 ℃的热稳定性较野生型提高 30%。于越[19]运用相同的方法对 α-L-鼠李糖苷酶构建随机突变库，借助 96 孔板从 3 000 多个突变体中筛选出了 5 株热稳定性提高的突变体。为了构建更加完备的突变体库，获得更接近预期的突变体，通常需要进行多轮进化或结合多种突变方法。Li 等[20]运用两轮定向进化策略 (先进行一轮易错 PCR，再进行一轮 DNA 改组)，结合刚果红平板筛选以及活性测定，得到了一株替换 3 个氨基酸 (Y233H、L264M 和 N343S) 的 β-甘露聚糖酶组合突变体，突变体的最适温度提高了 10 ℃，且表现出了良好的热稳定性。采用同样的组合定向进化策略，Ruller 等[21]筛选出了 80 ℃半衰期提高 60 min 的木聚糖酶突变体。

由于定向进化的突变体库容量大都在 103 之上，为了获得优良的耐热突变体，高效的高通量筛选体系非常关键。根据酶和底物反应的催化特性，开发一种简单、高效的筛选技术有助于提高筛选通量、快速锁定理想突变。近年来，虽不断有新的筛选方法涌出，但常用的方法依旧能满足大多数突变体的筛选：Liu 等报道的脂酶与三丁酸甘油酯水解后在琼脂糖平板上形成的透明圈大小筛选阳性克隆的琼脂平板筛选技术[22]；Zhou 等通过测定 α-葡萄糖苷酶与对硝基苯基-β-D-吡喃葡萄糖苷 (4-nitrophenyl-β-D-glucopyranoside，pNPG) 反应后释放的对硝基苯酚在 410 nm 处光

吸收值来筛选阳性克隆的分光光度法[23]；Fan 等利用谷氨酸脱羧酶在 pH 指示剂溴甲酚绿作用下向亮蓝变色的反应筛选阳性突变体的颜色反应法[24]；Goedegebuur 等直接将检测葡聚糖酶用高温孵育后的活性保留量作为筛选阳性克隆的活性测定法[25]；此外，一些表面展示技术也为高效的筛选提供了可能[26]。

随着 X 射线晶体衍射技术的发展，越来越多的蛋白质获得了晶体结构解析，通过分析蛋白质结构来改造特定位点且省时省力的理性设计方法越来越受到青睐[27]。

2 基于蛋白质结构与分子动力学模拟分析的理性设计与定点突变

理性设计是在对蛋白质的序列信息、三维结构、作用机制等有一定了解的基础上，对确定的位点进行突变来找寻优良突变体的方法。目前利用理性设计提高酶耐热性的研究大多从以下 3 个方面展开。

2.1 同源序列比对，分析潜在突变位点

中温酶和耐热糖苷酶的氨基酸序列同源比对可挖掘到与耐热性相关的保守氨基酸或者序列，作为潜在突变位点[28]。具有相同保守氨基酸的酶被认为是具有共同的进化关系，在同源序列比对中，增加比对的耐热酶数量，可能得到更多影响酶耐热性的潜在突变位点。Han 等[29]通过将 GH11 家族木聚糖 XynCDBFV 和 NCBI 数据库中同家族的所有木聚糖酶序列进行比对，发现 87-RGHT-90 这段序列在大多数木聚糖酶中表现得相对保守，结合 B-factor 分析，该酶 C 末端的氨基酸序列 87-QNSS-90 的 B-factor 值较高，因此对相应位点进行氨基酸突变，得到的突变体 N88G 在 65 ℃耐热性提高了 60%。同样也是从保守序列入手，Feng 等[30]将大肠杆菌表达的 β-葡萄糖醛酸苷酶 PGUS 与和其高度同源的 CAZY 数据库中 20 个同家族嗜热糖苷酶进行序列比对，找到 8 个潜在突变位点，最终获得突变体 F292L/T293K PGUS 在 65 ℃的耐热性提高了 30%。序列比对可以单独作为一种策略来设计耐热性突变体，但更多情况下，需要结合理性的结构分析以及其他算法支持，共同实现酶耐热性的改造提升[31]。

