Comparative Total Syntheses of Turkey Ovomucoid Third Domain by Both Stepwise Solid Phase Peptide Synthesis and Native Chemical Ligation

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Abstract: Turkey ovomucoid third domain, OMTKY3, is a potent protein inhibitor of most serine proteinases that prefer a neutral residue at the P1 position. It has been a target of increasingly intense scrutiny by site-directed mutagenesis in an attempt to elucidate the sequence-to-reactivity algorithm of the inhibitor. Here we report the total chemical synthesis of (6−56)OMTKY3 using both the stepwise solid phase peptide synthesis (SPPS) technique and the native chemical ligation approach. After refolding and affinity purification, the resultant products were characterized by electrospray ionization mass spectrometry, analytical ion exchange chromatography, measurement of association equilibrium constants with six different serine proteinases, and thermal denaturation studies. The two synthetic proteins were found to be functionally as well as structurally identical to their recombinant counterpart. Moreover, the native chemical ligation and stepwise SPPS techniques gave rise to comparable yields and similar product quality. The native chemical ligation strategy used here presents highly efficient synthetic access to novel analogs of OMTKY3 that can be used for understanding the molecular basis of enzyme−inhibitor recognition.

Introduction

Virtually all biological phenomena are simply reflections of molecular interactions, where proteins often play a central role. A large number of interacting systems have been intensely investigated in order to understand the molecular basis of protein−protein recognition. Among these interacting systems protein proteinase inhibitors interacting with their cognate enzymes represent one of the simplest examples.[1] Turkey ovomucoid third domain (OMTKY3) is a potent protein inhibitor of most serine proteinases that prefer a neutral residue at the P1 position (in Schechter & Berger notation[2]).[3] Three-dimensional structures of OMTKY3 in complex with several serine proteinases[4−6] have revealed a primary binding loop of nine residues in the inhibitor which make direct contact with the enzyme upon complex formation. In an effort to establish the first-order sequence-to-reactivity algorithm of OMTKY3,[7] enzymatic semisynthesis[8] and more recently systematic site-directed mutagenesis[9] have been used to construct hundreds of mutations in the primary enzyme-binding region of the inhibitor. As a result, a huge thermodynamic database involving hundreds of association equilibrium constants with eight different serine proteinases has been obtained, which has greatly enhanced our understanding of inhibitor−enzyme interactions.

While genetic engineering continues to enjoy tremendous success in probing protein structure/function relationships, the total chemical synthesis of proteins has become an increasingly important complementary tool in exploring the molecular basis of protein functions. The most important aspect of total chemical synthesis is its unique ability and versatility in incorporating into proteins unnatural amino acids and a variety of other novel structural and functional modifications in both side chains and the backbone of the polypeptide chain. Nevertheless, it was not until recently that the potential of total chemical synthesis was fully appreciated. Recent advances in Boc-chemistry solid phase peptide synthesis (SPPS) have dramatically increased the efficiency of assembly of protected peptide chains, which, as a result, has greatly augmented the number of proteins accessible by total chemical synthesis.[10] However, while stepwise synthesis of small proteins is now achievable,[11−16] synthesis of proteins of more than 100 amino acid residues by stepwise chain elongation still remains a

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(1) Bode, W.; Huber, R. *Eur. J. Biochem.* **1992**, *204*, 433−451.

(2) Schechter, I.; Berger, M. *Biochem. Biophys. Res. Commun*. **1967**, *27*, 157−162.

(3) Empie, M. W.; Laskowski, M., Jr. *Biochemistry* **1982**, *21*, 2274−2284.

(4) Read, R. J.; Fujinaga, M.; Sielecki, A. R.; James, M. N. G. *Biochemistry* **1983**, *22*, 4420−4433.

(5) Bode, W.; Wei, A. Z.; Huber, R.; Meyer, E.; Travis, J.; Neumann, S. *EMBO J.* **1986**, *5*, 2453−2458.

