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Enzymes for carbohydrate and peptide syntheses

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Abstract. New practical procedures have been developed for the enzyme-catalyzed synthesis of carbohydrates and peptides. Aldolases have been shown to be effective catalysts for the synthesis of uncommon sugars, particularly azasugars. Enzymatic methods for the large-scale synthesis of oligosaccharides have been developed with the use of glycosyltransferases coupled with the regeneration of sugar nucleotides. Engineered subtilisin variants that are stable and active in anhydrous dimethylformamide and in aqueous solution have been developed for peptide segment coupling.

As many enzymatic methods become available for the stereocontrolled synthesis of chiral synthons, attention has been extended to the enzymatic synthesis of molecules with increasing complexity. One class of such complex molecules are carbohydrates, especially glycoconjugates that exist on cell surfaces (1). These molecules are involved in many types of biochemical recognition phenomena. They have been difficult to isolate, characterize and synthesize, and are the least explored of the major classes of biomolecules. Carbohydrate-related structures therefore offer new opportunities for the study of molecular recognition and for the development of therapeutic agents. The recently identified tetrasaccharide sialyl Lewis x as the ligand of endothelial leukocyte adhesion molecule (2) stimulates new interests in the development of practical methods for the synthesis of carbohydrates and mimetics or analogs as ligands, antagonists or inhibitors of specific glycoenzymes or glycoreceptors. It is of our interest to develop enzyme-based technology for the synthesis of carbohydrates and related substances, to make these materials readily available to study their structure-function relationships and to evaluate their therapeutic potential.

SYNTHESIS OF UNCOMMON AND AZA SUGARS BASED ON ALDOLASES

Catalytic asymmetric aldol condensation is of current interest in synthetic organic chemistry. Enzyme-catalyzed aldol condensation holds great potential in this regard (3). More than 20 aldolases are known (Figure 1); many of them have been explored for synthesis. A particularly important application of aldolases is the synthesis of deoxyazasugars - a class of molecules useful as glycosidase inhibitors. We have examined five aldolases, i.e. fructose-1,6-diphosphate aldolase from rabbit muscle or *E. coli* (4), rhamnulose (Rham) and fuculose (fuc) 1-phosphate aldolases (5,6), 2-deoxyribose-5-phosphate aldolase (DERA) (5), and sialic acid aldolase (7), for their utility in asymmetric aldol condensations. All these aldolases possess two common features: first, they are highly specific for the donor substrate but flexible for the acceptor component; second, the stereoselectivity is controlled by the enzyme not by the substrate, with some exceptions observed in the sialic acid aldolase reactions. A general strategy was therefore developed for the synthesis of deoxyazasugars based on aldolase-catalyzed condensation of azidoaldehydes followed by Pd-mediated reductive amination. Both thermodynamic and kinetic approaches have been explored for the aldolase reactions. Figure 2 illustrates a general strategy for the synthesis of azasugars based on this combined chemical-enzymatic approach. With this straightforward stereocontrolled process, a number of azasugars have been synthesized and evaluated as glycosidase inhibitors. This research has led to a new direction of inhibitor design (4-6). The structure-inhibition studies indicate that the five-membered-ring aza sugars are good mimics of the transition state of glycosidic bond cleavage due to their positively charged half-chair conformation (6). We believe these aza sugars will be useful building blocks for the synthesis of many sequence-specific glycosidase and glycosyltransferase inhibitors, and the synthetic strategy illustrated in these processes should be applicable to the synthesis of other types of azasugars based on different aldolases.