2.2 通过结构分析确定潜在突变位点

酶分子的结构稳定性对酶的耐热性起着重要作用，通过增加酶分子的相互作用力、增强酶分子结构刚性、降低其柔性结构、引入分子伴侣蛋白、糖基化和引入二硫键等方法有助于不同程度地提高酶分子耐热性。

在糖苷酶的耐热性研究中，精氨酸的引入使得耐热性的提高体现出一定的优势，由于精氨酸侧链的胍基可以在不同方向发生相互作用，比其他氨基酸能形成更多的相互作用力，因此精氨酸一直是设计耐热突变的热点氨基酸[32]。Kaewpathomsri 等[33]通过分析 GH77 家族的淀粉麦芽糖酶结构，将远离活性中心的 27 位点谷氨酸突变为精氨酸，使其在酶分子表面形成了一个精氨酸簇 (R27-R30-R31-R34)，增强了糖苷酶表面的相互作用力，最终使得突变体在 80 ℃时的活性较野生型提高了 40%。精氨酸在糖苷酶的耐碱性改造中也发挥着巨大作用[34]。此外，甘氨酸具有最高的构象熵，因此甘氨酸突变可以降低展开熵，从而稳定糖苷酶结构；脯氨酸在侧链上有一个环状结构 (吡咯烷环) 使其具有独特的构象刚性，引入脯氨酸有利于提高糖苷酶分子的结构稳定性，对提高耐热性也发挥着巨大作用[35-36]。

在糖苷酶改造工程中，仅对部分氨基酸进行取代的“小改”对酶分子耐热性的提升大多是有限的，往往需要对酶分子进行“中改”，比如将糖苷酶分子部分结构域进行截除、替换或者引入其他结构域或伴侣蛋白。其中，通过截除柔性区域是“中改”最常见的方式之一。酶结构中的柔性区域通常被认为是影响酶结构稳定性的关键部分[37]，而柔性区域大多集中在结构的末端以及无规则卷

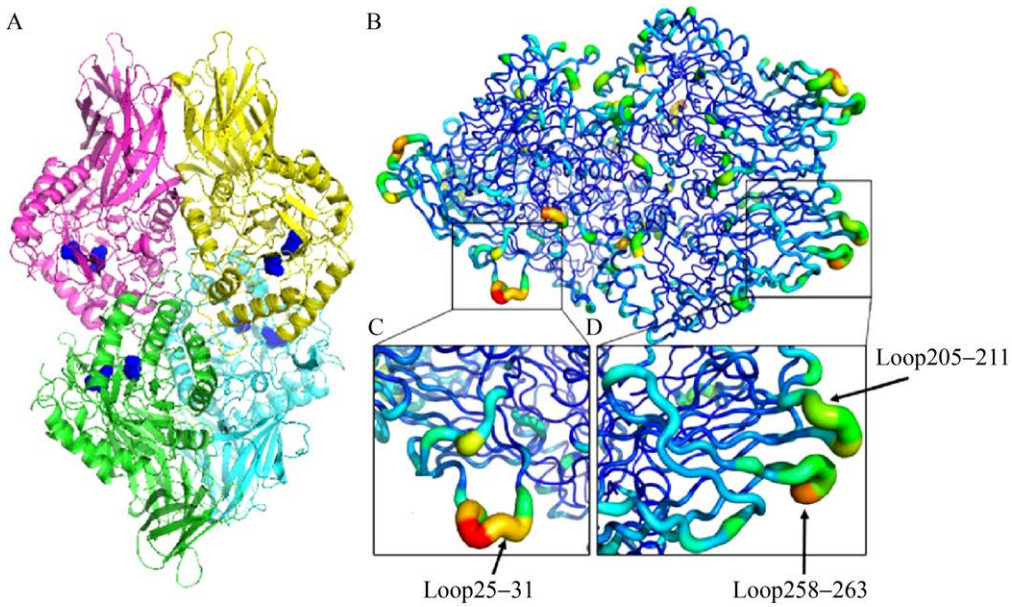

图 3 β-葡萄糖醛酸苷酶 PGUS-E 的柔性区域 (A：PGUS-E 四聚体结构，蓝色球形为催化位点；B：PGUS-E 整体柔性展示颜色越红，线条越粗，柔性越大，则刚性越小，结构越不稳定；C–D：部分 loop 环的局部图[40])

Fig. 3 The flexible region of β-glucuronidase PGUS-E. (A) The structure of PGUS-E tetramer. The catalytic sites are shown in blue spheres. (B) Flexible display of PGUS-E. Unstable regions with greater flexibility and lower rigidity are shown in thick lines and red color. (C–D) Partial view of part of loop ring[40].

