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·综 述·

固定化糖苷酶在糖苷类化合物合成中的应用

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摘 要：糖苷类化合物在医药、食品、表面活性剂和化妆品等领域应用广泛，通过糖苷酶催化糖苷类化合物合成具有原料成本低、反应条件温和等优点。糖苷酶催化过程可分为逆水解反应和转糖苷反应两大类，但反应体系中的水会限制反应的进行，而适当降低体系中的水活度可以有效提高糖苷酶的催化效率。但游离的糖苷酶在低水活度时容易失活，限制了糖苷酶在低水环境下的应用。固定化酶技术通过载体和酶的相互结合能够有效提高酶结构的稳定性，使得糖苷酶能够在低水环境下甚至有机溶剂体系中保持酶活，从而实现糖苷酶在低水活度环境下的应用，提升糖苷合成效率。从糖苷酶催化性质出发，文中归纳了近 30 年来糖苷酶固定化的相关研究，其中包括单一或综合的固定化技术，以及近些年发展的结合基因工程的固定化技术，为糖苷酶的固定化及糖苷合成提供了可借鉴的思路和方法。

关键词：糖苷类化合物，糖苷酶，固定化，非水相催化

Application of immobilized glycosidase in the synthesis of glycoside compounds

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Abstract: Glycoside compounds are widely used in medicine, food, surfactant, and cosmetics. The glycosidase-catalyzed synthesis of glycoside can be operated at mild reaction conditions with low material cost. The glycosidase-catalyzed processes include reverse hydrolysis and transglycosylation, appropriately reducing the water activity in both processes may effectively improve the catalytic efficiency of glucosidase. However, glucosidase is prone to be deactivated at low water activity. Thus,

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glucosidase was immobilized to maintain its activity in the low water activity environment, and even in neat organic solvent system. This article summarizes the advances in glycosidase immobilization in the past 30 years, including single or comprehensive immobilization techniques, and immobilization techniques combined with genetic engineering, with the aim to provide a reference for the synthesis of glycosides using immobilized glycosidases.

Keywords: glycoside compounds, glycosidase, immobilization, non-aqueous catalysis

1 糖苷类化合物的生物合成

1.1 糖苷类化合物

糖苷是指醛糖或酮糖半缩醛羟基上的氢被烷基或芳基所取代后得到的缩醛衍生物。糖苷结构由两部分组成，其中糖结构部分称为糖基(Glycone)，剩余结构部分则称为配基 (Aglycone)，当配基结构也为糖时，那样的特殊糖苷结构也被称为多糖。

糖苷类化合物在自然界中分布广泛，几乎存在于所有的生物体内[1]，并且在生物体内具有多种重要的功能，如调节植物生长、促进心肌生长、为生物提供能量等[2]。糖苷类化合物在生产和生活中的使用非常广泛，具有亲水糖苷主链和亲脂三萜衍生物的皂苷作为表面活性剂应用于环境污染治理中[3]，三萜苷类化合物还具有抗癌活性、抗病毒活性[4-6]、可以缓解月经疼痛和更年期疾病[7]；从植物中分离出来的糖苷具有广泛的药理活性，如葛根中提取的异黄酮苷具有退烧和醒酒作用[8-9]，而一些植物次生代谢天然产物所得的糖苷具有显著的抗真菌作用[10]；芳香 C-糖苷是治疗中风、癌症、心血管和糖尿病等疾病的候选药物[11]；从 β-D-糖苷类化合物出发，可以制备多种糖苷酶抑制剂用于治疗糖尿病、癌症和艾滋病[12]；对多种癌症及肿瘤都有较好治疗效果的氟尿苷经糖基化修饰后生物利用度和选择性增强，并且副作用减少[13]；环烯醚萜苷类化合物历来被用于治疗小儿癫痫、营养不良等多种疾病，在现代医学中用于治疗乙型肝炎和各种肝脏损伤，疗效显著[14]；在化妆品方面，α-熊果苷是一种有效的美白成分[15]，一些糖苷及其衍生物可增加皮肤弹性和预防治疗晒伤[16]；烷基糖苷作为一类新型的非离子型表面活性剂，具有低毒、可生物降解的优秀性质[17]。综上所述，糖苷类化合物具有广泛的应用范围和应用价值。

1.2 糖苷酶催化合成糖苷类化合物

糖苷类化合物的应用价值引起了人们的研究兴趣，人们提出了多种糖苷类化合物的合成方法。其中化学合成方法有了长足的发展，但目前化学法合成糖苷类化合物需要进行官能团保护和去保护步骤，或需要对异构体进行拆分提纯，成本较高，且影响了合成产量[18-19]，此外，化学法中所使用的重金属催化剂也会造成环保和健康问题[20]。因此，人们将眼光转向了绿色、温和、低成本的生物催化合成技术[21]。