(6) Fujinaga, M.; Sielecki, A. R.; Read, R. J.; Ardelt, W.; Laskowski, M., Jr.; James, M. N. G. *J. Mol. Biol.* **1987**, *195*, 397−418.

(7) Laskowski, M., Jr.; Park, S. J.; Tashiro, M.; Wynn, R. *Protein Recognition of Immobilized Ligands*; Alan R. Liss, Inc.: New York, 1989; pp 149−168.

(8) Bigler, T.; Lu, W.; Park, S.; Tashiro, M.; Wieczorek, M.; Wynn, R.; Laskowski, M., Jr. *Protein Sci.* **1993**, *2*, 786−799.

(9) (a) Lu, W. Energetics of the Interactions of Ovomucoid Third Domain Variants with Different Serine Proteinases. Ph.D. Thesis, Purdue University, 1994. (b) Lu, W.; Apostol, I.; Qasim, M. A.; Warne, N.; Wynn, R.; Zhang, W.; Anderson, S.; Chiang, Y.-W.; Ogin, E.; Rothberg, I.; Ryan, K.; Laskowski, M., Jr. *J. Mol. Biol.*, submitted for publication.

(10) Muir, T. W.; Kent, S. B. H. *Curr. Opin. Biotechnol*. **1993**, *4*, 420−427.

(11) Clark-Lewis, I.; Aebersold, R.; Ziltener, H.; Schrader, J. W.; Hood, L. E.; Kent, S. B. H. *Science* **1986**, *231*, 134−139.

(12) Heath, W. F.; Merrifield, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6367−6371.

(13) Schneider, J.; Kent, S. B. H. *Cell* **1988**, *54*, 363−368.

(14) Clark-Lewis, I.; Moser, B.; Walz, A.; Baggiolini, M.; Scott, G. J.; Aebersold, R. *Biochemistry* **1991**, *30*, 3128−3134.

(15) Zawadzke, L. E.; Berg, J. M. *J. Am. Chem. Soc.* **1992**, *114*, 4002−4003.

(16) Schumacher, T. N. M.; Mayr, L. M.; Minor D. L., Jr.; Milhollen, M. A.; Burgess, M. W.; Kim, P. S. *Science* **1996**, *271*, 1854−1857.