曲中，所以该区域的截除或截短有可能实现酶耐热性的提升[38]。Han 等[39]通过动力学模拟发现，GH2 家族 β-葡萄糖醛酸苷酶 PGUS 的 C 末端柔性较大，截除 C 末端 14 个氨基酸后，突变体在 70 ℃的耐热性提高了 30%左右；该课题组 Feng 等[40]用两个耐热酶的稳定无规则卷曲序列替换了 PGUS-E 的相应柔性序列 (图 3)，替换后的突变酶在 70 ℃的半衰期较出发酶提高了 11.8 倍[40]。

N-糖基化可以影响酶的热稳定性。对未经修饰的酶分子定量引入糖基化位点，有可能实现酶耐热性的提升；而对高度糖基化的酶，通过部分去糖基化，也可以提高酶的耐热性[41]。王小艳等[42]对毕赤酵母重组表达的 β-葡萄糖醛酸苷酶结构表面 loop/turn 区的 3 个目标序列进行人工糖基化位点设计，最终在 65 ℃下，N-糖基化后的突变体较野生酶活性提高了 10%左右。这些研究都不同程度证明了适度的糖基化对酶的热稳定性具有正向作用。

除此之外，在蛋白结构内部引入二硫键也能提高酶的热稳定性。Tang 等[43]在里氏木霉菌来源的 GH11 家族木聚糖酶的 N 末端与 α 螺旋到 β 片层核心处引入 2 对二硫键，使突变体在 60 ℃下的半衰期提高了 2.5 倍。Yang 等[44]对沙生梭孢壳菌来源的 GH45 家族纤维素酶结构分析，通过在该酶分子内部引入二硫键后，酶在 100 ℃的活性提升了 15%左右。二硫键的引入对酶耐热性提升有一定作用，但引入二硫键数目过多时可能出现二硫键成键不完全的现象，所以在设计二硫键时，需考虑周围氨基酸对二硫键成键的影响。

2.3 计算机辅助设计，找寻潜在突变位点

计算机辅助的蛋白质设计是指利用计算机算法或软件，对已知序列、结构和功能信息的蛋白质作相应的数据分析处理，最终确定潜在突变位点。

目前，针对蛋白质热稳定性设计的软件层出不穷，对理性设计突变位点带来了诸多便利，研

究者们也纷纷将其应用到不同的糖苷酶上，并得到了较好的效果[45]。Torktaz 等[46]利用 PoPMuSiC 软件在线预测了 GH5 家族的糖苷酶 Cel5，用非极性的色氨酸替换了位于活性位点附近 94 位点的极性天冬酰胺，扩大了疏水口袋，使得突变体在 60 ℃的热稳定较野生型提高 150%。根据不同能量状态与蛋白质稳定性的关系也可以用于突变位点的确定。Bu 等[47]借助 FoldX、Rosetta_ddg、ABACUS 这 3 个计算机算法初步选取了木聚糖酶中折叠自由能较小的位点 (图 4)，并结合分子动力学模拟剔除了其中不合理的突变，进一步实验验证得到了 10 个显著影响酶耐热性的潜在突变位点，组合后的优良突变体在 65 ℃的热稳定性较野生型提高了 60%。此外，分子动力学模拟也可进行突变位点的预测。Li 等对[48]细菌来源的嗜中温 GH11 家族木聚糖酶进行分子动力学模拟，结合 B-factor 值分析发现木聚糖酶 N 末端 41 个氨基酸与同源嗜热酶的 N 末端 42 个氨基酸进行替换后，RMSD 值降低，木聚糖酶稳定性得以提高，替换后的突变体较野生型在 60 ℃的半衰期延长了 28 min。

