Cross-Linked Enzyme Crystals (CLECs) of Thermolysin in the Synthesis of Peptides

Rose A. Persichetti,[†] Nancy L. St. Clair,[†] James P. Griffith,[‡] Manuel A. Navia,[‡] and Alexey L. Margolin*,[†]

Contribution from Altus Biologics Inc., 40 Allston Street, Cambridge, Massachusetts 02139, and Vertex Pharmaceuticals Incorporated, 40 Allston Street, Cambridge, Massachusetts 02139

Received September 13, 1994[⊗]

Abstract: Cross-linked enzyme crystals (CLECs) of thermolysin exhibit functional characteristics that are superior to those found in soluble or conventionally immobilized enzymes. Thermolysin-CLECs (T-CLECs) are more stable than the native enzyme in water-immiscible organic solvents and in mixtures of water-miscible organic solvents (DMF, THF, acetone) with water. The operational stability of T-CLECs in these solvents has been demonstrated by the repetitive batch synthesis in ethyl acetate of Z-L-Asp-L-PheOMe, the chiral precursor of the artificial sweetener aspartame. We have also found that T-CLECs are stable in ethanol saturated with salts such as LiCl or $CaCl_2$, which are useful solubilizing agents for the separation and purification of insoluble peptides. Peptides of increasing size have been synthesized with the T-CLECs, including coupling $PheNH_2$ to the oxidized B-chain of insulin, a 30 amino acid peptide. The initial rates of synthetic reactions catalyzed by T-CLECs (V_{CLEC}) compared with those catalyzed by native enzme (V_{sol}) are similar up to a heptapeptide. These data suggest that enzymatic peptide coupling using CLECs might present a feasible alternative to traditional methods both in the laboratory and in large scale applications.

The benefits of using enzymes in organic synthesis in general[1] and for the preparation of optically pure drugs in particular[2] are well understood. Yet, despite the enormous potential of enzymatic catalysis, only a tiny portion of enzymes is used in the synthesis of fine chemicals or pharmaceuticals on an industrial scale.[3] One of the major problems that precludes wider acceptance of enzymes as practical catalysts is their low stability under the conditions required for many reactions of interest.

We have recently found that cross-linked enzyme crystals (CLECs, microcrystals grown from aqueous solution and cross-linked with a bifunctional agent such as glutaraldehyde[4]) exhibit remarkable characteristics that are superior to both soluble and conventionally immobilized enzymes. Such CLECs remain active in environments that are otherwise incompatible with enzyme function, including prolonged exposure to high temperatures, near-anhydrous organic solvents, and aqueous-organic solvent mixtures. CLECs are also highly stable against autolysis and exogenous protease degradation. Collectively, these properties make CLECs attractive and broadly applicable as catalysts in organic synthesis.[5] Here we report on the synthesis of peptides by thermolysin-CLECs (T-CLECs) in aqueous and nonaqueous solvents and discuss the effect of substrate size on their effective catalytic activity.[6]

Results and Discussion

Thermolysin catalyzes coupling of amino acid derivatives or peptides according to the scheme[7]

$$\text{X-AA}_1\cdots\text{AA}_n\text{-COOH} + \text{H}_2\text{N-AA}_1'\cdots\text{AA}_n' \rightarrow \text{X-AA}_1\cdots\text{AA}_n\text{-AA}_1'\cdots\text{AA}_n'$$

where AA_n can be Phe, Trp, Tyr, Leu, Ile, Ala, Gly, Pro, Met, Asp, Asn, Glu, Gln, Arg, Lys, or Cys, and AA_1' can be Phe, Leu, Val, or Ala.

Like all other proteases used in peptide synthesis, thermolysin-catalyzed coupling is highly stereoselective for L-amino acids and is racemization free. However, compared to other proteases, the esterolytic activity of thermolysin is very low, which enables the use of esters of amino acids as NH_2 donors and also makes activation of the C-terminal carboxyl component unnecessary.

[†] Altus Biologics Inc.

[‡] Vertex Pharmaceuticals Incorporated.

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1995.

(1) Santaniello, E.; Ferraboschi, P.; Grisenti, P.; Manzocchi, A. *Chem. Rev.* **1992**, *92*, 1071-1140. Poppe, L.; Novák, L. *Selective Biocatalysis*; VCH Publishers: New York, 1992.

(2) Margolin, A. L. *Enzyme Microb. Technol.* **1993**, *15*, 266-280.

(3) Matsumoto, K. In *Industrial Application of Immobilized Biocatalysts*; Tanaka, A., Tosa, T., Kobayashi, T., Eds.; Marcel Dekker: New York, 1993; pp 67-88. Gerhartz, W., Ed.; *Enzymes in Industry*; VCH: Weinheim, 1990.

(4) St. Clair, N. L.; Navia, M. *J. Am. Chem. Soc.* **1992**, *114*, 7314-7316.

(5) For the early publications on the enzymatic catalysis in the crystalline state, see the following: Low, B. W.; Richards, F. M.; Berger, J. E. *J. Am. Chem. Soc.* **1956**, *78*, 1107. Doscher, M. S.; Richards, F. M. *J. Biol. Chem.* **1963**, *238*, 2399-2406. Quiocho, F. A.; Richards, F. M. *Biochemistry* **1967**, *5*, 4062-4076. Quiocho, F. A.; Bishop, W. H.; Richards, F. M. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *57*, 525-537. Spiburg, C. A.; Bethune, J. L.; Vallee, B. L. *Biochemistry* **1977**, *16*, 1142-1150. Alter, G. M.; Leussing, D. L.; Neurath, H.; Vallee, B. L. *Biochemistry* **1977**, *16*, 3663-3668. Kasvinsky, P. J.; Madsen, N. B. *J. Biol. Chem.* **1976**, *251*, 6852-6859. Tuchsen, E.; Ottesen, M. *Carlsberg Res. Commun.* **1977**, *42*, 407-420. Lee, K. M.; Blaghen, M.; Samama, J.-P.; Biellmann, J. F. *Bioorg. Chem.* **1986**, *14*, 202-210.

