Tetrahedron Letters, Vol.32, No.47, pp 6827-6830, 1991
Printed in Great Britain

0040-4039/91 $3.00 + .00
Pergamon Press plc

The Catalytic Formation of Peptide Bonds with Carbohydrate Protein Conjugates of Proteases [CPC(Proteases)]

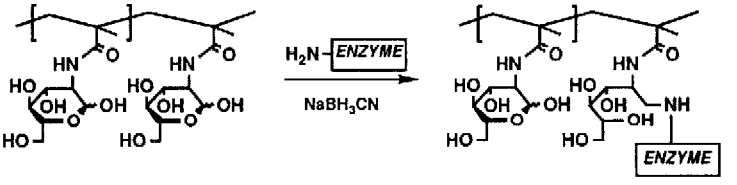
Peng Wang,‡,§ Tara G. Hill,† Mark D. Bednarski,‡,§,* and Matthew R. Callstrom†,§,*

Contribution from the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210,† the Department of Chemistry, University of California at Berkeley, Berkeley, California 94720,‡and the Center for Advanced Materials, Lawrence Berkeley Laboratory, Berkeley, California 94720.§

Abstract: This paper describes the use of carbohydrate protein conjugates of proteases [CPC(proteases)] for the catalytic formation of peptide bonds. We have found that CPC(proteases) are stable in organic solvents and perform at truly catalytic levels and have demonstrated a basis set of transformations that allow for convergent peptide synthesis.

The discovery of catalysts that can selectively couple unprotected peptide fragments would revolutionize protein chemistry by allowing convergent polypeptide synthesis.[1-4] Barriers that limit the use of proteases as catalysts for convergent peptide synthesis include (i) the stability of proteolytic enzymes in organic solvent systems;[5-10] (ii) a simple and effective C-terminal and N-terminal protecting group strategy; and (iii) the isolation of the polypeptide product from the reaction mixture.[5] In the previous paper we reported the stabilization of enzymes by the covalent attachment of proteins through their ε-lysine residues to a series of carbohydrate-based macromolecules.[11] In this paper we report the use of carbohydrate protein conjugates of proteases [CPC(proteases)] as catalysts for peptide bond synthesis and a general strategy for convergent oligopeptide synthesis.

We have prepared carbohydrate protein conjugates of α-chymotrypsin [**1**-CPC(CT)], thermolysin [**1**-CPC(Th)], and subtilisin BPN' [**1**-CPC(BPN')] to evaluate the use of these materials as catalysts for the synthesis of peptide bonds in organic solvents.[2, 3, 9-12] We choose to use acetonitrile, dioxane and tetrahydrofuran as solvents for this chemistry to demonstrate the catalytic efficiency and generality of the use of CPC(proteases) in organic media and because oligopeptides are soluble in these solvents or mixtures of these solvents with water (Table I).[9m, 13] Entries 1, 2 and 3 of Table I show that **1**-CPC(CT) is catalytically active in these solvents and that high yields of dipeptide can be



1 **1-CPC(*ENZYME*)**

synthesized using 90% acetonitrile containing 5% water and 5% triethylamine. The V_{max} for the formation of peptide bonds in acetonitrile is approximately 0.1 - 1 μmol/min/mg of **1**-CPC(CT) which is of the same order of catalytic efficiency as that for the cleavage of peptide bonds in aqueous systems.[14] The other immobilized enzymes, **1**-CPC(Th) and **1**-CPC(BPN'), could also be used in acetonitrile and acetonitrile-water mixtures with high catalytic efficiency (Table I, Entries 4-8).

Table I. Peptide Bond Formation Using Carbohydrate Protein Conjugates of α-Chymotrypsin [**1c**-CPC(CT)], Subtilisin BPN' [**1c**-CPC(BPN')], and Thermolysin [**1c**-CPC(Th)] in Organic Solvents.