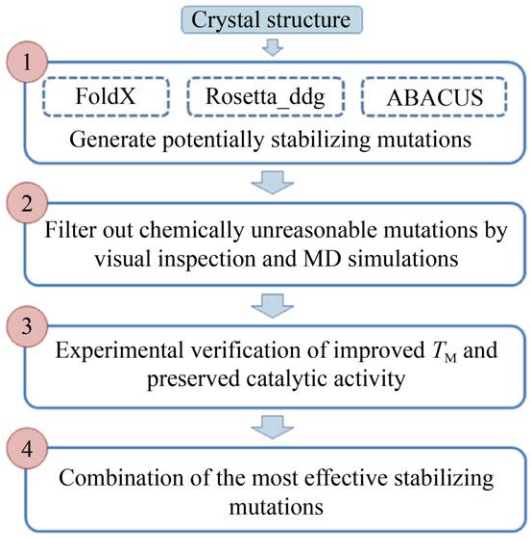

图 4　结合计算机软件辅助设计木聚糖酶耐热突变体[47]
Fig. 4　Computer software-aided design of thermostable mutants of xylanase[47].

3　糖苷酶的半理性设计

半理性设计结合了定向进化和理性设计的优点，将突变限制到一个或几个位点之上，可以在较小的库容量中得到好的突变体。一般包括两个步骤：1) 通过蛋白质序列、结构和功能信息以及计算机算法等预判潜在的目标位点；2) 对目标位点进行定向进化或饱和突变，进而筛选具有期望特性的突变体。

通过分析糖苷酶结构的 B-factor 可以指示酶的柔性区域，B-factor 值越大，柔性越强，以此作为突变依据，通过提高柔性区域的刚性，进而实现糖苷酶耐热性提升[49]。Li 等[50]对 GH11 家族木聚糖酶进行分子动力学模拟，选择 B-factor 较高的 21 位点甘氨酸进行饱和突变，得到的突变体 G21I 半衰期较野生型提高了 11.8 倍。同样，除了根据特征参数选取潜在突变位点外，对分子的氨基酸序列和结构信息的分析，也可获得影响酶耐热性的潜在突变位点。Xu 等[51]利用软件分析了几丁质酶的序列和结构，发现 S244 和 I319 可形成二硫键且 S259 位点可能影响酶的耐热性，因此额外设计二硫键并对 259 位点饱和突变后，突变体在 50 ℃的半衰期较野生型提高了 26.3 倍。随着计算机软件和算法的发展，基于酶结构分析的计算机辅助设计为改造酶的耐热性提供了极大便利。Song 等[52]通过计算机辅助分析黑曲霉来源的 GH10 家族木聚糖酶，确定了 5 个氨基酸位点并进行了 4 轮迭代饱和突变，最终得到的五突变体 (R25W/V29A/I31L/L43F/T58I) 在 60 ℃的半衰期较野生型延长了 60 倍。

4　不同策略改造糖苷酶耐热性的比较

不同蛋白质改造策略提高糖苷酶的耐热性的实例见表 1。由表 1 可知，定向进化广泛应用于糖苷酶特性改造，具体用哪种策略取决于对酶的认识和对策略的解读。定向进化的突变体库容量一般较大，需要高效的筛选方法。对于还未解析

表 1 糖苷酶耐热性改造不同策略的比较

Table 1 Comparison of different strategies for engineering the thermo-stability of glycosidase