(6) To the best of our knowledge the only large-scale catalytic application of a crystalline enzyme is with glucose isomerase in the production of high-fructose corn syrup: Visuri, K. European Patent 0341503, 1989.

(7) (a) Miranda, M. T. M.; Tominaga, M. *Int. J. Pept. Protein Res.* **1991**, *37*, 128-133. (b) Kitaguchi, H.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 9272-9274. (c) Sakina, K.; Kawazura, K.; Morihara, K.; Yajima, H. *Chem. Pharm. Bull.* **1988**, *36*, 4345-4354. Cheng, E.; Miranda, M. T. M.; Tominaga, M. *Int. J. Pept. Protein Res.* **1988**, *31*, 116-125. Wayne, S.; Fruton, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3241-3244. Oka, T.; Morihara, K. *J. Biochem.* **1980**, *88*, 807-813. Isowa, Y.; Ohmori, M.; Ichikawa, T.; Kurita, H.; Sato, M.; Mori, K. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 2762-2765. Isowa, Y.; Ichikawa, T. *Ibid.* **1979**, *52*, 796-800.

0002-7863/95/1517-2732$09.00/0

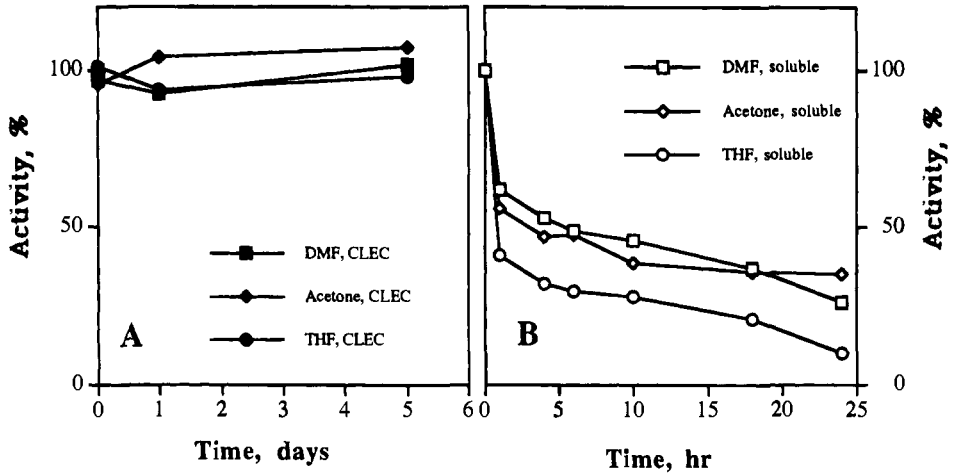

Figure 1. Stability of T-CLEC (A) and soluble thermolysin (B) in mixed aqueous–organic solutions. T-CLECs or soluble thermolysin (10 mg/mL for both) was incubated in the indicated organic aqueous mixture (50:50, v/v) at 40 °C. Aqueous solution contained 20 mM calcium acetate and 100 mM Tris-HCl (pH 7). Activity was measured by FAGLA assay (see Experimental Section).

Organic solvents are advantageous in enzyme-catalyzed peptide synthesis, both to solubilize substrates and products and to manipulate reaction kinetics and equilibrium to increase product yield.[8] The use of water-miscible organic solvents, however, leads to the rapid inactivation of enzymes;[9] the addition of DMF, THF, acetone (Figure 1), or even a mild solvent such as glycerol[10] rapidly inactivates soluble thermolysin. While soluble enzyme loses 50% of its activity in less than 6 h when exposed to 50% mixtures of aqueous organic solvents at 40 °C, T-CLECs are completely active after 5 days of incubation under the same conditions (Figure 1). In addition, T-CLECs are extremely stable in near-anhydrous organic solvents such as ethyl acetate at elevated temperature.

We decided to exploit the stability and productivity of T-CLECs in nearly neat organics by conducting a repetitive batch synthesis of the artificial sweetener aspartame precursor (Z-AspPheOMe)[11] in ethyl acetate at 55 °C (Figure 2 and Table 1). This was possible because T-CLECs are sturdy insoluble particles that can be easily recovered from the reaction mixture and reused (Figure 3).[12] T-CLECs retained full activity over the 18 cycles. Under the same conditions free crude enzyme lost all activity by the fourth reaction cycle. Moreover, the high stability and activity of T-CLECs combined with product solubility in both ethyl acetate and *tert*-amyl alcohol[13] make it possible to design an efficient continuous process for the synthesis of the aspartame precursor.[14]

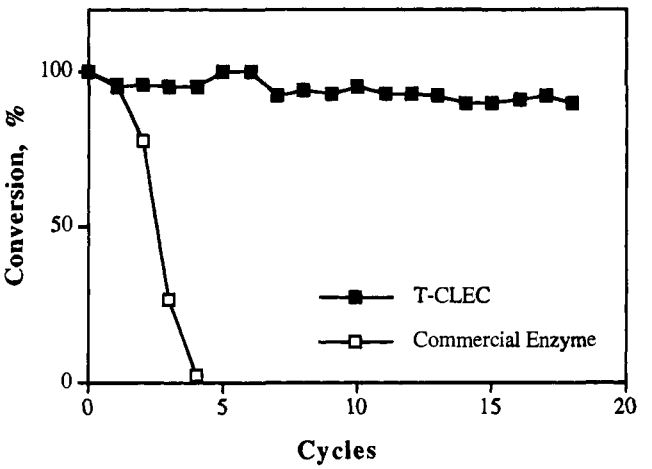

Figure 2. Multicycle synthesis of the aspartame precursor. Conditions: 80 mM Z-Asp; 240 mM PheOMe; concentrations of both T-CLECs and soluble enzyme were 2.5 mg of protein/mL; ethyl acetate, cycle time 20 h, 55 °C.