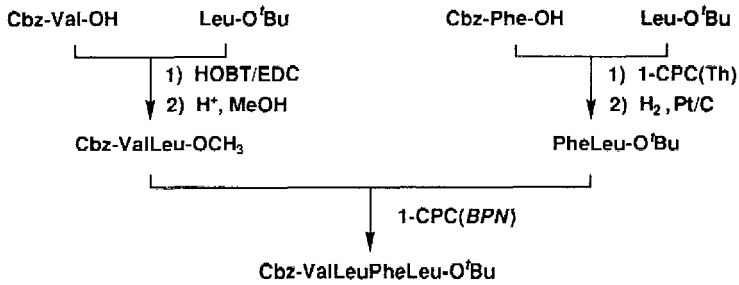
Entry	Enzyme	Solvent[a]	Time (h)	Acceptor Amino Acid	Donor Amino Acid[b]	Product[c]	Yield (%)
1	**1c**-CPC(CT)	THF	24	Ac-Phe-OEt	Ala-NH_2	Ac-PheAla-NH_2	98
2	**1c**-CPC(CT)	Dioxane	24	Ac-Phe-OEt	Ala-NH_2	Ac-PheAla-NH_2	98
3	**1c**-CPC(CT)	CH_3CN	12	Ac-Phe-OEt	Ala-NH_2	Ac-PheAla-NH_2	100
4	**1c**-CPC(BPN')	CH_3CN	24	Cbz-LeuLeu-OMe	PheLeu-O^{t}Bu	Cbz-LeuLeuPheLeu-O^{t}Bu	95
5	**1c**-CPC(BPN')	CH_3CN	24	Cbz-ValLeu-OMe	PheLeu-O^{t}Bu	Cbz-ValLeuPheLeu-O^{t}Bu	90
6	**1c**-CPC(Th)	CH_3CN	48	Cbz-Phe-OH	Leu-OMe	Cbz-PheLeu-OMe	95
7	**1c**-CPC(Th)	CH_3CN	48	Cbz-Phe-OH	Leu-O^{t}Bu	Cbz-PheLeu-O^{t}Bu	90
8	**1c**-CPC(Th)	CH_3CN	48	Boc-MetLeuPhe-OMe	PheLeu-NH_2	Boc-MetLeuPhePheLeu-NH_2	70

(a) Reactions were carried out at 37°C. Each solvent, unless noted, contains 5% (v/v) triethylamine and <5% (v/v) water.
(b) Two equivalents of donor amino acid, relative to the acceptor amino acid, were used in each reaction.
(c) All compounds were fully characterized by ^1H and ^{13}C NMR and high resolution mass spectrometry.
(d) The solvent was distilled from calcium hydride.

The use of CPC-proteases allows for the simple synthesis of dipeptides and their rapid isolation. The coupling of 100 mg (0.42 mmol) of Ac-Phe-OEt and 113 mg (0.90 mmol) of Ala-NH_2 with 30 mg of **1**-CPC(CT) in 10 mL of acetonitrile with 5% (v/v) of triethylamine and 5% water was carried out at 37 °C in a shaker bath (Table I, entry 3). The reaction was monitored by HPLC analysis on a C_{18} column using a 25% aqueous solution of acetonitrile and 0.1% trifluoroacetic acid as the mobile phase. After 12 h, the coupling was greater than 99% complete. The solution was filtered through a 0.2 μ membrane filter, the solvent was removed in vacuo, and then redissolved in ethyl acetate. The organic solution was washed with 0.1 *N* hydrochloric acid, dried with anhydrous magnesium sulfate, and the solvent removed in vacuo to give a 97% isolated yield of Ac-PheAla-NH_2.[15] Entries 6 and 7 of Table I demonstrate the use of **1**-CPC(Th) and acceptor groups in the form of a free acid for the synthesis of the peptides Cbz-PheLeu-OMe and Cbz-PheLeu-O^{t}Bu.

In order to prepare oligopeptides using convergent methodology, we required a protecting group strategy for the C-terminus of the peptide fragments.[6] We found that *tert*-butyl esters, which are available directly from solid-phase peptide synthesis,[16] are not substrates for **1**-CPC(Th) or **1**-CPC(BPN') (Entries 4, 5, and 7).[17] Conversion of the *tert*-butyl esters to either the methyl ester (for **1**-CPC(BPN')) or the free acid (for **1**-CPC(Th)) provides a simple method for activation of the peptide for further reaction. We synthesized a tetrapeptide, Cbz-ValLeuPheLeu-O^{t}Bu, to demonstrate this strategy. One of the required dipeptide fragments was synthesized by the chemical coupling of Cbz-Val-OH with Leu-O^{t}Bu using the EDC-HOBt method to give Cbz-ValLeu-O^{t}Bu in a 90% isolated yield.[18] The *tert*-butyl ester was converted quantitatively to the methyl ester, Cbz-ValLeu-OMe, using a catalytic amount of hydrochloric acid in methanol. The second dipeptide fragment was synthesized by the **1**-CPC(Th) catalyzed coupling of

Cbz-Phe-OH with Leu-O^{t}Bu in acetonitrile to give, after hydrogenation, a 92% isolated yield of H_2N-PheLeu-O^{t}Bu. The coupling of these dipeptide fragments using 1-CPC(BPN') as the catalyst in acetonitrile gave the desired tetrapeptide in a 85% isolated yield for an overall yield of 70% for this convergent synthesis.