自然生物合成糖苷的途径需要糖基转移酶或磷酸活化的糖苷中间体[16,22]，但该方法价格昂贵，不适合在体外进行糖基化[23]，在工业生产中，使用的一大类生物催化剂是糖苷水解酶。糖苷水解酶通常被认为是降解酶[24]，但在特定的条件下，可以催化反向水解或转糖基反应，实现糖苷键的合成[25] (图 1)。逆水解反应是指在低水活度环境下 (单) 糖与糖基受体在糖苷酶催化作用下缩合形成糖苷键并脱去一分子水，过程如图 1A 所示；而转糖基反应是指多糖在糖苷酶的作用下离去一个或部分糖基基团与糖苷酶形成复合体，复合体再和糖基受体发生反应生成糖苷，总体糖苷键数量没有增加，总反应过程如图 1B 所示。糖苷酶催化合成糖苷过程稳定、原料廉价易得、立体选择性高、底物适用性广，是合成复杂糖苷类化合物的理想方法[19]。但这种方法也有其局限

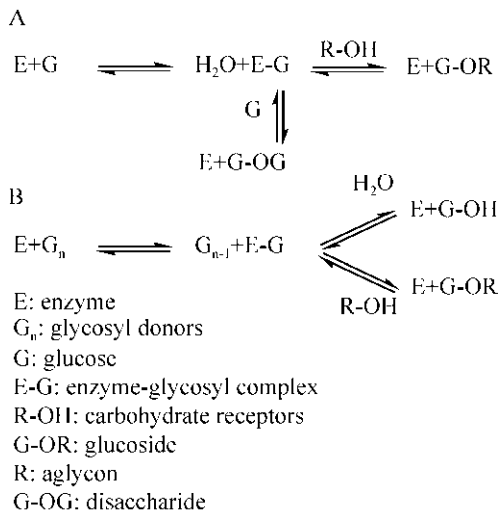

图 1　糖苷酶催化糖基化反应的机理[26]

Fig. 1　The mechanisms of glycosylation catalyzed by glycosidase[26]. (A) Reversed hydrolysis. (B) Transglycosylation.

性，由于酶促反应大多数发生在水溶液环境中(酶在水相中更稳定)，而催化环境中的水活度对糖苷酶的逆水解和转糖基作用分别造成了热力学和动力学的限制，影响了糖苷合成的效率[24,26]。

在糖苷酶的催化机制中，先是形成共价的糖基-酶催化中间体，它不仅能和水反应，还能和其他亲核试剂反应，生成糖苷。逆水解反应由于糖基供体与水解产物相同，因此受热力学控制，反应需在高底物浓度与低水环境下进行[22]。然而，少量水的存在对于保持糖苷酶催化活性又是必需的。当反应体系中存在有机溶剂时，酶的活性和选择性高度依赖于水活度和有机溶剂的种类和浓度[21,27]，虽然很难用单一的溶剂参数来预测有机溶剂对酶活性的影响[28]，但通常来说酶的活性容易受到极性有机溶剂的影响。极性有机溶剂会带走酶分子表面必要的结合水，破坏酶分子骨架的氢键，使酶失去活性[29-32]。Wang 等[33]在一项研究中提出当亲水溶剂分配系数的对数值 log P 小于 2 时会导致糖苷酶的失活。Ljunger 等[34]也提出当水活度低于 0.65 时，催化体系中存在极少量的水，酶几乎完全失活。另外酶失活还可能是由于溶剂粘度较高，阻碍了酶与底物的有效相互作用[35]。

转糖基反应由于底物与产物中不含有水，故不受水的热力学限制，可在富水环境中进行，并且利于酶活保持。然而，水在催化过程中可进攻糖基-酶中间体，引起产物的水解副反应[36]，因此反应受到水解过程的动力学控制，糖苷合成效率受到转糖苷和水解反应选择性的 (r_s/r_h) 影响。因此，在转糖基过程中水同样起到双刃剑的作用，既能保持酶活，又是影响催化选择性的关键因素。Gruz-Guerrero 等[37]在研究中发现，随着反应体系中水活度的增加，用 β-半乳糖苷酶通过转糖苷反应催化合成低聚半乳糖 (Galactooligosaccharides，GOS) 的生成量先逐渐增加，然后减少。然后采用响应面法研究了不同的水活度和不同的乳糖浓度对 GOS 的合成及其长度的影响，证明对 GOS 合成影响最大的变量是水活度。Hansson 等[38]通过比较不同水活度对 4 种糖苷酶转糖基催化合成己醇糖苷的影响发现，硫磺矿硫化叶菌的 β-半乳糖苷酶 r_s/r_h 值随着水活度的增加而降低，解糖热纤维菌的 β-半乳糖苷酶 r_s/r_h 值基本保持在一个恒定水平，强烈火球菌 β-半乳糖苷酶 r_s/r_h 值先降后升，杏仁 β-半乳糖苷酶 r_s/r_h 值则逐渐上升。