The value of the high operational stability of T-CLECs in the mixtures of water-miscible organic solvents and water was also demonstrated in the synthesis of both a tetrapeptide in 20% EtOH at elevated temperature (Figure 4A) and a heptapeptide in 90% EtOH (Figure 4B). In both cases the T-CLEC-catalyzed reactions gave a higher yield of the peptide product than was the case with soluble enzyme.

The high stability of T-CLECs in organic solvents also facilitates the isolation and purification of reaction products. One common feature of enzyme-catalyzed peptide synthesis and of most of the syntheses listed in Table 1 is precipitation of the reaction product. Precipitation is one approach to shifting the equilibrium of a hydrolase-mediated reaction toward peptide synthesis in both aqueous and organic media.[15] Product precipitation makes the recovery of immobilized enzyme difficult, however, and often involves the treatment of the precipitate by organic solvents at elevated temperature, conditions that can inactivate both soluble and immobilized enzyme. As a result, immobilized enzymes are rarely used in peptide synthesis.[15]

(8) Homandberg, G. A.; Mattis, J. A.; Laskovski, M., Jr. *Biochemistry* **1978**, *17*, 5220. Margolin, A. L.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 3802–3804. Margolin, A. L.; Tai, D.-F.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 7885–7887. Barbas, C. F.; Matos, J. R.; West, J. B.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 5162–5166. Gill, I.; Vulfson, E. N. *J. Chem. Soc., Perkin Trans. 1* **1992**, 667–668. Chen, S.-T.; Chen, S.-Y.; Wang, K.-T. *J. Org. Chem.* **1992**, *57*, 6960–6965. Sears, P.; Schuster, M.; Wang, P.; Witte, K.; Wong, C.-H. *J. Am. Chem. Soc.* **1994**, *116*, 6521–6530.

(9) Reissmann, S.; Greiner, G. *Int. J. Pept. Protein Res.* **1992**, *40*, 110–113.

(10) Durrant, I.; Beynon, R. J.; Rodgers, P. R. *Arch. Biochem. Biophys.* **1986**, *250*, 280–285.

(11) Isowa, Y.; Ohmori, M.; Ichikawa, T.; Mori, K. *Tetrahedron Lett.* **1979**, *28*, 2611–2612. Oyama, K.; Kihara, K.; Nonaka, Y. *J. Chem. Soc., Perkin Trans. 2* **1981**, 356–360. Oyama, K.; Nishimura, S.; Nonaka, Y.; Kihara, K.; Hoshimoto, T. *J. Org. Chem.* **1981**, *46*, 5241–5242. Ooshima, H.; Mori, H.; Harano, Y. *Biotechnol. Lett.* **1985**, *7*, 789–792. Nakanishi, K.; Kamikubo, T.; Matsuno, R. *Bio/Technology* **1985**, *3*, 459–464. Nakanishi, K.; Matsuno, R. *Eur. J. Biochem.* **1986**, *161*, 553–540. Nakanishi, K.; Kimura, Y.; Matsuno, R. *Ibid.* **1986**, *161*, 541–549. Lee, K. H.; Lee, P. M.; Siaw, Y. S.; Morihara, K. *Biotechnol. Lett.* **1992**, *14*, 1159–1164. Oyama, K. In *Chirality in Industry*; Collins, A. N., Sheldrake, G. N., Crosby, J., Eds.; John Wiley & Sons: 1992, pp 237–247.

(12) T-CLECs are available from Altus Biologics under the trade name PeptiCLEC-TR.

(13) K. Nakanishi. Personal communication.

(14) Nagayasu, T.; Miyanaga, M.; Tanaka, T.; Sakiyama, K.; Nakanishi, K. *Biotechnol. Bioeng.* **1994**, *43*, 1118–1123.

(15) Kullmann, W. *Enzymatic Peptide Synthesis*; CRC Press: Boca Raton, FL; 1987.

Table 1. Peptide Synthesis Catalyzed by Thermolysin-CLECs

substrate	product[a]	solvent[b]	V_{CLEC}[d]	V_{sol}[d]	V_{CLEC}/V_{sol}	yield[e] (%)
Z-Asp	Z-AspPheOMe	EtOAc	41	140	0.3	99
		buffer[c]	575	600	1.0	
GlyGlyPhe	GlyGlyPhePheNH$_2$	50% MeOH	75	78	1.0	91
Z-GlyGlyPhe	Z-GlyGlyPhePheNH$_2$	90% EtOH	78	48	1.6	98
Z-GlyProGlyGlyProAla	Z-GlyProGlyGlyProAlaPheNH$_2$	90% EtOH	23	9	2.6	83
oxidized insulin B	oxidized insulin B-Phe^{31}NH$_2$	90% DMF/EtOH (1:1)	0.44	10.8	0.04	46

[a] For the reaction conditions see Experimental Section. [b] All reactions were conducted at room temperature unless specified otherwise. [c] 30 °C. [d] V_{CLEC} and V_{sol} are initial rates of the product formation for T-CLEC and soluble thermolysin, respectively, in nmol/min per mg of protein. In each case the initial rates of the product formation were measured by HPLC (see Experimental Section). V_{CLEC} was proportional to enzyme concentrations in the range 2−20 mg/mL. [e] Determined by HPLC.