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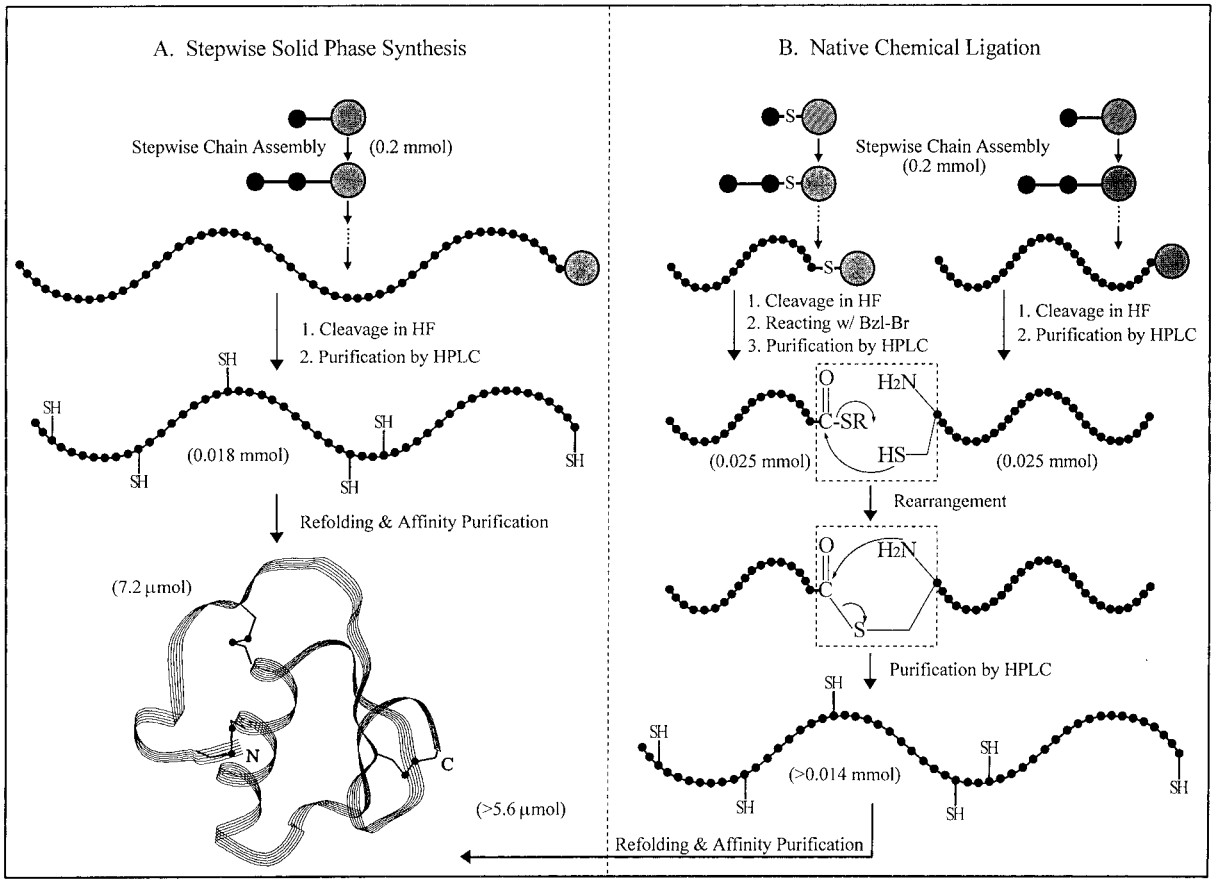
Scheme 1



daunting task due to an exponential increase in the number of side products accumulated over the many synthetic steps. The size barrier for synthetic targets has been effectively overcome by the development of chemoselective ligation strategies that made proteins of up to 200 amino acid residues accessible by total chemical synthesis.[17] Among many chemoselective ligation methods is the powerful native chemical ligation technique[18] developed for two fully unprotected peptide fragments to form a native peptide bond by forming a thioester ligation product followed by a spontaneous acyl-S to acyl-N transfer. Several proteins of moderate size have been successfully synthesized by a rapid single-step ligation.[18−20]

In this paper we report on the total chemical synthesis of wild type (6−56)OMTKY3 using both the stepwise SPPS and the native chemical ligation techniques (Scheme 1), in an effort to rigorously compare the two different synthetic approaches and to establish a reliable and efficient synthetic access to unnatural analogs of OMTKY3 for studies of enzyme−inhibitor interactions.

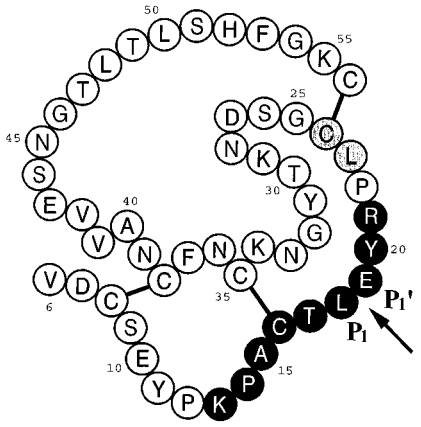


Figure 1. Amino acid sequence of (6−56)OMTKY3. The residues in black are involved in direct contact with cognate enzymes. The arrow indicates the reactive site scissile peptide bond. The shaded residues represent the site for native chemical ligation.