有很多研究针对糖基化催化过程水活度这一问题进行了研究，例如使用离子液体作为糖苷酶催化反应介质，酶在这种反应介质中保持了较好的活性和稳定性[39-40]，或是通过对糖苷酶进行改造来降低甚至消除酶的水解活性，并改用氟糖作为糖基供体[41-48]，但上述方法成本过高，不适合用于规模化生产糖苷类化合物。还有一种方法是将糖苷酶应用在水-有机溶剂体系中，糖苷酶在有机溶剂中的使用有以下优点：1) 增加了疏水性底物的溶解度[49]；2) 提高糖苷酶的糖基化/水解催化选择性[26]。然而游离酶在有机溶剂体系中的应用受限于其结构稳定性，有机溶剂可能会进入到酶的活性中心改变催化中心结构[50]，或是诱导酶蛋白解折叠，导致失活或变性。对此的策略包括

在耐有机溶剂的微生物中直接筛选糖苷酶[23,51]，糖苷酶的固定化以及在有机溶剂中的预孵育形成分子记忆[52-53]。

其中糖苷酶固定化的优势在于将酶与载体相结合，使酶的三维结构具有一定的刚性，或是阻止了酶的活性位点与有机溶剂直接接触，从而不易在极端催化环境下失活。另外，酶固定化有助于实现酶的回收和重复利用，并且不污染产物。而且固定化酶制剂可以应用于连续性生物反应器中，增加了生产的可操作性和持久性。本综述总结了近 30 年各种固定化糖苷酶用于糖苷合成的应用，举例说明固定化酶相比游离酶的优势，特别是在水-有机溶剂两相体系中进行催化反应，从而扩大糖苷类化合物合成的生产规模和潜在的商业化价值。

2 固定化对酶稳定性的影响

糖苷酶在进行 (转) 糖基化过程时，当糖基配体在水溶液中的溶解度不高，或水活度太高时，反应很难朝着期望的方向进行。而在有机溶剂-水介质中，或者纯有机溶剂中进行酶促反应，可以推动反应平衡向糖苷合成方向进行[52]。但糖苷酶在有机溶剂中性质不稳定容易失活，这是限制游离糖苷酶在有机溶剂应用的一个重要因素[54]。固定化酶是指酶被限制或定位在一个特定的物理空间区域内，该技术能够保留酶的催化活性，并实现酶的重复使用[55]。此外，由于固定化载体能为酶提供一个受保护的微环境，且酶与载体之间的相互作用有助于酶三维结构的保持，固定化酶在与不溶性载体结合后往往具有更高的稳定性，这有助于糖苷酶在低水环境下高效催化糖苷合成[56-57]。

相比于游离酶，固定化酶的一个显著优势在于可以进行回收再利用，这样不会污染产物，还减少了分离的工作并降低了成本，特别是通过最近比较新颖的一些磁性纳米材料能够很方便地将酶和产物分离[58-60]，并且经固定化后，酶可以在一个很长的储存周期内保持较高的活性，有些用于实际生产的固定化 β-糖苷酶甚至可以在几个月中保持酶活几乎没有损失[61]。表 1 中列举了部分糖苷酶经固定化后稳定性提高的例子，固定化使糖苷酶的半衰期延长了许多，与游离酶几天的半衰期相比能提升至几周甚至几个月，并且经过固定化后，糖苷酶在实际生产中对高温、高底物产物浓度和过酸或过碱的环境中的稳定性也有显著的提升。

3 固定化糖苷酶合成糖苷的应用

大部分应用糖苷酶催化合成糖苷反应的产率都较低，限制糖苷酶应用在于游离酶的稳定性较差。而通过固定化后，酶在催化介质中的热稳定性、有机溶剂的耐受性以及耐酸碱性的变化可能有利于催化合成糖苷的反应。即使在固定化过程中会有不可避免的酶活损失[74]，但固定化酶可以多次回收利用，积累产率最终都会高于只能使用一次的游离酶。表 2 中列举了一些游离和固定化糖苷酶在最适条件下合成糖苷类衍生物的研究，固定化酶的生产效益普遍优于游离酶。

烷基糖苷是目前为止报道较多的通过固定化糖苷酶催化合成的糖苷类化合物，烷基糖苷是两亲性分子，其中亲水的糖基通过糖苷键与疏水的烷基链共价连接，是一种良好的表面活性剂。烷基糖苷的合成过程中，一方面为保证烷基糖苷产量，催化过程中通常需要加入过量糖基配体；另一方面，由于糖基配体本身以有机溶剂的形式存在，容易造成糖苷酶失活。而采用固定化糖苷酶能够进一步提升糖苷酶在有机溶剂-水两相介质中的稳定性，防止糖苷酶受糖基配体影响导致失活。表 3 中举出了部分通过固定化糖苷酶生产烷基糖苷的例子，这些固定化糖苷酶可以在既作为溶剂又作为糖基配体的高浓度有机溶剂中保持一定的催化活性，虽然有机溶剂会影响酶三维结构的稳定性，使得酶活相较于水相中降低，但高浓度的底物更利于反应朝催化糖苷合成方向进行。例如 Vera 等[83]分别通过共价连接和交联聚合的方式固