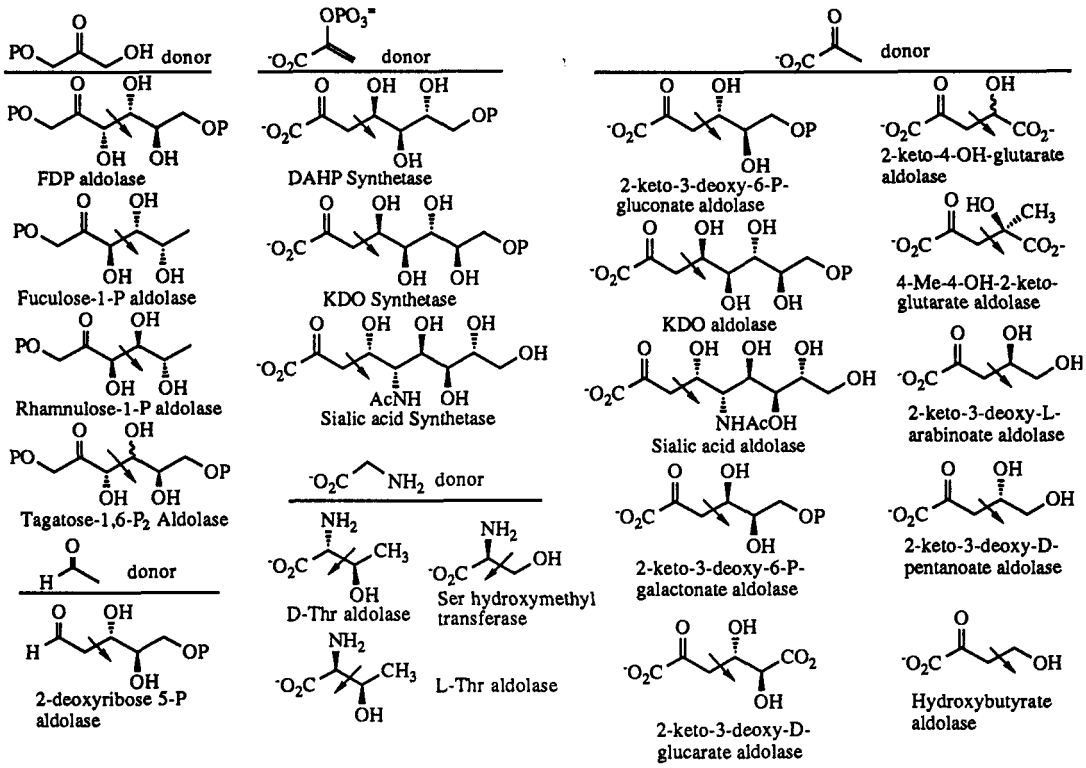


Figure 1. Enzymes that catalyze aldol reactions.

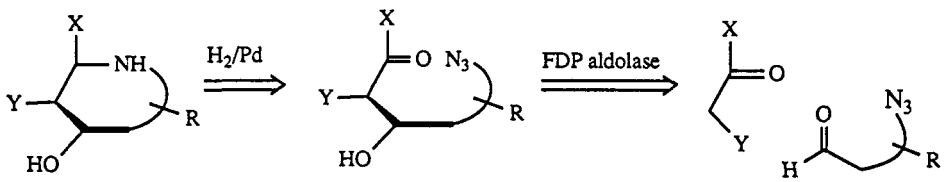


Figure 2. Retrosynthesis of azasugars. Enzyme-catalyzed aldol condensation and Pd-mediated reductive amination.

X	Y
-H	H, CH_3, $ClCH_2$
$-CH_3$	CH_3
$-CH_2OPO_3^=$	OH
$-CO_2^-$	CH_3

REGENERATION OF SUGAR NUCLEOTIDES FOR OLIGOSACCHARIDE SYNTHESIS

Glycosidases and glycosyltransferases have been used in oligosaccharide synthesis (3,8-11). The sugar nucleotide-dependent glycosyltransferases seem to be more suitable for the synthesis of complex oligosaccharides as the enzymatic reactions are stereo- and regioselective for various complex acceptor structures. The major problems are that glycosyltransferases are not readily available and that sugar nucleotides are too expensive to be used as stoichiometric reagents. Furthermore, the reactions often exhibit product inhibition caused by the released nucleoside phosphates. A simple solution to these problem is to regenerate the sugar nucleotide during the enzymatic reaction (Figure 3). It has been demonstrated previously that UDP-glucose and UDP-galactose can be regenerated *in situ* in a 50-g synthesis of a disaccharide (12). We have recently developed new procedures for the regeneration of CMP-sialic acid in a sialyltransferase-catalyzed synthesis of sialyl oligosaccharide (13), and for the regeneration of GDP-fucose from GDP and mannose 1-phosphate (Figure 4). We have also demonstrated that two glycosyltransferases can be used in a one-pot reaction, coupled with the regeneration of more than two sugar nucleotides, for the formation of two glycosidic bonds starting from three unactivated monosaccharides. No product inhibition was observed in this reaction (14). The enzymes can be immobilized on a polymer support and recovered for reuse. We believe the strategies and principles