We have found that CPC-proteases are stable in organic solvents and catalyze the formation of peptide bonds. These materials perform at truly catalytic levels, retain their specificity in organic solvents, and allow for the simple isolation of the product and recovery of the enzyme. We have also demonstrated a basis set of transformations that allow for convergent peptide synthesis. We are currently investigating the use of CPC-proteases for the synthesis of other peptides and proteins.

Acknowledgement. We are grateful to Cargill, Incorporated (Minneapolis, MN) and to the Director, Office of Energy Research, Office of Basic Energy Sciences, Divisions of Materials Sciences and also Energy Biosciences of the U.S. Department of Energy under contract DE-AC03-76SF00098 to the Lawrence Berkeley Laboratory for their financial support of this work. M.D.B. thanks the American Cancer Society for a Junior Faculty Award. The laser light-scattering detector was purchased under a grant from the Campuswide Materials Research Program of The Ohio State University (M.R.C.). We thank Dr. Patrick Gruber (Cargill, Inc.) and Dr. Mark Alper (Lawrence Berkeley Laboratory) for useful discussions and encouragement. We also thank Professor George M. Whitesides (Harvard University, Cambridge, MA) whose interdisiplinary environment brought M.D.B. and M.R.C. together to help foster the idea of this research.

References and Footnotes

1. Nakatsuka, T.; Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1987**, *109*, 3808. Kaiser, E. T.; Mihara, H.; Laforet, G. A.; Kelly, J. W.; Walters, L.; Findeis, M. A.; Sasaki, T. *Science* **1989**, *243*, 187.

2. Barbas, C. F.; Matos, J. R.; West, J. B.; Wong, C. -H. *J. Am. Chem. Soc.* **1988**, *110*, 5162.

3. Kitaguchi, H.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 9272.

4. Kemp, D. In *The Peptides*; Underfriend, S.; Meienhofer, J. Eds.; Academic Press: San Diego; 1979; Vol. 1.

5. Bergmann, M.; Fraenkel-Conrat, J. *J. Biol. Chem.* **1937**, *119*, 707. Bergmann, M.; Fraenkel-Conrat, J. *Ibid.* **1938**, *124*, 1.

6. Kullman, W. *Enzymatic Peptide Synthesis*; CRC Press: Boca Raton; 1987. Jakubke, H-D. In *The Peptides*; Underfriend, S.; Meienhofer, J. Eds.; Academic Press: San Diego; 1987; Vol. 9, Chapter 3.

7. Jakubke, H. D.; Kuhl, P.; Konnecke, A. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 85.

8. Sakina, K.; Kawazura, K.; Morihara, K.; Yajima, H. *Chem. Pharm. Bull.* **1988**, *36*, 3915. Sakina, K.; Kawazura, K.; Morihara, K.; Yajima, H. *Ibid.* **1988**, *36*, 4345. Morihara, K. *Trends Biotechnol.* **1987**, *5*, 164. Morihara, K.; Oka, T. *Biochem. J.* **1977**, *163*, 531.