表 1　糖苷酶固定化对稳定性的影响

Table 1　Effect of glycosidase immobilization on stability

Glycosidase	The source of the enzyme	Support	Hydrolyzation/ Glycosylation	Immobilization method	Long period residual enzyme activity (%)	Other stability enhancement	References
β-glucopyranosidase α-arabinofuranosidase α-rhamnopyranosidase	*Aspergillus niger*	Silanized bentonite	Glycosylation	Covalent bonding		pH, thermal, substrate and product inhibition	[62]
β-glucosidase	*Almonds*	Acrylamide	Glycosylation	Cross-linking polymerization	48 h more than 95%	Thermal, substrate inhibition	[63]
β-glucosidase	*Sulfolobus solfataricus*	Chitosan	Hydrolyzation	Adsorption	30 days at 70 °C 50% 56 days at 60 °C 50%	Thermal	[64]
β-glucosidase	*B. circulans*	Eupergit C	Hydrolyzation	Covalent bonding	4 months without appreciable deactivation (less than 4%)	Thermal	[65]
α-L-rhamnopyranosidase	*Aspergillus niger*	Chitosan	Hydrolyzation	Cross-linking	50 days more than 60%	Thermal, substrate inhibition	[66]
β-glucosidase	*Archaeon Pyrococcus*	Eupergit C	Hydrolyzation	Covalent bonding	In reactor at 70 °C 5 days 50%	Thermal	[67]
β-glucosidase	*Talaromyces thermophilus*	Eupergit C	Hydrolyzation	Covalent bonding	27h at 50°C 50%	pH, thermal	[68]
β-glucosidase	*Pyrococcus furiosus*		Hydrolyzation and Glycosylation	Entrapment Cross-linking	150 h in loop reactorat 60 °C 50%	Thermal, substrate inhibition	[69]
α-glycosidase		Magnetic nanospheres	Hydrolyzation	Covalent bonding	5 days 97% 10 days 94.7%		[70]
Rhamnulose-1-phosphate aldolase		Agarose	Hydrolyzation	Adsorption	37 days 50%	Solvent resistance	[71]
glycosidase	*Microbacterium oxydans*	DEAE-52 cellulose	Glycosylation	Adsorption	Reused 10 times retained 88%	pH, thermal	[72]
β-glucosidase	*Sulfolobus solfataricus*	Chitosan	Hydrolyzation	Covalent bonding	5 months without appreciable deactivation	Acid pH tolerance, thermal	[61]
β-glucosidase	*Thermotoga petrophila*	Polyethylenimine	Hydrolyzation	Covalent bonding	Reused 10 times retained 68%	pH, thermal	[73]

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表 2　糖苷酶固定化对催化合成糖苷类衍生物产率的影响

Table 2　Effect of glucosidase immobilization on catalytic synthesis of glycoside derivatives

Glycosidase	The source of the enzyme	Product	Support	Immobilization method	Immobilized enzyme yield (%)	Free enzyme yield (%)	References
β-galactosidase	*Aspergillus oryzae*	Galactosyl alcohol	Agarose	Covalent bonding	85		[75]
β-galactosidase	*Bacillus circulans*	Galactosylkojic Acid	Modified sintered porous ceramic (SM10-C2)	Covalent bonding	29.8		[76]
β-glycosidase	Almonds	Chloramphenicol-*β*-glucoside	Acrylamide	Cross-linking polymerization	6.3	less than 3.15	[63]
β-glycosidase	*Sulfolobus solfataricus*	hexyl-*β*-D-glucoside	Egg whites immobilize cells	Cross-linking	65		[77]
β-glycosidase	*Pyrococcus furiosus*	5-phenylpentyl-*β*-D-glucopyranoside	Gelatin	Entrapment	41	16	[78]
β-galactosidase	*Kocuria rhizophila*	5′-O-β-D-galactosyl-floxuridine	Alginate	Entrapment	80		[79]
β-galactosidase	*Aspergillus oryzae*	Ethyl-β-D-galactopyranoside	Polyvinylalcohol (PVA) hydrogel	Entrapment	Analogy		[80]
β-glycosidase	Almonds	But-3-en-2-yl-glycosides	Ion exchange resin XAD-4	Adsorption	27.0	24.1	[81]
β-glycosidase	Bitter almonds	Hexyl-1-β-glycosides	Ion exchange resin XAD-4	Adsorption	10	8	[20]
Puerarin glycosidase	*Microbacterium oxydans*	Puerarin-7-O-glucoside	DEAE-52 cellulose	Adsorption	63.1		[72]
α-glycosidase	Rice	Deoxynojirimycin glycoside derivative	Chitosan BCW-3570	Adsorption	Analogy		[82]
α-glycosidase	*Rhodotorula lactosa*	Deoxynojirimycin glycoside derivative	Chitosan BCW-3507	Adsorption	The transglycoside activity was significantly decreased compared with the free enzyme		[82]
β-glycosidase	*Rhodotorula lactosa*	Deoxynojirimycin glycoside derivative	Chitosan BCW-3507	Adsorption	69	73	[82]