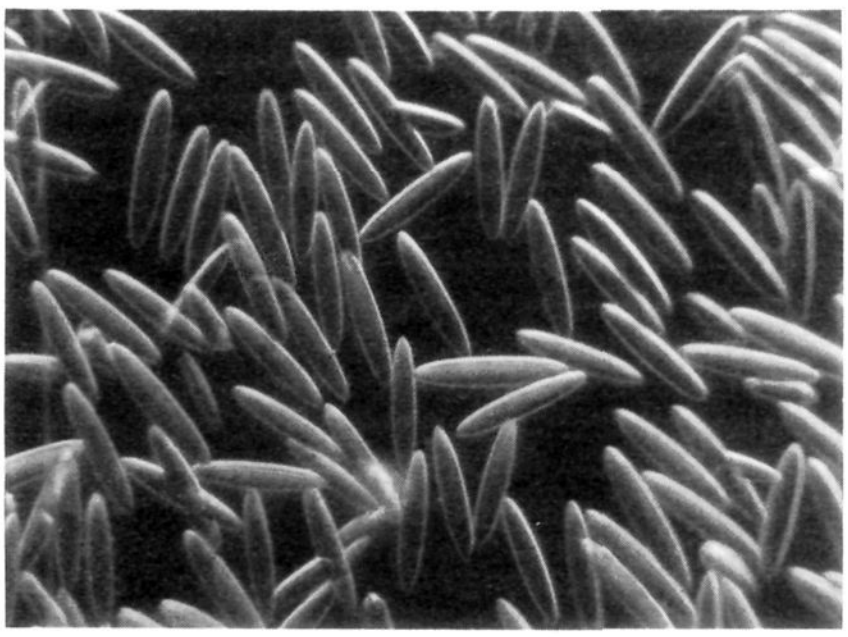

Figure 3. Photo of T-CLECs (400×) (reproduced at 81% of the original size).

In order to develop an efficient peptide synthesis process one needs to devise a solvent system which will easily solubilize the peptide product but will not inactivate the enzyme. The stability of T-CLECs permits the use of water-miscible organic solvents for that purpose. In addition, peptide solubility in certain organic solvents sharply increases by the addition of salts.[16] The low stability of enzymes in such mixtures, however, has prevented the exploitation of this phenomenon in conjunction with enzymatic synthesis. We have found that ethanol saturated with salts such as LiCl or $CaCl_2$ is a useful solubilizing agent for the separation and purification of insoluble peptides. The addition of salts increases the solubility of the hydrophobic tetrapeptide Z-GlyGlyPhePheNH$_2$ by 10-fold from 0.14 mg/mL in EtOH to 1.2 mg/mL in EtOH saturated by either LiCl or $CaCl_2$, yet remains compatible with the catalytic activity of T-CLECs. We have been able to recycle T-CLECs in the synthesis of Z-GlyGlyPhePheNH$_2$ in these mixtures at least three times without any loss of enzymatic activity[17] (see Experimental Section).

In addition to the higher stability of T-CLECs in mixtures of water-miscible organic solvents, their stability against proteolysis[4] is beneficial for peptide synthesis. Indeed, many reactions, especially those involving unnatural amino acids, are slow and require large amounts of enzyme in order to complete the processes in reasonable time. The increase in protease concentration inevitably leads to extensive autolysis and contamination of the reaction mixture by the autolysis products. The problem of contamination of the reaction mixtures by the products of protein self-digestion is especially serious in the synthesis of peptides or other compounds of pharmaceutical interest since these impurities can cause anaphylactic shock. In these situations a thorough purification of the product is required. Since CLECs are highly stable against proteolysis, they can easily circumvent this problem.

The source of T-CLEC stability may be 2-fold: the crystallinity of the material and the covalent cross-linking of enzyme molecules. It is known that the unfolding of proteins can be prevented by multipoint attachment of a protein to a support. Moreover, observed stabilization effects correlate with the number of contacts involved.[18] In the crystal lattice, where the concentration of protein is close to the theoretical limit, a large number of protein−protein interactions can be realized (Figure 5). Proteins in the crystal are held together by hydrophobic interactions and by electrostatic forces.[19] An increase in the number of both polar (electrostatic) and hydrophobic interactions among the protein molecules when a protein goes from a free to a crystalline environment may significantly enhance stability of proteins against heat and other denaturants[20] by preventing unfolding, aggregation, or dissociation of the proteins.[21] In addition, stability of CLECs against exogenous proteolysis may be explained by the exclusion of protease due to the size of the solvent channels (Figure 5) and by overall restriction of protein−protein interactions.[18] The crystal structure is absolutely required for the CLEC stability. Neither cross-linked soluble thermolysin nor its precipitate, both lacking the crystal structure, exhibits stability beyond the level of the soluble enzyme (data not shown).

Intermolecular cross-linking is certainly necessary to maintain the crystal structure in environments different from the crystallization liquor. (When non-cross-linked crystals are removed from the crystallization solution, they quickly dissolve.) The intramolecular cross-linking of proteins by glutaraldehyde and other cross-linking agents[22] may, in turn, lead to thermostabilization of proteins by preventing their unfolding. Cross-linking of proteins of glutaraldehyde is a widely used albeit poorly understood process. What is well-established is that glutaraldehyde reacts with ϵ-amino groups of lysine.[23] In the case of T-CLECs approximately eight of the 11 lysines in the thermolysin molecule are modified as determined by the amino acid analysis. The ability of glutaraldehyde to stabilize T-CLECs may stem from the fact that in aqueous solution glutaraldehyde

(16) Seebach, D.; Thaler, A.; Beck, A. K. *Helv. Chim. Acta* **1989**, *72*, 857−867. Seebach, D. *Aldrichimica Acta* **1992**, *25*, 59−66.

(17) When the product was extracted by an EtOH/$CaCl_2$ mixture, the activity of thermolysin-CLEC was 5.76, 5.28, and 5.28 μmol/mg of solid after each repetitive cycle. When the same product was extracted with EtOH/LiCl, the activity of thermolysin-CLEC was 5.70, 5.64, and 5.10 μmol/mg of solid after each repetitive cycle.

(18) For recent reviews, see: Mozhaev, V. V. *TIBTECH* **1993**, *11*, 88−95 and references therein.

(19) Islam, S. A.; Weaver, D. L. *Proteins: Struct., Funct. Genet.* **1990**, *8*, 1−5.

(20) Guisan, J. M. *Enzyme Microb. Technol.* **1988**, *10*, 375−382.

(21) Klibanov, A. M. *Anal. Biochem.* **1979**, *93*, 1−25.

(22) Wong, S. S. *Chemistry of Protein Conjugation and Cross-Linking*; CRC Press: Boca Raton, FL; 1993.

(23) Stability of the cross-links to acid rules out the simple Shiff's base formation and points out a more complex cross-linking mechanism. See, for example: Hardy, P. M.; Nicholls, A. C.; Rydon, H. N. *J. Chem. Soc., Perkin Trans. 1* **1976**, 958−962.