Echnology	Enzyme (Resource)	Strategy	Result (Compare with the starting enzyme)	Pros and cons	References
Directed evolution	α-L-rhamnosidase (*Aspergillus niger* JMU-TS528)	Error-prone PCR	The half-life of the thermostable mutant at 60 °C is doubled	**Pros**: no restriction of enzyme structure	[53]
	α-glucosidase (*Thermus thermophilus* TC11)	Error-prone PCR	The thermostable mutant is almost activity loss when incubate at 70 °C for 7 h	**Cons**: the mutant library is large and screening is difficult	[23]
	Xylanase (*Geobacillus stearothermophilus*)	Error-prone PCR and family DNA shuffling	The half-life of the thermostable mutant at 75 °C is 52 times that of the wild type		[54]
	β-glucuronidase (*Penicillium purpurogenum* Li-3)	Error-prone PCR	The activity of the thermostable mutant increased by 10% when treated at 65 °C for 120 min		[55]
Rational design	Xylanase (*Aspergillus oryzae*)	N-terminal sequence replacement	The thermostable mutant still retains 85% of the initial activity when treated at 60 °C for 100 min	**Pros:** fast point selection and high accuracy	[56]
	Endoglucanase (*Penicillium verruculosum*)	Sequence alignment and free energy calculation	The half-life of the thermostable mutant at 80 °C increased by 1.3 times	**Cons**: may miss the best mutant	[57]
	β-glucuronidase (*Aspergillus terreus* Li-20)	C-terminal non-conservative gene sequence editing	The thermostable mutant was treated at 65 °C for 30 min, and retained 30% of the initial activity higher than that of the wild type		[58]
	β-glucuronidase (*Aspergillus oryzae* Li-3)	Design sugar bridges and sugar clips	The half-life of the thermostable mutant at 70 °C is 7.1 times that of the wild type		[59]
	Chitosanase (*Bacillus ehimensis*)	Artificially designed disulfide bonds	The half-life of the thermostable mutant at 50 °C is 58.8 min longer than that of the wild type		[60]
	Xylanase (*Talaromyces leycettanus* JCM12802)	Structure-based N-terminal modification	The dissolution temperature of the thermostable mutant is 7.8 °C higher than that of the starting enzyme		[61]
	β-mannanase (*Bacillus subtilis* TJ-102)	Molecular dynamics simulation	The half-life of the thermostable mutant at 60 °C is 24 times that of the wild type		[62]
	β-mannanase (*Aspergillus usamii*)	Computer-assisted protein fusion	The temperature required to maintain the same residual activity increased by 8 °C		[63]
	Pullulanase (*Bacillus acidopullulyticus*)	PoPMuSiC-2.1 Software-aided prediction	The half-life of the thermostable mutant at 60 °C is 11 times that of the wild type		[64]
Semirational design	Xylanase (*Psychrobacter* sp. strain 2-17)	Error-prone PCR and saturation mutation	The half-inactivation temperature of the thermostable mutant increased by 4.3 °C	**Pros**: the small mutant library is not easy to miss good mutants	[65]
	Endoglucanase (*Clostridium thermocellum*)	Structural analysis and saturation mutation	The thermostable mutant has a 10-fold increase in the half-life at 86 °C	**Cons**: it is still necessary to construct a mutant library through experiments	[66]
	β-glycosidase (*Thermus thermophiles*)	Structure and sequence analysis, saturation mutation	The half-life of the thermostable mutant at 93 °C increased by 4.7 times		[67]
	β-glucuronidase (*Talaromyces pinophilum* Li-93)	Random mutation and the introduction of arginine on the TIM barrel	The half-life of the thermostable mutant at 55 °C increased by 2.9 times		[68]

结构的酶来说，查找公共数据库中已被鉴定和注释的同源耐热酶序列，或借用可分析序列的软件可以得到有关信息。而对于具有晶体结构的糖苷酶，从关键位点出发，通过构建小的突变体库来筛选优良突变的半理性设计极大地减少了工作量，这是相对高效的改造方法。由于改造糖苷酶稳定性的方法通常费时费力，所以利用计算技术辅助预测酶的功能和活性成为近年来改造糖苷酶的新选择。

5 总结与展望

随着糖苷键水解产生的重要衍生物被广泛应用，糖苷酶的工业应用价值受关注。然而，由于大多天然中温糖苷酶在工业高温条件下活性较低，直接从耐热生物体内挖掘可工业应用的嗜热酶又并非易事，所以蛋白质改造技术是目前最温和有效的方法[69]。

除此之外，晶体结构的解析对于快速精准地改造糖苷酶分子结构、提升糖苷酶的耐热性也是非常关键的。但是对一些晶体结构解析较为困难的糖苷酶，除了了解酶的氨基酸序列信息外，使用定向进化、同源建模和序列比对来确定突变位点是最常用的改造策略。在未来蛋白质改造中，电镜技术和蛋白质晶体学的快速发展会让越来越多的糖苷酶分子结构得到解析，为快速高效提升糖苷酶的催化特性提供了坚实的理论基础。针对酶分子的氨基酸序列特性，开发更精确的结构和功能预测软件，有望实现通过糖苷酶的序列分析计算对酶进行更准确的突变位点预测。同时，开发更精确的计算机辅助蛋白质修饰也将是未来蛋白质耐热性改造的一大趋势。

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