表 3　固定化糖苷酶在烷基糖苷合成中的应用

Table 3　Application of immobilized glycosidase in the synthesis of alkyl glycosides

Glycosidase	The source of the enzyme	Support	Immobilization method	Maximum enzyme loading rate	Specific activity / maximum enzyme activity rete	Types of alkyl	Organic solvent concentration (*V*/*V*)	References
β-glycosidase	*Thermotoga neapolitana*	Modified off-stoichiometric thiol-ene (OSTE) particles	Covalent bonding		13.6 μmol/(h·g)	1-propanol	5%	[27]
α-glycosidase/ *β*-glycosidase	Baker's yeast/ Almonds	Modified polyacrylamide-type bead support (Acrylex C-100)	Covalent bonding	6.2 mg/g	73 μmol /min	n-pentanol	10%	[84]
β-glycosidase	*S. sclerotiorum*	DEAE-sepharose gel	Adsorption	91%	83.2%	Isopentanol	80%	[85]
β-glycosidase	Almonds	Ion exchange resin XAD-4	Adsorption	80%–100% 194.5 mg/g	2.1mmol/(min·mg)	1-octanol/DMF (cosolvent)	Low water activity	[86]
β-glycosidase	Bitter almonds	Ion exchange resin XAD-4	Adsorption		0.07–0.09 g/(l·h)	Hexyl alcohol	95%	[20]
β-glycosidase	Almonds	Ion exchange resin XAD-4	Adsorption		8.27 μmol/(h·g)	Hexyl alcohol	92.6%	[21]
β-glycosidase	Bitter almonds	Polyamine microspheres (PA-M)	Adsorption, covalent bonding	84.8% 0.266 U/g	58.4%.	Octanol	80%	[33]
β-galactosidase	*Aspergillus oryzae*	Polyvinylalcohol (PVA) hydrogel	Entrapment		35%	Ethanol/propanol	15%/10%	[80]
β-galactosidase	*Aspergillus oryzae*	Glyoxyl-agarose beads	Covalent bonding	33%	26.5% 2030 IU/g	1-propanol	70%	[83]
β-galactosidase	*Aspergillus oryzae*	Amino-glyoxyl-agarose beads	Covalent bonding	29.8%	56% 3983 IU/g	1-propanol	70%	[83]
β-galactosidase	*Aspergillus oryzae*		Aggregation and cross-linking	99.9%	30% 10376 IU/g	1-propanol	70%	[83]

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定了 β-半乳糖苷酶，并在含有 70%正丙醇的有机溶剂体系中进行催化反应，虽然相对于游离酶来说固定化后保留酶活分别为 26.5%、56%和 30%，但在最适条件下游离酶催化得到产物为 0.47 mol 丙基-β-半乳糖苷/mol 乳糖，而 3 种固定化的糖苷酶分别能得到 0.75、0.81 和 0.87 mol 丙基-β-半乳糖苷/mol 乳糖，因此，通过固定化的方法牺牲了一部分的酶活性，但是增加了酶在有机溶剂中的耐受性，这有利于难溶于水相的底物催化反应。

此外，固定化糖苷酶在催化转糖基过程的应用也有所报道，糖苷酶的选择性也可以通过固定化载体实现优化，实现转糖基效率的提升。Hoffmann 等[27]将 β-糖苷酶通过烯丙醇(Allyl alcohol，AA)、烯丙基丙二酸(Allyl malonic acid，AMA)、丙烯酸八氟戊酯(Octafluoropentyl acrylate，OFPA)、1-乙烯基咪唑 (1-vinylimidazole，Vim) 和聚乙烯基咪唑 (Polyethylene imidazole，PVim) 这 5 种间隔臂固定在相同的载体上，实验表明具有生物相容性的 PVim 连接的 β-糖苷酶酶活性保留最高，这个活性包括反式糖基化和水解的起始反应速率，具有亲水性羧基基团的 AMA 连接的 β-糖苷酶转糖苷/水解催化选择性比最高 (r_s/r_h= 9.2)，而 PVim 和 Vim 效果类似排在第二，但另一具有亲水性基团羟基的 AA 催化选择性比最低，并且这种催化选择性随着醇浓度的增加而提高，这说明间隔臂亲水性/疏水性对固定化酶的影响并不是很简单的，与酶上的氨基酸残基的连接方式也有可能会影响酶的活性，但可以证明固定化酶的载体以及间隔臂产生的微环境的变化可以改变糖苷酶进行转糖苷/水解的催化选择性。