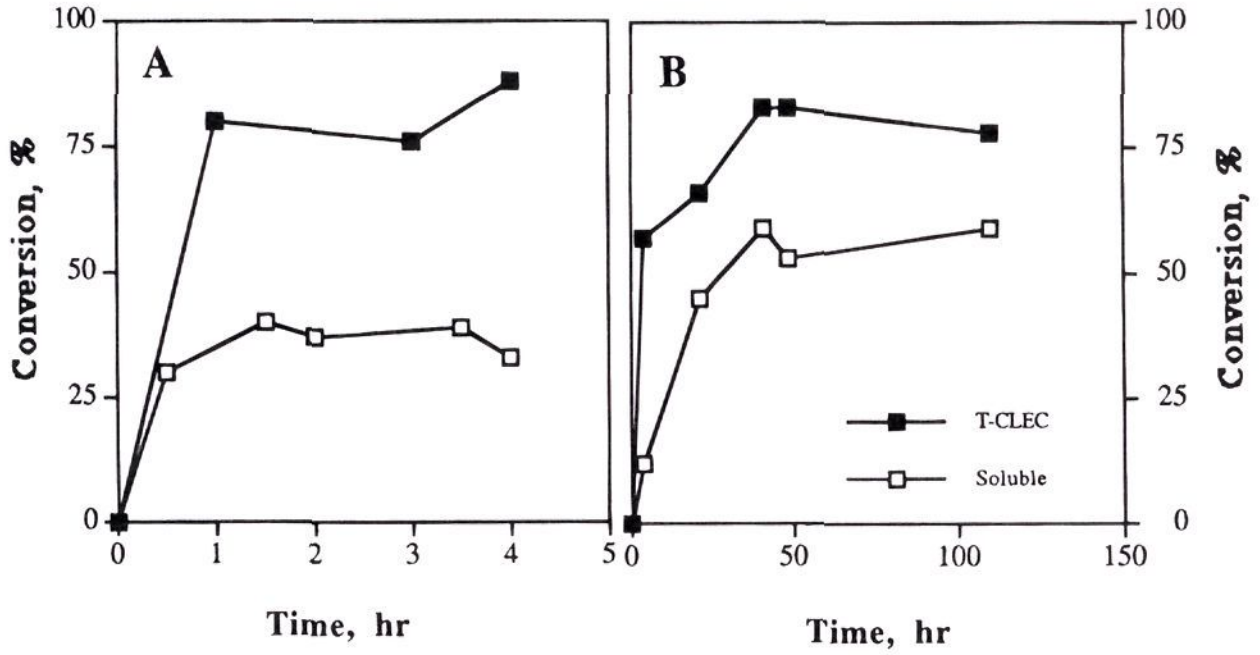

Figure 4. Synthesis of Z-GlyGlyPhePheNH$_2$ in 20% EtOH (A) and Z-GlyProGlyGlyProAlaPheNH$_2$ in 50% MeOH (B). Conditions: (A) 60 mM Z-GlyGlyPhe; 80 mM PheNH$_2$; 20% EtOH; concentrations of both T-CLECs and soluble enzyme were 2.6 mg of protein/mL; 45 °C; (B) 60 mM Z-GlyProGlyGlyProAla; 80 mM PheNH$_2$; concentrations of both T-CLECs and soluble enzyme were 5 mg of protein/mL, 25 °C.

