REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	20 Apr 99	Final, 2 June 94 - 2 June 97

4. TITLE AND SUBTITLE

Designing Novel Enzymes for Homogeneous Catalysis in Organic Solvents

5. FUNDING NUMBERS

N00014-91-J-1652

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8. PERFORMING ORGANIZATION REPORT NUMBER

Not applicable

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800 N. Quincy Street
Arlington, VA 22217

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

Not applicable

11. SUPPLEMENTARY NOTES

Distribution Unlimited

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Distribution Unlimited

12b. DISTRIBUTION CODE

Not applicable

13. ABSTRACT *(Maximum 200 words)*

Several mutants derived from the thermostable subtilisin 8397 were produced in order to create an enzyme that is more stable toward organic solvents, or more effective for peptide coupling in aqueous solution. Mutants were created with higher stability (8397/K256Y), broader substrate specificity (8397/M222A/Y217W), and better aminolysis:hydrolysis ratio (8397/C206Q/S221C). Engineered subtilisins were shown to be useful in the coupling of glycopeptides, and in tandem with the use of glycosyltransferases, subtilisin was shown to be useful for synthesizing homogeneous glycoproteins. X-ray crystallographic and NMR structural studies of the subtilisin active site in 50% DMF support a nearly 180° flip in the ring of the active site histidine, which may help explain the large changes in catalysis under these conditions.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Enzymes, Organic Synthesis, Mutagenesis, Peptide Synthesis, Glycoprotein Synthesis	6
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

FINAL REPORT

GRANT #: N00014-91-J-1652

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GRANT TITLE: Designing Novel Enzymes for Homogeneous Catalysis in Organic Solvents

REPORT PERIOD: June 2, 1994 - June 2, 1997

AWARD PERIOD: June 2, 1994 - June 2, 1997

OBJECTIVE: To engineer enzymes with improved properties (stability, substrate specificity, catalytic activity) for organic synthesis; to demonstrate the utility of enzymes for organic chemistry; to investigate the effects of solvent on enzyme structure and function.

APPROACH: Enzymes, in particular the serine protease subtilisin, are mutated at specific sites chosen based on analysis of the available crystal structure. Mutagenesis is performed using standard molecular biology techniques. The mutant enzymes are isolated and investigated with regard to (a) their stability in various solvent systems and at various temperatures, by monitoring their rates of deactivation under various conditions; (b) their substrate specificity, by following the rate of hydrolytic/aminolytic reactions with various ester and amide substrates; (c) their utility in synthesis, by investigating their ability to catalyze novel and synthetically useful reactions, either alone or in series with standard synthetic techniques.

Many of the reactions of interest have hydrolytic side reactions. For example, the aminolysis of an ester to form the amide will have a competing hydrolytic side reaction to form the acid. For this reason it is frequently desirable to minimize the amount of water present. Organic cosolvents have been included in the reaction mixture to accomplish this, and this approach has produced excellent results. One goal of this project has been to investigate the active site of subtilisin in the presence of high concentrations of organic cosolvent, to see if variations in the active site structure may account for some of the observed effects on catalysis. Although our original plan was to deduce structural changes using X-ray crystal structures of a stabilized subtilisin mutant in buffer with various concentrations of DMF (a collaborative effort with Prof. G. Farber of Pennsylvania State University), ^{1}H NMR is also used in this study to confirm the crystal structure

results. The strong downfield shift of the active site histidine δ1 N-H (~18ppm in imidazolium, ~15ppm in the imidazole) allows the active site histidine to be studied with one-dimensional NMR.

Finally, we are studying the potential utility of immobilizing subtilisin and other enzymes on a temperature-sensitive acrylamide-based copolymer which is soluble at low temperature and insoluble at high termperature (in collaboration with Prof. D. Bergbreiter at Texas A&M). Immobilization to such a support should allow us to conduct reactions at low temp., where the support is soluble, then allow recovery of the catalyst by mild heating to precipitate it.

ACCOMPLISHMENTS. Several mutants derived from the thermostable subtilisin 8397 were produced in order to create an enzyme that is more stable toward organic solvents, or more effective for peptide coupling in aqueous solution. One of three surface charges was removed each time to create the variants Lys43→Asn, Lys256→Tyr, and Asp181→Asn. The stabilities of these mutants appeared little different from that of 8397 in anhydrous DMF. When these variants were dissolved in water containing various concentrations of DMF, we found that in most cases, the K256Y mutant was more stable than, the D181N mutant less stable than, and the K43N mutant about the same stability as the 8397 variant.

The S1′ subsite specificity of subtilisin was modified by site directed mutagenesis of subtilisin 8397 to provide an enzyme (Met222→Ala/Tyr217→Trp) with broader residue tolerance in that subsite.