4 糖苷酶的固定化方法

自从 1916 年 Nelson 和 Griffin 首次证明蔗糖转化酶被吸附到木炭上时仍能保持其活性以来，各种酶固定化技术得到了发展[87]。固定化方法主要包括共价结合、交联、包封包埋、物理吸附等，这些方法又可分为可逆固定化和不可逆固定化。可逆固定化方法通常不易改变酶的活性中心，这样得到的固定化酶保留活性高，但容易发生泄露，导致保存时间短、循环使用次数少，有些可以通过补充新的酶载体来保持酶活[88]。不可逆固定化方法通常将载体功能性基团和酶上的氨基酸残基相结合，或使用双功能交联试剂如戊二醛或碳二亚胺来连接。这样得到的固定化酶通常稳定性好，但容易造成酶的空间构象改变从而影响酶活，当然这也不是绝对的[66,89]。酶的固定化可以增加酶对极端环境的耐受性，保持稳定构象，并且能够多次循环使用，保存时间也大大增长。各种糖苷酶经固定化后结合膜反应器、微流体反应器、毛细管反应器和柱式反应器在高效生产、蛋白质组学分析和生物芯片中取得了很好的应用[67,90-100]，有希望进行更大规模的工业应用。糖苷酶固定化用来进行合成糖苷的研究相比水解的研究比较有限，但后者的固定化方法和载体仍可以作为参考。

4.1 共价结合

共价结合固定化就是酶表面的活性氨基酸残基与适当载体上的官能团发生化学反应并通过共价键结合，这种结合方式是强作用力，是酶固定化中的一种重要的方法[88]。一些载体可能本身并不具有能够与酶共价结合的功能性基团，但可以通过化学反应修饰、功能化得到，其中较为常用的方法是碳化二亚胺法、硅烷偶联剂法和琥珀酰亚胺法等[62,94,96,101-103]。载体通常具有生物相容性且对酶活抑制作用弱[104]，有很多载体功能性基团可以参与反应，例如环氧基、羟基、氨基、羧基和巯基等。绝大部分共价结合固定化酶是由载体、间隔臂、共价键和酶组成的。在一定条件下，酶表面的各种游离初级氨基酸基团都能与载体反应，而提高酶和载体之间的结合密度可以进行多点结合，得到酶蛋白的结构具有更强的刚性和稳定性，不易因为外界条件改变而失活[105-106]。例如 Gonzalez-Delgado 等[107]将一种商用棘孢曲霉

β-半乳糖苷酶多点固定在乙醛酰基改性的大孔介孔氧化硅支架上，为了验证固定化的稳定性，在59 ℃下与 Sipernat 50S 生物催化剂反应 24 h 后，上清液依然没有检测到任何 β-半乳糖苷酶活性，表明共价固定在乙醛酰改性载体与酶具有较强的连接能力。Piñuel 等[108]将一种支顶孢属的双苷酶——α-鼠李糖-β-糖苷酶，多点固定在乙醛活化琼脂糖载体上，拥有高度稳定性，能够在极端环境下进行催化反应 (10% *V*/*V* DMSO，60 ℃)，连续分批反应 20 个周期后仍能保持 80%的酶活性。但同时这样的多点结合也更容易使得酶活性中心的氨基酸残基与载体反应而失去活性，例如，将来源于米曲梅的 β-半乳糖苷酶固定在部分氨基改性的环氧树脂上酶活性基本保持不变，而直接固定在疏水性环氧树脂上时固定化酶大部分失活[109]。在最近的一篇报道中已证明在酶稳定性的增加和活性的减少之间存在一种内在的平衡，而这种平衡依赖于共价结合的数量[56]。

酶经过多点共价结合后还会缺少适当流动性，这也会导致酶活性下降[110]，这个问题可以通过引入间隔臂来改善。间隔臂可将固定化酶推离载体表面，从而防止酶和载体之间的强相互作用影响酶活[111]，例如 Arica 等[112]的研究中用乙二胺作为间隔臂的葡糖淀粉酶活性相比直接固定在聚2-甲基丙烯酸羟乙酯-乙二醇二甲基丙烯酸(Poly(2-hydroxyethylmethacrylate-ethyleneglycol dimethacrylate，poly(HEMA-EGDMA)) 微球表面的提升了一倍；Khan 等[113]将新阿波罗栖热袍菌的 β-糖苷酶通过多种方法固定在 3 种丙烯酸载体上，其中以戊二醛为间隔臂固定在 Eupergit C 上的酶在 80–95 ℃催化反应时，比活都高于直接固定得到的固定化酶；Park 等[114]将黑曲霉菌的 β-糖苷酶以不同分子量的聚乙二醇 (Polyethylene glycol，PEG) 为间隔臂固定在磁性纳米粒子表面，固定在最接近磁性粒子表面的酶热稳定性下降最大，热稳定性的增加与间隔臂 PEG 长度的增加呈正相关。间隔臂对固定化酶活性的改变是多方面的，一方面提供固定化酶类似游离酶的柔性，从而降低酶与底物的空间位阻[115]，例如牛血清白蛋白(Bovine serum albumin，BSA)就是一种成本低且降低结合蛋白空间位阻的材料[111]；另一方面，间隔臂相比直接固定降低了底物的扩散阻力，使底物更容易接近酶的活性位点从而影响固定化酶的催化性质[88]。但也有很多文献得出了不同的结论，El-Aassar 等[116]的研究中把 β-半乳糖苷酶以聚乙烯亚胺 (Polyethyleneimine，PEI) 为间隔臂固定在共聚物纳米纤维上时，固定化 β-半乳糖苷酶对温度失活的抗性优于游离酶，但米氏常数为游离酶的 1.6 倍，说明酶对其底物的亲和力下降，并且最大反应速率 V_{max} 值也同样下降，这可能是因为间隔臂本身的亲水和疏水性质改变了酶的微环境，以及与载体结合方式引起酶的构象变化，从而影响催化活性[117-118]。所以间隔臂对固定化酶的影响还缺少系统的研究和理论支持，这可能是未来的一个研究方向。