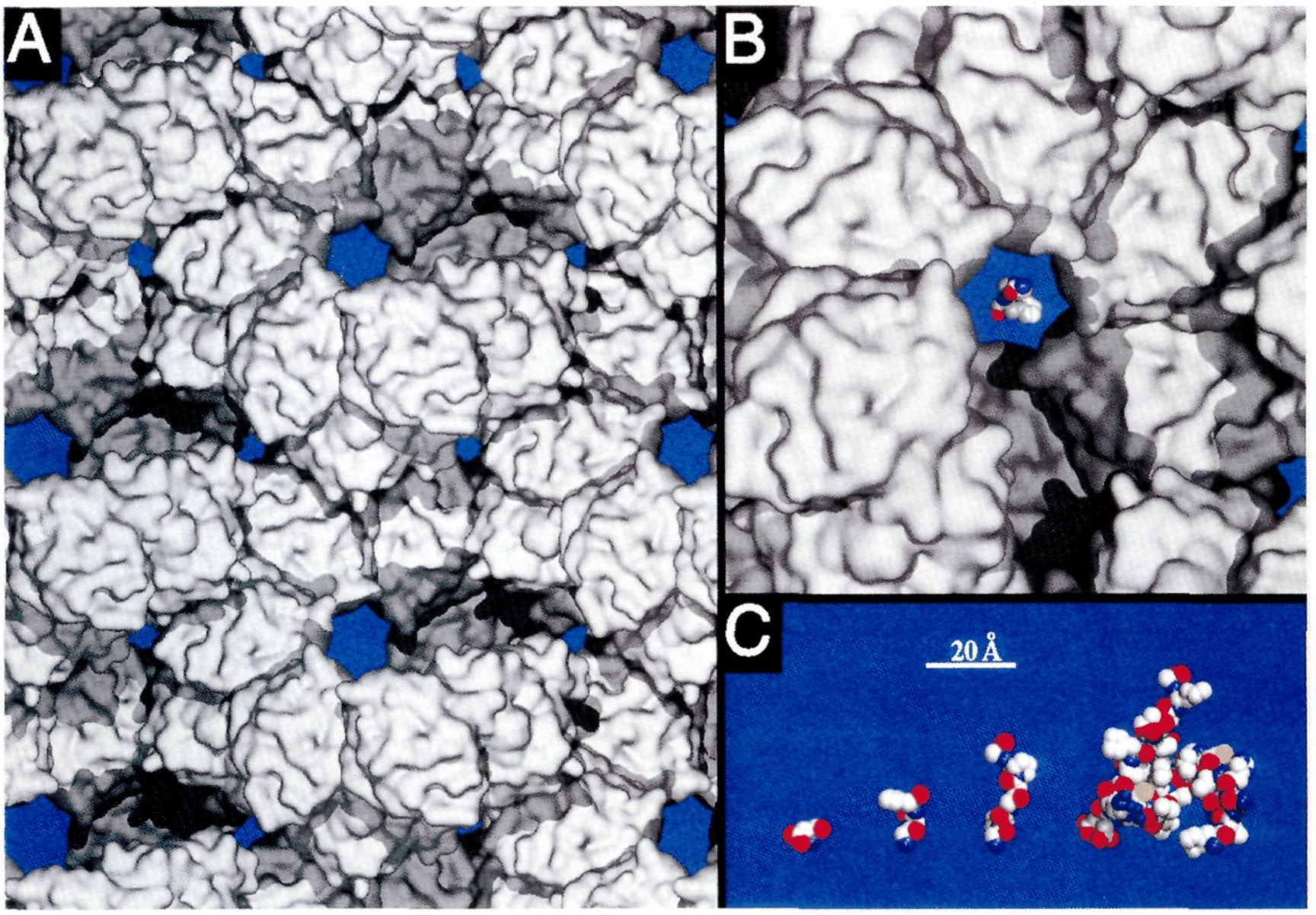

Figure 5. (A) Computer-generated view of a thermolysin crystal lattice. Thermolysin coordinates are taken from entry 3TLN in the Brookhaven Protein Data Bank.[33] The enzyme molecules are represented by an isovalue surface derived from the calculated model electron density using two-Gaussian atoms. Thermolysin molecules pack systematically around the 6-fold axis of symmetry to form large continuous pores that traverse the entire length of the crystal. (B) Close-up view of one of the pores, showing a tripeptide thermolysin substrate (Z-GlyGlyPhe) to scale. The bar, corresponding to 20 Å, establishes the absolute scale of the figure. Because the thermolysin molecules are arranged in a spiral around the 6-fold symmetry axis, the effective diameter of the pore through the crystal is larger than the approximate 25 Å diameter seen in the projection in blue. (C) Relative sizes of space-filling models of various T-CLEC substrates, including the blocked amino acid Z-Asp, tripeptide Z-GlyGlyPhe, hexapeptide Z-GlyProGlyGlyProAla, and the 30 amino acid oxidized insulin B-chain (Table 1). All are on the same scale as B.

forms a mixture of oligomers of different lengths and structures,[24] and therefore a crystal itself can "choose" the most appropriate cross-linking species.[25]

Encouraged by the successful use of T-CLECs in the synthesis of small peptides, we decided to investigate the limits of this technology in terms of substrate size. To this end, we have compared the initial rates of synthetic reactions catalyzed by T-CLECs (V_{CLEC}) with those catalyzed by native enzyme (V_{sol})

(24) Tashima, T.; Imai, M.; Kuroda, Y.; Yagi, S.; Nakagawa, T. *J. Org. Chem.* **1991**, *56*, 694−697.

(25) Martinek, K.; Torchilin, V. P. *Methods Enzymol.* **1983**, *137*, 615−624.

in the synthesis of peptides of increasing length. Under the high substrate concentrations normally used in peptide synthesis, the activity of T-CLECs is very similar to that of purified soluble thermolysin in the synthesis of small peptides up to a heptapeptide.[26] The ratio of initial rates of synthetic activity by T-CLECs and a soluble enzyme (V_{CLEC}/V_{sol}) lies in the range from 0.3 for the dipeptide in ethyl acetate to 2.6 for the synthesis of the heptapeptide in 90% EtOH (Table 1).

The results presented in Table 1 can be better visualized on the basis of the thermolysin crystal structure.[27] Large channels are formed by the packing of thermolysin molecules around the hexagonal axis of symmetry of the crystal that traverse the entire body of the macroscopic crystal and facilitate the diffusion of substrates, solvents, and products. In fact, thermolysin crystals (Figure 5) contain about 50% solvent by volume, a value typical of protein crystals.[28] Given the size of the channels (~25 Å in diameter), one can see (Figure 5) that small substrates will easily penetrate the body of the crystal to react with the active sites within. As substrate size increases toward the diameter of the channels, however, the effective specific activity of the crystal is expected to decrease, until eventually substrate is excluded from the crystal interior altogether and only the crystal surface is involved in catalysis.

Surprisingly, we found that a substrate as large as oxidized insulin B, a 30 amino acid peptide, was also a suitable substrate for T-CLECs, with an initial rate of 4% of that observed for native catalyst.[29] The insulin B chain is depicted (Figure 5C) in a globular conformation, consistent with its expected structure in an aqueous environment. However, its molecular conformation in a 90% DMF–ethanol mixture (Table 1) may be more linear, thus facilitating diffusion into the crystal.

Enzyme-catalyzed peptide synthesis offers many advantages to the synthetic chemist, including the absence of racemization and minimal protection and activation requirements.[30] These advantages are rarely exploited in preparative synthesis because of limited catalyst stability. T-CLECs, by virtue of their high specific activity and resistance to inactivation by organic solvents, elevated temperatures, and proteolysis, make enzymatic peptide coupling a feasible alternative to traditional methods, both in the laboratory and in large scale applications.