The active site serine of subtilisin 8397/C206Q was converted chemically to cysteine, forming thiosubtilisin 8397/C206Q, and the aminolysis:hydrolysis ratio of the acyl enzyme was found to be several orders of magnitude higher than for subtilisin BPN' and comparable to that of thiosubtilisin BPN'. We demonstrated that this stable variant is useful for peptide and, importantly, glycopeptide synthesis in aqueous solution by using it to couple a number of short glycopeptide esters. Thiosubtilisins acylate slowly, and it is typically necessary to use activated ester substrates. Because thiosubtilisin 8397 is thermostable, however, it can be used at elevated temperature to couple poorly activated substrates.

Subtilisins 8397 and BPN′ have also been investigated with regard to their synthetic utility. The ability of these enzymes to couple glycopeptides in 70% DMF was investigated systematically, placing the glycan of the glycopeptide in each of the enzyme's 8 subsites (S1-S4, S1′-S4′) and observing the resulting coupling yield. Generally, the further the saccharide was from the scissile bond, the better the coupling yield. Peptides glycosylated at residues immediately adjacent to the scissile bond were

not substrates, nor was the site one position toward the N-terminus (S2).

The ability of subtilisin to catalyze the condensation of glycopeptides, along with the known ability of subtilisin to cleave and religate ribonuclease A (RNase A) prompted us to demonstrate the use of an enzymatic approach in the preparation of homogeneous glycoproteins. In order to study the effects carbohydrates have on glycoprotein structure and function it is imperative to be able to synthesize the appropriate natural and non-natural glycoprotein variants in a single form. Available *in vivo* techniques provide only heterogeneous mixtures of different glycoforms, however. Using the N-glycoprotein RNase B (glycosylated version of RNase A) as a model system, the oligosaccharide was removed leaving only the N-acetyl glucosamine as a "tag" to the site of glycosylation. Glycosyltransferases were then used to build a unique carbohydrate moiety. A new RNase glycoform containing the branched oligosaccharide, sialyl Lewis X, was synthesized enzymatically to demonstrate the feasibility of the method. In addition, the monoglycosylated protein was digested into smaller pieces by subtilisin BPN'. These fragments were re-ligated by the protease to the full length RNase by addition of glycerol; this method points to a new chemical-enzymatic process for the synthesis of glycoproteins using synthetic peptides and glycopeptides as substrates for enzymatic ligation followed by further enzymatic glycosylations.

The active site structure of the stabilized 8397/Lys256→Tyr ("8397+1") was investigated by X-ray crystallography in various concentrations of DMF. Crystallographic data indicated that the catalytic histidine may have flipped nearly 180°, and NMR studies were used to confirm this. The histidine δ1-NH has a very downfield (~18ppm) resonance, due to a putative low barrier hydrogen bond with the aspartic acid of the catalytic triad. Disruption of this hydrogen bond by rotation of the histidine would be expected to make this 18ppm peak disappear. This was, in fact, observed, and adds strength to the argument that the histidine has flipped in 50% DMF.

Studies are currently ongoing to establish the utility of immobilization to the thermosensitive copolymer. Enzymes are linked via amide bonds to an N-hydroxysuccinimide activated copolymer. The resulting immobilized enzyme is washed several times by precipitation, centrifugation, and resuspension, and the enzyme's stability and kinetic parameters are measured.

CONCLUSIONS: Enzymatic approaches can offer an excellent alternative to chemical techniques for synthesizing complex molecules such as glycoproteins. Where available enzymes have poor characteristics for synthesis such as low stability or undesirable substrate specificity/catalytic activity, enzyme engineering and the use of orgnic

cosolvents can be used to tailor the enzyme's stability and activity.

SIGNIFICANCE: Enzymatic techniques offer improved methods for synthesizing complex molecules that are difficult to synthesize using standard chemical methodology. These studies provide examples of synthesis using the enzymes we have developed. Some of the molecules synthesized, in particular the sLe^x-RNase, would be extremely difficult to synthesize by other means.

The studies into the subtilisin active site structure in 50% DMF should help provide a structural explanation for the improvement in catalytic properties in cosolvent.

The immobilization studies may provide a useful technique for separating and recycling enzymes, which are typically the most expensive reagents in an enzymatic reaction.

AWARD INFORMATION:

1995: ACS Melville L. Wolfrom Award in Carbohydrate Chemistry.

1995: Member of the NIH Bioorganic and Natural Products Chemistry Study Section

1996: Elected Member of The American Academy of Arts and Sciences

1998: ACS Harrison-Howe Award in Chemistry

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