4.2 吸附

吸附法来固定化酶是最早的固定化方法之一，这种工艺简单、经济，与共价化结合酶相比，吸附法避免了化学修饰对酶构象的改变，不阻碍活性位点的空间分布，可以保持更高的酶活。例如，Carpio 等[119]将 β-半乳糖苷酶和淀粉转葡糖苷酶通过酶与基质蛋白组分 (主要是胶原蛋白) 之间的相互作用固定在骨粉上时，在远低于饱和酶复合的情况下，所有的酶活性都被保留。物理结合中使用多孔径材料和离子交换技术进行固定化较为普遍，载体通常都是多孔材料，通过大大小小、形状各异、互相贯通的孔穴增大比表面积。

有多篇研究参考了 Vulfson 等[21]于 1990 年提出的方法，将葡糖苷酶固定在 Amberlite XAD-4 大孔树脂上。XAD-4 树脂是一类含离子交换基团的交联聚合物，它的理化性质稳定，不溶于酸、碱及有机溶剂，不受无机盐类及强离子低分子化合物的影响，通过范德华力和较大的比表面进行物理吸附。并且 XAD-4 是一种相对吸湿性较好的载体，

在实验中吸收了所有添加的水,因此不存在明显的富水相，有利于烷基糖苷的合成。Papanikolaou[20]比较了固定化酶和游离酶催化烷基糖苷合成的速率和产率，得出两者初始反应速率类似，但固定化可以提高终产量 (游离酶己基糖苷产量为 1.6 g/L，转化率为 8%；固定化酶己基糖苷产量为 2 g/L，转化率为 10%)。Vulfson 等[21]还将不同型号的 XAD 树脂做比较，所获得的数据表明，载体的种类主要影响反应速率，对产品的最终收率几乎没有影响。Ducret 等[86]报道使用 XAD-4 载体的酶泄漏率约为 20%，这种树脂材料便于从反应混合物中分离相对昂贵的糖苷酶，并在后续的实验中重复使用，有希望进行生物反应器连续生产[20,81]。不同种类糖苷酶吸附在相同树脂上酶的性质也不同，将 β-木糖苷酶吸附在 Duolite A 568 树脂上，固定化效率为 67.3%，但几乎无活性，而 β-半乳糖苷酶吸附在 Duolite A 568 树脂上时，固定化 10 min 就能达到 70%的保留酶活，120 min 甚至可以达到几乎 100%的保留酶活[85,120]。

有一些固定化方法可以与酶某种结构或区域特异性结合，例如通过固定化金属螯合亲和层析法 (Immobilized metal-chelate affinity chromatography，IMAC) 来选择性吸附有组氨酸标签 (His-tag) 的半乳糖苷酶[121]，这样得到的固定化酶活性保留相对较高，并且可以使用粗酶液进行固定化，把纯化和固定化一步进行，降低了生产成本[122-123]。然而，静电相互作用不是很强，大多数蛋白质在中等离子强度(0.2-0.3 mol/L NaCl) 或 pH 值发生变化时会发生完全解吸，这会导致固定化酶的失活，并污染产品[124]。现在有些研究先是通过材料的电负性或是多孔表面来吸附酶，再使用交联剂例如戊二醛等进行进一步的固定化，也得到了很好的效果[125-127]。