Experimental Section

General Methods. Melting points were obtained with an Electrothermal capillary melting point apparatus and are uncorrected. NMR spectra were recorded on a Bruker 500 spectrometer. ^{1}H chemical shifts are reported relative to methanol at δ 3.35 or chloroform at δ 7.24. Mass spectra were obtained from a Finnegan TSQ700 triple quadrupole mass spectrometer under electrospray ionization mass spectrometry (ESIMS) conditions. HPLC analyses were conducted with a Hewlett-Packard 1050 chromatograph using a Microsorb 5μ C_{18} column: 4.6 × 250 mm (300 Å pore size) for insulin; 4.6 × 150 mm (100 Å pore size) for all other samples. The identities of all products were verified by HPLC comparison with authentic samples.[7b,c] T-CLEC and soluble thermolysin activity was assayed by hydrolysis of the substrate 3-(2-furylacryloyl)-Gly-L-Leu amide (FAGLA), supplied by Schweizerhall, by monitoring decrease in absorbance at 345 nm.[31]

Materials. Amino acids and peptide starting materials were purchased from Sigma Chemical Co. Analytical grade solvents from J. T. Baker Inc. were used without further purification. Thermolysin was purchased from Daiwa Kasei K. K. Japan as a powder containing 30% protein. Thermolysin bound to agarose (12.5 units/mg of solid) was a product of Sigma. Enzyme crystallization with DMSO and cross-linking with glutaraldehyde were described previously.[4] Alternatively, thermolysin was crystallized by suspending crude enzyme powder in calcium acetate buffer.[32] The enzyme was solubilized by the gradual addition of NaOH, the pH being maintained at 11.0–11.5. Following complete solubilization the solution was neutralized by addition of acetic acid. Rod-shaped crystals appeared overnight. Both crystallization procedures gave the same hexagonal prism (Figure 3).

Multiple-Cycle Synthesis of Aspartame Precursor Z-Asp-PheOMe. A mixture of Z-L-Asp (0.406 g, 1.52 mmol) and L-PheOMe (0.812 g, 4.56 mmol) was stirred with 19 mL of buffer-saturated ethyl acetate (0.05 M MES–NaOH, 5 mM $CaCl_2$, pH 6.0). T-CLEC (47.5 mg) was added, and the reaction mixture was stirred at 55 °C. Nineteen 20 h cycles were completed. Catalyst was recovered for recycling by low-speed centrifugation. Reaction progress was monitored by HPLC (eluent, $CH_3CN/H_2O/CF_3CO_2H$ (60:40:0.1%); flow rate = 0.8 mL/min; λ = 215 nm; t_R (Z-AspPheOMe) = 3.5 min, t_R (Z-Asp) = 2.6 min). The product (at least 612 mg; 1.43 mmol; 95% yield) was isolated after washing the ethyl acetate layer with 1 N HCl, drying the organic layer over $MgSO_4$, and vacuum drying to an off-white solid: mp 137.5–138.8 °C; ^{1}H NMR ($CDCl_3$) δ 10.75 (bs, 1H), 7.34–7.08 (m, 10H), 6.08 (d, J = 8.5 Hz, 1H), 5.09 (s, 2H), 4.81 (dd, J_1 = 14.0 Hz, J_2 = 6.0 Hz, 1H), 4.61–4.59 (m, 1H), 3.65 (s, 3H), 3.10–3.01 (m, 2H), 2.94 (dd, J_1 = 17.5 Hz, J_2 = 4.5 Hz, 1H), 2.72 (dd, J_1 = 17.5 Hz, J_2 = 6.0 Hz, 1H).

Z-GlyGlyPhePheNH$_2$. $PhNH_2$·HCl (32.1 mg, 0.16 mmol) was stirred with 1.8 mL of EtOH, 150 μL of buffer (0.2 M NaOAc, 50 mM $Ca(OAc)_2$, pH 6.5), and 53.3 μL of 3 N NaOH, and addition of Z-GlyGlyPhe (49.6 mg, 0.12 mmol) followed. Insoluble material was dissolved by heating momentarily at 45 °C. PeptiCLEC-TR (6.6 mg) was added, and the reaction mixture was stirred at room temperature for 72 h (98%). Reaction progress was monitored by HPLC (eluent, $CH_3CN/H_2O/CF_3CO_2H$ (35:65:0.1%); flow rate = 1.2 mL/min; λ = 210 nm; t_R (Z-GlyGlyPhePheNH$_2$) = 9.2 min, t_R (Z-GlyGlyPhe) = 4.3 min, t_R ($PheNH_2$) = 1.3 min). PeptiCLEC-TR was isolated and reused after dissolving the product in $CaCl_2$-saturated EtOH, filtering, and then washing the CLECs with pure EtOH. The product was purified after removal of EtOH *in vacuo* by washing the resultant solid consecutively with 1 N HCl, 5% $NaHCO_3$, and then H_2O and vacuum drying to afford 62.4 mg (93%) of a white powder: mp 191.4–192.4 °C; ^{1}H NMR (CD_3OD) δ 7.42–7.15 (m, 15H), 5.11 (s, 2H), 4.71 (bs, 1H), 4.59 (dd, J_1 = 10.0 Hz, J_2 = 5.0 Hz, 1H), 4.44 (dd, J_1 = 10.0 Hz, J_2 = 5.0 Hz, 1H), 4.01 (d, J = 16.5 Hz, 1H), 3.92 (s, 2H), 3.81 (d, J = 16.5 Hz, 1H), 3.21 (dd, J_1 = 13.5 Hz, J_2 = 4.5 Hz, 1H), 3.06 (dd, J_1 = 13.5 Hz, J_2 = 10.0 Hz, 1H), 2.98 (dd, J_1 = 14.0 Hz, J_2 = 4.5 Hz, 1H), 2.89 (dd, J_1 = 13.5 Hz, J_2 = 10.5 Hz, 1H); ESIMS m/z 559.3 $(M)^+$.

GlyGlyPhePheNH$_2$. $PheNH_2$·HCl (50 mg; 0.25 mmol) was stirred with 0.5 mL of buffer (0.2 M NaOAc, 50 mM $Ca(OAc)_2$, pH 6.5) 0.5 mL of MeOH, and 83 μL of 3 N NaOH. The tripeptide, GlyGlyPhe (14 mg; 0.05 mmol), was added and addition of 5 mg of PeptiCLEC-TR followed. The mixture was stirred at room temperature for 16 h (91%). Reaction progress was monitored by HPLC (eluent, $CH_3CN/H_2O/CF_3CO_2H$ (20:80:0.1%); flow rate = 1.0 mL/min; λ = 210 nm; t_R (GlyGlyPhePheNH$_2$) = 3.5 min, t_R ($PheNH_2$) = 1.3 min). After 16 h the catalyst was removed by centrifugation and the mother liquor

(26) At low acid concentration (6 mM) the activity ratio V_{CLEC}/V_{sol} was 1.2 for GlyGlyPhePheNH$_2$, 0.43 for Z-GlyGlyPhePheNH$_2$, and 0.87 for Z-GlyProGlyGlyProAlaPheNH$_2$.