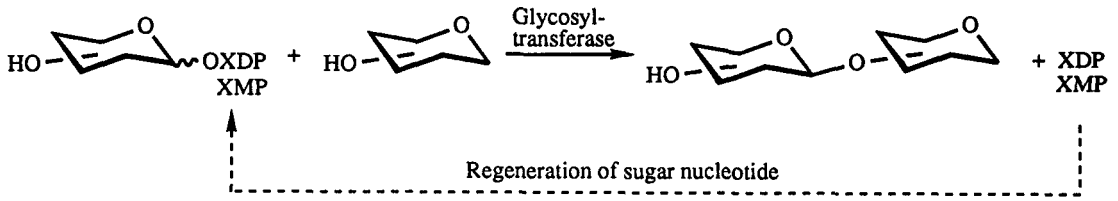


Figure 3. Regeneration of sugar nucleotides in glycosyltransferase-catalyzed synthesis of oligosaccharides.

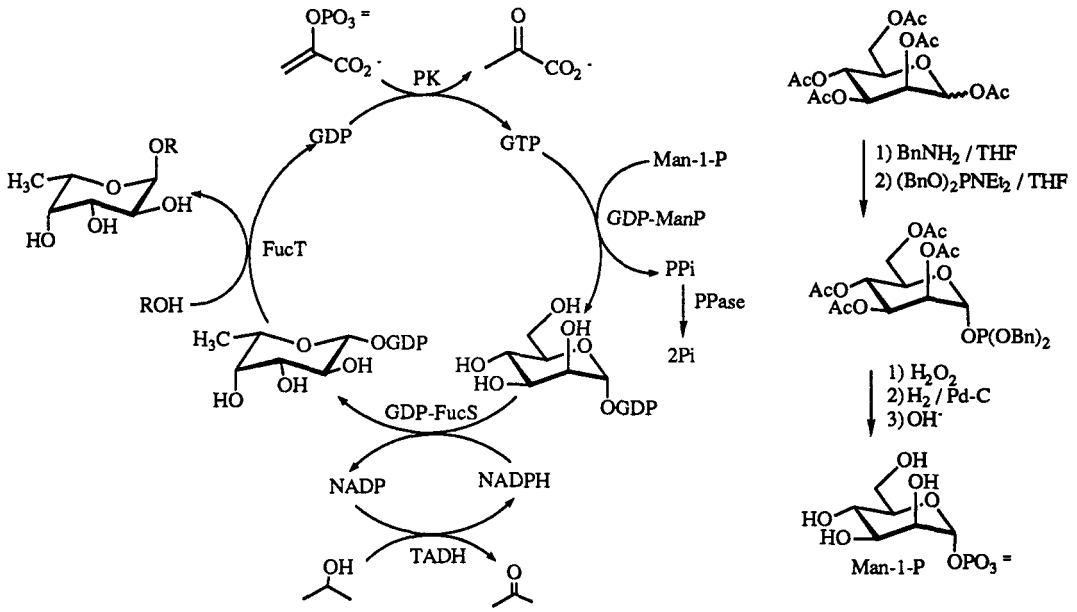


Figure 4. (Left) Regeneration of GDP-Fuc and GDP-Man for Enzymatic Fucosylation. (Right) Synthesis of Mannose 1-Phosphate. Abbreviations: FucT, fucosyltransferase; PK, pyruvate kinase; GDP-Man, GDP-Mannose pyrophosphorylase; PPase, Pyrophosphatase; GDP-FucS, GDP-Fucose synthesizing enzymes; TADH, *Thermoanaerobium brockii* alcohol dehydrogenase.

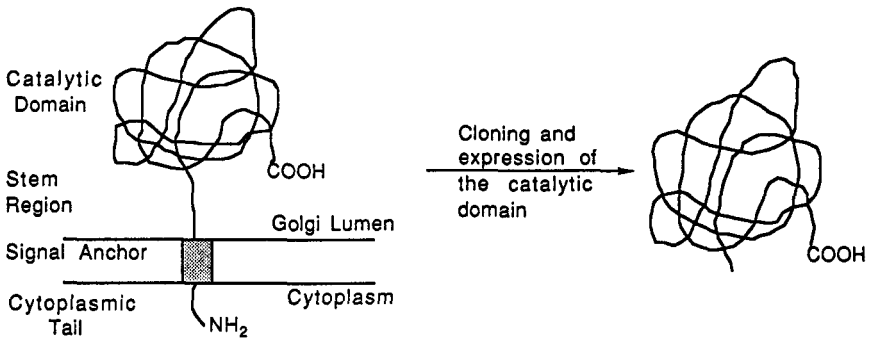


Figure 5. Cloning and expression of the catalytic domain of glycosyltransferases in microorganisms.