Results and Discussion

Stepwise Synthesis of (6−56)OMTKY3. Ovomucoid, an abundant protein in avian egg whites, is composed of three homologous domains, each of which is a serine proteinase inhibitor of the Kazal family.[21] Turkey ovomucoid third domain, OMTKY3, consists of 56 amino acid residues. As part of the connecting peptide between the second and the third domains of turkey ovomucoid, the first five residues of OMTKY3 have been found to have no effect on the inhibitory activity,[22] and therefore the recombinant variants have been prepared without these residues.[9] It has been demonstrated by X-ray crystallographic studies and binding assays that the recombinant (6−56)OMTKY3 is structurally and functionally identical to the naturally occurring OMTKY3.[9,23] Figure 1 is a schematic representation of the amino acid sequence of (6−56)OMTKY3.

(17) Canne, L. E.; Ferré-D'Amaré, A. R.; Burley, S. K.; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, *117*, 2998−3007.
(18) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779.
(19) Liu, C-F.; Rao, C.; Tam, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 307−312.
(20) Dawson, P. E.; Churchill, M.; Ghadiri, M. R.; Kent, S. B. H. Submitted for publication to *J. Am. Chem. Soc.*
(21) Laskowski, M., Jr.; Kato, I. *Annu. Rev. Biochem.* **1980**, *49*, 593−626.
(22) Wieczorek, M.; Park, S. J.; Laskowski, M., Jr. *Biochem. Biophys. Res. Commun.* **1987**, *144*, 499−504.
(23) Huang, K.; Lu, W.; Anderson, S.; Laskowski, M., Jr.; James, M. N. G. *Protein Sci.* **1995**, *4*, 1985−1997.

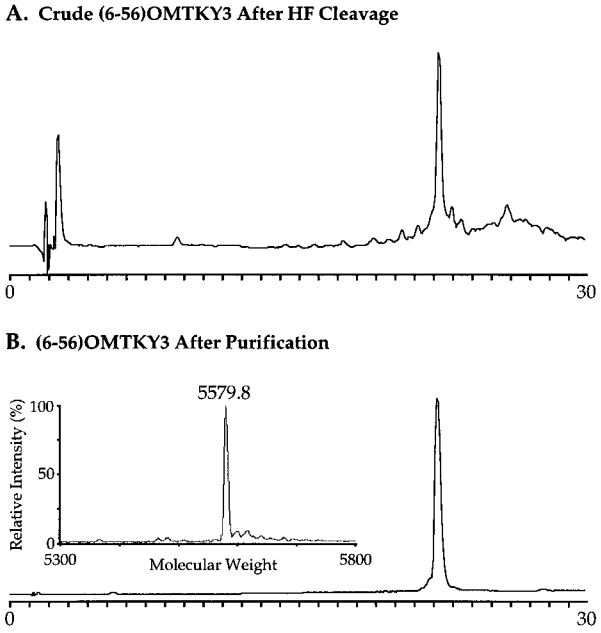


Figure 2. (A) Chromatogram of crude (6−56)OMTKY3 synthesized by stepwise SPPS, after HF cleavage. (B) Chromatogram of (6−56)-OMTKY3 after purification on a preparative C18 reversed-phase HPLC column. The observed mass determined by ES-MS was 5579.8 ± 0.5 Da (calculated 5579.3 Da, average isotope composition). Both chromatograms were obtained on a narrow-bore C18 reversed-phase HPLC column with a gradient of 0−42% B performed over 24 min.

Synthesis of the 51-residue OMTKY3 by machine-assisted stepwise solid phase methods using the 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation*/in situ N,N*-diisopropylethylamine (DIEA) neutralization coupling protocol for Boc chemistry[24] was relatively straightforward. Boc-amino acids (2.2 mmol) were activated by HBTU in the presence of DIEA, and were coupled in stepwise fashion on 0.2 mmol of preloaded Boc-Cys-(4-CH_3-Bzl)OCH_2-Pam-resin on a custom-modified ABI 430 peptide synthesizer (5 cycles/h). TFA (100%) was used for removal of *N*-Boc groups; DMF was used for flow washes throughout the entire synthesis. The optimized Boc-chemistry SPPS has been demonstrated to dramatically improve the efficiency of assembly of protected peptide chains.[24] *In situ* neutralization overcomes sequence-dependent incomplete acylation that can arise from intermolecular aggregation in the neutralization step *prior to* coupling. While the DMF flow wash maximizes resin swelling, 100% TFA, a superb peptide solvent, effectively disrupts the formation of secondary structures, therefore preventing peptides from aggregating. Moreover, use of a maximal concentration of activated Boc-AA-OBt ester gives high reaction rates and near-quantitative coupling yields.