9. For examples, see: (a) Cabaret, D.; Maillot, S.; Wakselman, M. *Tetrahedron Lett.* **1990**, *31*, 2131. (b) Wong, C. -H.; Chen, S. -T.; Hennen, W. J.; Bibbs, J. A.; Wang, Y. -F.; Lui, J. L. -C.; Pantoliano, M. W.; Whitlow, M.; Bryan, P.N. *J. Am. Chem. Soc.* **1990**, *112*, 945. (c) Gaertner, H.; Puigserver, A. *Eur. J. Biochem.* **1989**, *181*, 207. (d) Ricca, J. M.; Crout, D. H. G. *J. Chem. Soc., Perkin Trans. I* **1989**, 2126. (e) Cassells, J. M.; Halling, P. J. *Biotechnol. Bioeng.* **1989**, *33*, 1489. (f) Scholten, J. Stolowich, N, J.; Hogg, J. L.; Scott, A. I.; Wong, C. -H. *J. Am. Chem. Soc.* **1988**, *110*, 3709. (g) Barbas, C. F., III; Matos, J. R.; Wong, C. -H. *J. Am. Chem. Soc.* **1988**, *110*, 5162. (h) Cassells, J. M.; Halling, P. J. *Enzyme Microb. Technol.* **1988**, *10*, 486. (i) Ferjancic, A.; Puigserver, A.; Gaertner, H. *Biotechnol. Lett.* **1988**, *10*, 101. (j) Cheng. E.; De Miranda, M. T. M.; Tominaga, M. *Int. J. Peptide Protein Res.* **1988**, *31*, 116. (k) Kamihira, M.; Taniguchi, M.; Kobayashi, T. *Agric. Biol. Chem.* **1987**, *51*, 3427. (j) Barbas, C. F., III; Wong, C. -H.; *J. Chem. Soc., Chem. Commun.* **1987**, 533. West, J. B.; Wong, C. -H. *Tetrahedron Lett.* **1987**, *28*, 1629. (l) Margolin, A. L.; Tai, D. F.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 7885. (m) Margolin, A. L.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 3802. (n) De Miranda, M. T. M.; Cheng, E.; Muradian, J.; Seidel, W. F.; Tominaga, M. *Bioorg. Chem.* **1986**, *14*, 182. (o) Riechman, L.; Kasche, V. *Biochim. Biophys. Acta* **1986**, *872*, 269. (p) Nakanishi, K.; Matsuno, R. *Eur. J. Biochem.* **1986**, *161*, 533. (q) Nakanishi, K.; Kamikubo, T.; Matsuno, R. *Bio/Technology* **1985**, *3*, 459. (r) Ooshima, H.; Mori, H.; Harano, Y. *Biotechnol. Lett.* **1985**, *7*, 789. (s) West, J. B.; Luthi, P.; Luisi, P. L. *J. Am. Chem. Soc.* **1984**, *106*, 7285. (t) Oyama, K.; Nishimura, S.; Nonaka, Y.; Kihara, K.; Hashimoto, T. *J. Org. Chem.* **1981**, *46*, 5241. (u) Oyama, K.; Kihara, K.; Nonaka, Y. *J. Chem. Soc., Perkin Trans. II* **1981**, 356. (v) Oka, T.; Morihara, K. *J. Biochem.* **1978**, *84*, 1277. (w) Morihara, K.; Oka, T. *Biochem. J.* **1977**, *163*, 531.

10. Organic solvent systems are required to prevent enzymatic hydrolysis of the starting peptide fragments and also to promote peptide bond formation.[5]

11. Hill, T. G.; Wang, P. W.; Oehler, L. M; Huston, M. E.; Wartchow, C. A.; Smith, M. B.; Bednarski, M. D.; Callstrom, M. R. previous paper in this issue.

12. The **1**-CPC(*ENZYME*) materials were purified by gel filtration chromatography using 0.05 M sodium borate solution at pH 8 on Sephacryl HR-200 at a flow rate of 1.5 mL/min. Alternatively, isolation by dialysis of the reaction solution using Spectra Por CE 100K MWCO membrane against 2 x 250 mL of 0.05 *M* sodium borate at pH 8 for approximately 12 h gave approximately 40% yields for α-chymotrypsin (E.C. 3.4.21.1, Sigma) and thermolysin (Type X, Sigma) conjugates and approximately 10% yields (80% recovered activity) for subtilisin BPN' (Type XXVII, Sigma) conjugates. The yields were determined by measurement of their relative activity with *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide as the substrate in 0.05 *M* sodium borate at pH 8 containing 10% methanol.

13. All compounds were fully characterized by ^{1}H and ^{13}C NMR and high resolution mass spectroscopy.

14. In some cases the use of a large excess of the protein catalyst can compensate for its low catalytic activity and high rate of decompositon in organic solvents. We felt that this approach is not practical for the synthesis of large peptides since the isolation of the polypeptide from the enzyme would be difficult. In addition, we observed low turnover numbers for proteases in acetonitrile and dioxane, even at high enzyme concentrations, which led to a unacceptable yields of coupled peptides (0-10%).

15. An analytically pure sample was obtained by HPLC using a C_{18} column with a 40% aqueous solution of methanol as the the mobile phase.

16. DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258.

17. Isowa, Y.; Ohmori, M.; Ichikawa, T.; Kurita, H.; Sato, M.; Mori, K. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 2762. Isowa, Y.; Ichikawa, T.; Ohmori, M. *Ibid.* **1978**, *51*, 271. Isowa, Y.; Ichikawa, T. *Ibid.* **1979**, *52*, 796. Isowa, Y.; Ohmori, M.; Ichikawa, T.; Mori, K.; Nonaka, Y.; Kihara, K.; Oyama, K. Satoh, H.; Nishimura, S. *Tetrahedron Lett.* **1979**, 2611.

18. Hudson, D. *J. Org. Chem.* **1988**, *53*, 617.

(Received in USA 15 May 1991; accepted 21 August 1991)