illustrated in these syntheses can be utilized in the synthesis of many oligosaccharides. The remaining issues will be to make the glycosyltransferases available and to explore their substrate specificity. Although several cloning systems have been developed, there is still a need for the development of practical expression systems other than those based on CHO cells (15). Since the catalytic domain of human galactosyltransferase was expressed in *E. coli* (16), the cloning and expression of the active domains of glycosyltransferases has been an interesting subject for investigation (Figure 5). We are currently applying the phage vector system used for the overproduction of CMP-sialic acid synthetase (13) to express the active domains of several glycosyltransferases in *E. coli*. With regard to substrate specificity, it appears that glycosyltransferases accept many modified substrates, including acceptors and donors, albeit at a very slow rate, as indicated in the substrate specificity study of β-1,4-galactosyltransferase (Figure 6) (17,18).

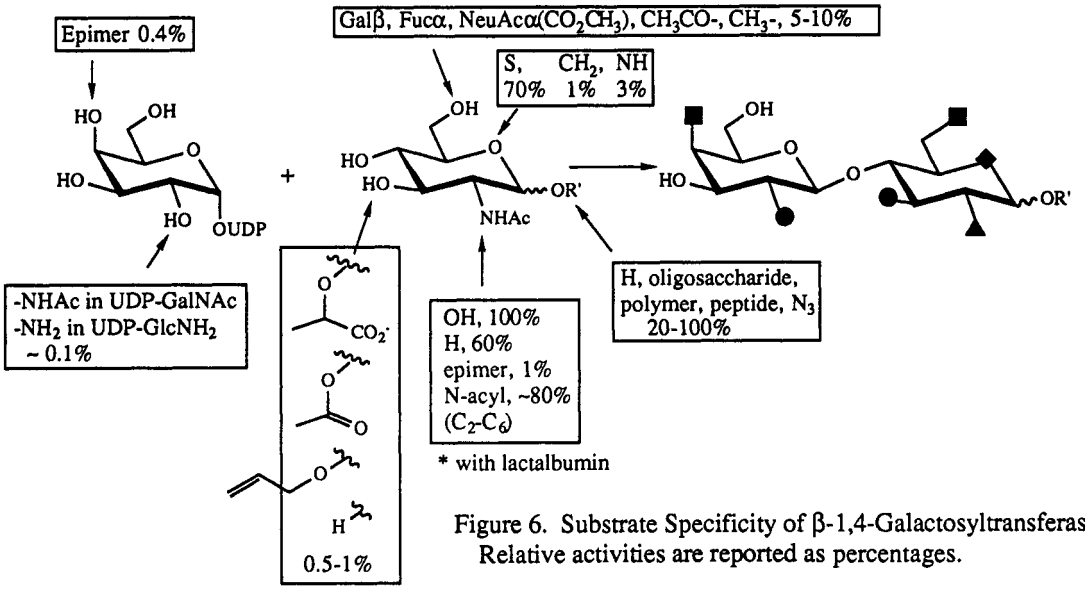


Figure 6. Substrate Specificity of β-1,4-Galactosyltransferase. Relative activities are reported as percentages.