After the chain assembly was complete, crude peptides were deprotected and cleaved from the resin by treatment with anhydrous HF in the presence of *p*-cresol/*p*-thiocresol as scavengers, and were purified on a preparative C18 reversed-phase HPLC column (Figure 2). Fractions were analyzed by electrospray ionization mass spectrometry (ES-MS), and the desired product of correct mass was pooled and lyophilized. The observed mass of the reduced (6−56)OMTKY3 was 5579.8 ± 0.5 Da (calculated 5579.3 Da, average isotope composition). Typically, 100 mg (0.018 mmol) of purified product in the reduced form was obtained from a 0.2 mmol scale synthesis. Notably, the final product was found to be in the fully reduced state because of a low pH environment throughout the entire workup and purification.

(24) Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H *Int. J. Peptide Protein Res.* **1992**, *40*, 180−193.

It is worth noting that synthesis of OMTKY3, using Fmoc-chemistry SPPS, was first attempted by the Purdue group, and wild type (6−56)OMTKY3 as well as a number of unnatural analogs was made.[25] In addition, syntheses of bovine pancreatic trypsin inhibitor (BPTI), an inhibitor of similar size from the Kunitz family, have also been reported.[26]

Synthesis of (6−56)OMTKY3 by Native Chemical Ligation. Native chemical ligation is initiated by a chemoselective nucleophilic attack of the thiol of Cys at the N terminus of one peptide on a C-terminal thioester moiety of another peptide, forming a thioester ligation product, which undergoes spontaneous rearrangement, through an acyl transfer, to generate a native peptide bond at an Xxx-Cys site. Three cysteines, Cys^{24}, Cys^{35}, and Cys^{38}, in the middle of the sequence of (6−56)OMTKY3 could serve as the potential ligation site. However, because the primary binding loop of the inhibitor, which will be the target region for chemical mutations, is located near the N terminus, the ligation site Leu^{23}-Cys^{24} seemed optimal.

The C-terminal segment, (24−56)OMTKY3, was manually synthesized in stepwise fashion on Boc-Cys-(4-CH_3Bzl)OCH_2-Pam-resin using the optimized *in situ* neutralization/HBTU protocol for Boc-chemistry.[24] Prior to its use in the ligation reaction, the deprotected peptide was purified on a preparative C18 reversed-phase HPLC column, and its mass was ascertained by ES-MS (observed mass 3513.0 ± 0.6 Da; calculated 3512.9 Da, average isotope composition). The typical yield of the purified (24−56)OMTKY3 in the reduced form was 0.05 mmol on a 0.2 mmol scale synthesis. The N-terminal peptide, (6−23)αCOSH, was manually synthesized on Boc-Leu-S-(linker)-aminomethyl-resin that was made according to the published protocols.[27] All the amino acids were coupled as described above. After cleavage and lyophilization, the fully unprotected peptide-αCOSH (observed mass 2100.0 ± 0.1 Da; calculated 2100.4 Da, average isotope composition) was treated with excess benzyl bromide to form the benzyl thioester peptide, (6−23)αCOSBzl (observed mass 2191.0 ± 0.4 Da; calculated 2190.4 Da, average isotope composition), which was then purified on preparative C18 reversed-phase HPLC. A typical yield of the purified (6−23)αCOSBzl on a 0.2 mmol scale was 55 mg (0.025 mmol).