4.3 基因工程在固定化中的应用

固定化酶与游离酶相比也存在一些缺点，例如通常需要纯度高的酶进行固定化，这就增加了分离提纯的时间和成本，并且固定化缺少一种通用性强、可操作性强、步骤简单、成本低廉的载体制造策略[128]。而基因工程的引入，可以将特定片段和酶的氨基酸序列一起表达，通过特异性识别将重组蛋白整合到廉价易得的载体上，并且可以有选择性地控制酶蛋白和载体的结合方向和位点[129]。Merlo 等[130]使用了一种基于锚定和自识别功能的蛋白标签 (Anchoring and self-labelling protein tag，AGT) 将 β-糖苷酶固定在大肠杆菌表面，在不影响酶催化活性的情况下，构建了 1 种集催化、荧光定量、分离纯化和锚定功能的融合蛋白，一步完成了酶的表达和自固定化。Choi 等[131]将一段纤维素结合域 (Cellulose-binding domain，CBD) 基因融合到栖热菌属 β-糖苷酶 (BglA) 上一起表达，经破碎分离可得到不溶性的蛋白颗粒，融合蛋白保留了 90%以上的酶活，然后再通过戊二醛固定在纤维素滤纸上，酶的再利用率大于 95%。这种应用催化蛋白颗粒的优势在于粒径为亚微米大小，这使底物的扩散限制较小，质量比表面积和酶负荷量较大。

巯基-二硫键的可逆特异性结合也是一种常用的锚定蛋白的方法，通过分析酶的晶体结构或同源建模可以更好地特异性结合酶上的任意一个区域，设计突变位点并进行定点突变，插入外源片段来改造酶蛋白，以及通过化学方法修饰酶蛋白或载体都能实现酶和载体之间巯基-二硫键的锚定。Mateo 等[132]报道了巯基改性的环氧树脂来固定化，通过巯基-二硫键可逆连接锚定酶，再通过长时间孵育使酶表面氨基与环氧基团实现多点共价连接，得到的大肠杆菌 β-半乳糖苷酶保留酶活达到 100%。Lafleur 等[133]用类似方法将 PNGase F 锚定在巯基-烯(Thiol-ene，TE)聚合物上来制备微流控设备。Zimmermann 等[134]还报道在氨基硅烷改性的载体表面使用琥珀酰亚胺-聚乙二醇-马来酰亚胺 (N-hydroxy succinimide-poly (ethylene glycol)-maleimide，NHS-PEG-maleimide) 作为间

隔臂也能连接巯基。

纳米材料现在是固定化酶的研究热点之一，但因高成本和需要长时间的制备限制了其应用。在最近的一项研究中 Zhang 等[135]报道了 1 种使用 T4 噬菌体衣壳作为纳米载体的自组装固定化系统大大降低了成本。用敲除了 *soc* 基因的 T4 噬菌体侵染表达了糖苷酶-Soc 融合蛋白的大肠杆菌，通过自组装装配在衣壳上。这种固定化策略定点产生特异性相互作用[136-137]，使酶尽量保持原有构象，得到的 β-糖苷酶、PNGase F 都和商品酶比活在同一个数量级。同时，制备时间与使用纯酶的方法相比，由 2 d 缩短到 7 h 内。Patterson 等[138]用类似方法，将重组 P22 类病毒颗粒的外壳蛋白(Coat protein，CP)、支架蛋白(Scaffolding protein，SP) 和超嗜热菌的 β-糖苷酶 (CelB) 通过自组装的过程包埋到 P22 噬菌体内，结果表明，P22 能够包封大量的四聚体蛋白 CelB (每个衣壳有 80 个单体) 并对其整体活动没有任何影响。该小组在后续的扩展性研究中[139]将 2 到 3 种酶 (超嗜热菌 β-糖苷酶 CelB、半乳糖激酶 GALK 和葡糖激酶 GLUK) 同时共包埋在 P22 中，模拟了细胞环境下的偶联酶系统催化糖代谢途径也得到了很好的效果。证明了噬菌体可用于定点固定化糖苷酶来构建复杂的生物催化材料，这种结合方式是值得继续研究的。

5 总结与展望

由糖苷酶催化的逆水解和转糖基反应可以实现糖苷类化合物的绿色生物法合成，但在富水环境下，逆水解和转糖基过程受到水活度和底物溶解度的影响，反应过程分别受到了热力学和动力学限制。通过降低催化体系内的水活度，如引入有机溶剂，不但能够抑制糖苷酶的水解作用，而且能够提升疏水性糖基受体的溶解度，从而提升糖基化效率。然而受自然进化过程的影响，糖苷酶在低水活度环境中普遍表现出较差的稳定性，限制了糖苷酶在低水活度下催化合成糖苷类化合物的应用。通过选择合适的载体和连接方式可以实现酶的固定化，固定化酶与载体的相互作用以及载体对于酶的空间限制可以实现酶稳定性的提升，且固定化酶具有可重复利用性，是一种理想的稳定糖苷酶并实现糖苷类化合物工业化生产的方法。目前对于糖苷酶的固定化技术已有了一定的研究基础，例如传统的物理吸附、共价结合、包封包埋等固定化方法均可以实现糖苷酶的固定化及酶稳定性的提升。较为新颖的如利用基因工程技术，可以实现糖苷酶与载体的特异性结合。目前对于合成糖苷类化合物的固定化糖苷酶的种类的研究仍具有较大的局限性，如大多数研究使用的是来源于苦杏仁的 β-糖苷酶，未来对于其他来源的糖苷酶仍有待进一步的研究。

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