DEVELOPMENT OF STABLE AND ACTIVE ENZYMES IN POLAR ORGANIC SOLVENTS

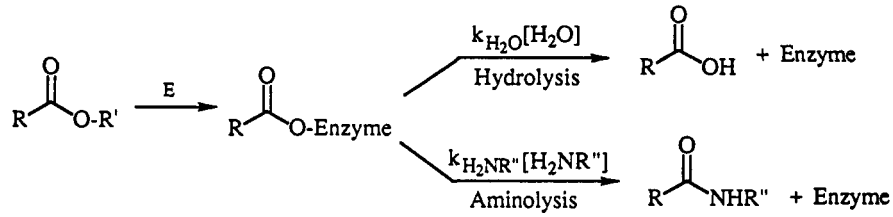
In our work with the aldolase reactions, O-acylated monosaccharides were used as acceptors for sialic acid aldolase-catalyzed reactions. The acylated sugars were prepared via subtilisin-catalyzed transesterification in anhydrous dimethylformamide (DMF) using enol esters as acylating reagents (19). We also conducted the condensation of peptide fragments in DMF or a mixture of DMF and water via the subtilisin-catalyzed aminolysis of N-protected peptide esters. This latter process is aimed at the development of new methods for the enzymatic coupling of glycosylpeptides. A major problem encountered in this work is the instability of the enzyme in polar organic solvents which are often required for the reactions. The half life of subtilisin BPN' in dry DMF, for example, is about 20 minutes, which is not long enough for large-scale processes. An improvement of the enzyme stability in DMF has been accomplished with the use of site-directed mutagenesis to increase the half life to about one day at room temperature (20). A second variant of subtilisin BPN' with a half life of about 14 days in dry DMF has recently been prepared (21). Table 1 lists the relative stabilities of several subtilisin variants we have prepared. This work has provided a substantial amount of experimental information useful for the design of enzymes to be used in polar organic solvents. Based on the subtilisin work, we feel that several modifications must be carried out simultaneously to make enzymes stable and active in polar organic solvents. These include the minimization of surface charges, the enhancement of internal polar interactions (e.g. H-bonding, ionic, and metal-coordinating forces), the optimization of internal hydrophobic interactions, and the enhancement of conformational restrictions. In the case of subtilisin, surface charge minimization (e.g. Lys217→Tyr) and conformational restriction (e.g. Gly169→Ala) have proven to be the most effective. Each of the changes improves the enzyme stability in DMF corresponding to approximately 4 kcal/mol as measured by its irreversible inactivation. Surface charge minimization has also proven to be effective for the stabilization of α-lytic protease (21) and subtilisin E (22) in aqueous DMF. Active-site modification of serine proteases also change the nature of catalysis in water. The ratio of aminolysis to hydrolysis, for example, increases substantially when the active-site His is methylated at the ε-2 N, or when the active-site Ser is converted to Cys (Table 2).

TABLE 1. Half-lives of subtilisin BPN' and variants at 25°C in phosphate buffer (0.1 M, pH8.4) and in anhydrous dimethylformamide. The stability was measured by taking aliquots of the enzyme solution at different times for activity assays in an aqueous solution (0.1 M phosphate, pH8.4). The half-life was thus determined based on the irreversible inactivation.

Enzyme	$t_{1/2}$ H_2O (pH 8.4)	DMF
BPN'	15 h	0.3 h
8397	1600 h	350 h
8350	1500 h	22 h
8399	1000 h	43 h

8399: Met50Phe, Ans76Asp, Gln206Cys, Asn218Ser
8350: Met50Phe, Gly169Ala, Ans76Asp, Gln206Cys, Tyr217Lys, Asn218Ser
8397: Met50Phe, Gly169Ala, Ans76Asp, Gln206Cys, Asn218Ser

TABLE 2. Ratios of aminolysis to hydrolysis of modified serine proteases. Activities were determined at 25°C in aqueous solution (0.1 M phosphate, pH8.4) in the presence or absence of dimethylsulfoxide (50% v/v). Methylchymotrypsin: the active-site His was methylated at the ε2-nitrogen group. Thiosubtilisin: the active-site Ser was converted to Cys.



Enzyme	$\frac{k_{H_2NR"}}{k_{H_2O}}$
Chymotrypsin[a]	
aqueous	38.6
50% DMSO	256
Methylchymotrypsin[b]	
aqueous	1.78×10^3
50% DMSO	4.91×10^4
Subtilisin BPN'[a]	
aqueous	55.7
50% DMSO	88.5
Thiosubtilisin[a]	
aqueous	1.7×10^4
50% DMSO	1.01×10^5

[a] Cinnamoyl enzyme was prepared and used as acyl donor.
[b] Suc-Ala-Ala-Pro-Phe-SBzl was used as the acyl donor. Gly-NH_2 was used as the nucleophile.

CONCLUSION

We have demonstrated the use of aldolases, glycosyltransferases and engineered subtilisin in the synthesis of carbohydrates and related substances. This enzyme-based technology is complementary to non-enzymatic technology, and is well-suited for the synthesis of polyhydroxylated structures. It is expected that the large-scale synthesis of various monosaccharides, oligosaccharides, and their related substances and analogs will become a routine process in the near future.

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