The ligation of (6−23)αCOSBzl (0.025 mmol) and (24−56)-OMTKY3 (0.025 mmol) was carried out in the presence of 6 M GuHCl, 1% benzyl mercaptan, and 3% thiophenol, pH 7.5.[20] The reaction went to completion in 36 h, after which the resultant ligation product was purified on a preparative C18 reversed-phase HPLC column (Figure 3). The mass was determined by ES-MS to be 5579.8 ± 0.8 Da (5579.3 Da calculated, average isotope composition). The yield of the purified (6−56)OMTKY3 in the reduced form was typically 75 mg (∼0.014 mmol).

Benzyl mercaptan present in the ligation reaction acted as a reducing agent to prevent the formation of both intermolecular and intramolecular disulfide bonds. Its presence would also help convert nonproductive ligated thioesters back to the starting materials. However, it was found that benzyl mercaptan alone did not always give rise to a fast and productive ligation reaction, particularly when the C-terminal residue at the ligation site had a bulky side chain, in which case the steric hindrance could effectively slow the nucleophilic attack by the N-terminal Cys. As was found previously, addition of excess thiophenol

(25) (a) Warne, N. Ph.D. Thesis, Purdue University, 1990. (b) Ojunjobi, O. M.; Laskowski, M., Jr. Unpublished results.

(26) (a) Tan, N. H.; Kaiser, E. T. *J. Org. Chem.* **1976**, *41*, 2787−2793. (b) Ferrer, M.; Woodward, C.; Barany, G. *Int. J. Pept. Protein Res.* **1992**, *40*, 194−207.

(27) Canne, L. E.; Walker, S. M.; Kent, S. B. H. *Tetrahedron Lett.* **1995**, *36*, 1217−1220.

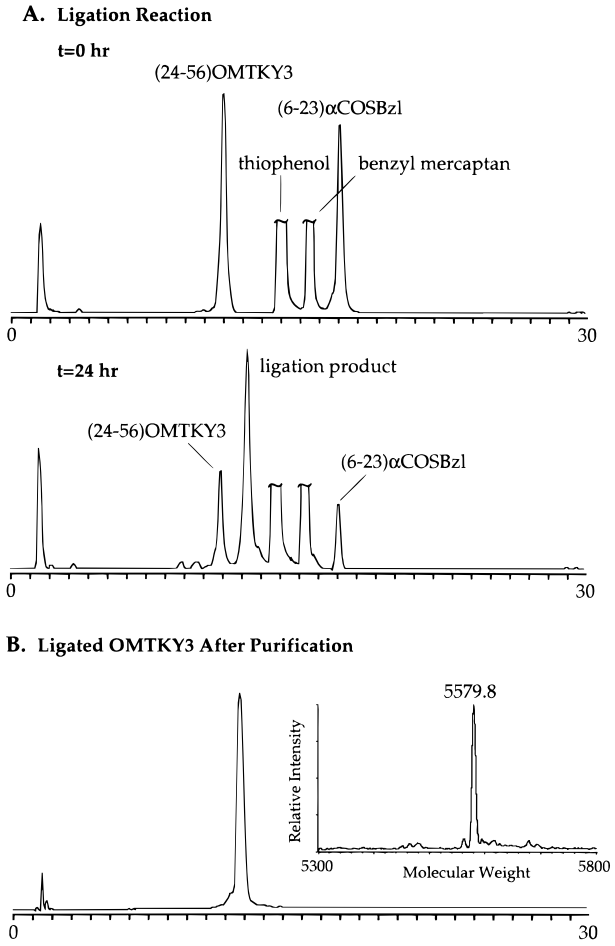


Figure 3. (A) Chromatogram demonstrating ligation of (6−23)αCOSBzl and (24−56)OMTKY3. The starting materials were dissolved at 8 mg/mL in 0.1 M phosphate buffer containing 6 M GuHCl, pH 7.5, to which 1% benzyl mercaptan and 3% thiophenol had been added. After 24 h, more than 65% of the reactants were converted to the product. The ligation reaction was monitored by injecting a 1 μL aliquot onto a narrow-bore C18 reversed-phase HPLC column running a gradient of 20−55% B, at a flow rate of 0.3 mL/min, over 24 min. (B) Chromatogram of ligated (6−56)OMTKY3 after purification on a preparative C18 reversed-phase HPLC column. The observed mass of 5579.8 ± 0.8 Da (calculated 5579.3 Da, average isotope composition) was determined by ES-MS.