(27) Holmes, M. A.; Matthews, B. W. *J. Mol. Biol.* **1982**, *160*, 623–639. Matthews, B. W. *Acc. Chem. Res.* **1988**, *21*, 333–340.

(28) Matthews, B. W. *J. Mol. Biol.* **1968**, *33*, 491–497.

(29) In contrast, thermolysin immobilized on agarose (Sigma) at a concentration as high as 109 mg/mL was more than 30 times less active than T-CLECs at a concentration 10 times lower (8.7 mg/mL). A low specific activity for immobilized thermolysin is expected since immobilized enzyme is <5% catalyst by weight. CLECs, on the other hand, are pure catalysts. The entire volume of a CLEC consists of active enzyme and not, as in the case of immobilized enzymes, inert carrier. Enzyme concentrations within the crystal approach the theoretical packing limit for molecules of a given size. Thus, the activity of CLECs (for a given volume of enzyme) is significantly higher than that of conventionally immobilized enzymes, which serves to reduce both reaction times and volume of enzyme required.

(30) Schelleberg, V.; Jakubke, H.-D. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1437–1449.

(31) Feder, J.; Schuck, J. M. *Biochemistry* **1970**, *9*, 2784–2791.

(32) Endo, S. *J. Ferment. Technol.* **1962**, *40*, 346–353.

(33) Bernstein, F. C.; Koetzle, T. F.; Williams, G. J. B.; Meyer, E. F., Jr.; Brice, M. D.; Rodgers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. *J. Mol. Biol.* **1977**, *112*, 535–542.

was concentrated *in vacuo*. The product was dissolved in $CHCl_2$ (2 mL) and washed with 5% $NaHCO_3$ (3 × 2 mL). The organic layer was dried over $MgSO_4$ and then concentrated *in vacuo* to yield 17.0 mg (80%) of an off-white residue: ^{1}H NMR (CD_3OD) δ 7.29−7.05 (m, 10H), 4.95−4.78 (m, 4H), 4.19−4.05 (m, 2H), 3.80 (s, 2H), 3.15−3.09 (m, 1H), 2.96−2.89 (m, 2H), 2.68−2.62 (m, 1H); ESIMS *m/z* 425.3 $(M)^+$.

Z-GlyProGlyGlyProAlaPheNH$_2$. A suspension of Z-GlyProGlyGlyProAla (35.3 mg, 0.06 mmol) and $PheNH_2$·HCl (16.1 mg, 0.08 mmol) in 900 μL of EtOH, 73 μL of buffer (0.2 M NaOAc, 50 mM Ca(OAc), pH 6.5), and 26.7 μL of 3 N NaOH was stirred at room temperature followed by addition of 5 mg of CLECs. Stirring was continued at room temperature, and reaction progress was monitored by HPLC (eluent, $CH_3CN/H_2O/CF_3CO_2H$ (35:65:0.1%); flow rate = 1.2 mL/min; λ = 210 nm; t_R (Z-GlyProGlyGlyProAlaPheNH$_2$) = 2.5 min, t_R ($PheNH_2$) = 1.3 min, t_R (Z-GlyProGlyGlyProAla) = 1.7 min). The yield of desired heptapeptide after 40 h was 83% (by HPLC). No further reaction was seen after 40 h. The CLECs were removed as described for Z-GlyGlyPhePheNH$_2$, and the remaining solution was concentrated *in vacuo* to a solid, which was then washed consecutively with 1 N HCl, 5% $NaHCO_3$, and finally water. The resulting solid was dried *in vacuo* to yield 34.1 mg of the heptapeptide as a white solid (77%): mp 190.4−190.9 °C; ^{1}H NMR (CD_3OD) δ 7.37−7.15 (m, 10H), 5.09 (s, 2H), 4.55 (dd, J_1 = 9.0 Hz, J_2 = 5.0 Hz, 1H), 4.37 (dd, J_1 = 8.0 Hz, J_2 = 5.0 Hz, 1H), 4.32 (dd, J_1 = 8.0 Hz, J_2 = 5.0 Hz, 1H), 4.20 (d, J = 15.5 Hz, 1H), 4.15 (q, J = 9.3 Hz, 1H), 4.03 (d, J = 15.5 Hz, 1H), 3.96 (d, J = 22.0 Hz, 1H), 3.95 (d, J = 22.0 Hz, 1H), 3.88 (d, J = 13.5 Hz, 2H), 3.71−3.46 (m, 4H), 3.20 (dd, J_1 = 13.5 Hz, J_2 = 5.0 Hz, 1H), 2.98 (dd, J_1 = 13.5 Hz, J_2 = 5.0 Hz, 1H), 2.29−1.84 (m, 8H), 1.23 (d, J = 9.3 Hz, 3H); ESIMS *m/z* 734.6 $(M)^+$.

Insulin-B-PheNH$_2$. Oxidized insulin B-chain, (10.5 mg, 3.0 μmol) and $PheNH_2$·HCl (12.0 mg, 60.0 μmol) were dissolved in 60 μL of H_2O and 540 μL of DMF/EtOH, 1:1. The pH was adjusted to 6.5 with 7% NH_4OH followed by the addition of 10 mg of CLEC. The reaction was monitored by HPLC and then terminated after 3 h (46% yield) by the addition of 1 mL of 1 N HCl. After centrifugation, the mother liquor was applied to a column (10 × 250 mm, Microsorb C_{18}, 5 mm, 100 Å pore) and eluted with $CH_3CN/H_2O/CF_3CO_2H$ (34:66:0.1%).

Acknowledgment. Support for this research by the National Science Foundation (Grant III-9306424) is greatly appreciated. We thank Sam Pazhanisamy of Vertex Pharmaceuticals Incorporated for fruitful discussions.

JA943024C