significantly enhanced the ligation reaction rate[20] probably through nucleophilic catalysis, where displacement of benzyl mercaptan by thiophenol ultimately yielded a better leaving group. Interestingly, the thioester intermediate that rearranges to the amide product in native chemical ligation[18] has never been observed by HPLC. In the case of OMTKY3, the rearrangement must have taken place in order to regenerate the sulfhydryl group of Cys[24], which is involved in disulfide bond formation.

It might be anticipated that the thioester peptide would undergo facile racemization at the C-terminal residue via an oxazolone mechanism.[28] In order to address this concern, a model study of the ligation site Leu[23]-Cys[24] was carried out. Two 10-residue peptide segments, (14−23)αCOSBzl and (24−33)OMTKY3, were reacted under the same ligation conditions as discussed above to generate (14−33)OMTKY3. To identify the possible epimerized product, the reference compound (14−33)-D-Leu[23]-OMTKY3 was manually synthesized by SPPS on MBHA resin. Purified (14−33)-D-Leu[23]-OMTKY3 was added as an internal standard into the ligation mixture, which was then analyzed on an analytical C18 reversed-phase HPLC column. The two diastereomers were separated to baseline under a gradient of 20−40% B over 24 min (data not shown). By analyzing the ligation mixture alone, we were able to conclude that no detectable amount of racemization at Leu[23] (<1%) had occurred under the ligation conditions used.

Protein Folding and Purification. The folding of (6−56)-OMTKY3 was initiated by a rapid dilution of the reduced polypeptide chain dissolved in 6 M GuHCl to a final concentration of 0.2 mg/mL in 1 M GuHCl, pH 8.7. The formation of disulfides was further aided by air oxidation in an open container with gentle stirring. The folding was complete in 8 h, as monitored by detecting the inhibitory activity of the solution against chymotrypsin. It should be pointed out, however, that the refolding of (6−56)OMTKY3 is pH-dependent. At pH lower than 7.5, the initial folding rate was significantly slower presumably because of protonation of the sulfhydryl group of Cys. In addition, refolding at higher concentration tended to cause aggregation, possibly of folding intermediates that had more hydrophobic residues exposed. One consequence of the dilution approach for protein refolding is that it results in large volumes that often complicate subsequent recovery of folded protein. Affinity chromatography is particularly useful in solving this problem. OMTKY3 is a strong inhibitor of bovine α-chymotrypsin ($K_a = 1.8 \times 10^{11}$ M^{-1}). His[57] of chymotrypsin can be specifically methylated by *p*-nitrobenzenesulfonic acid methyl ester,[29] yielding 3-methyl-His[57] chymotrypsin that is inactive yet retains binding ability. Methylated chymotrypsin covalently linked to Sepharose gel has been widely used for isolation of chymotrypsin inhibitors, and proved to be useful in this case. Although the conditions used for the refolding of (6−56)OMTKY3 are by no means optimal, a decent and highly reproducible recovery yield was obtained after affinity purification on a methylchymotrypsin−Sepharose affinity column (typically 40% by weight). However, one should be cautious about using purification procedures solely based on functional selection, particularly when the target protein is functionally unpredictable. For this reason, we thoroughly characterized the synthetic protein products with respect to purity and covalent structure.

The recombinant (6−56)OMTKY3 that served as a reference sample in this work was expressed in the periplasmic space of *Escherichia coli*, where the formation of the disulfide bonds was catalyzed by a strong oxidizing protein, DsbA.[30] X-ray crystallographic studies have demonstrated that the recombinant OMTKY3 is structurally identical to the naturally occurring inhibitor isolated from turkey egg whites.[23] To our surprise, however, both the synthetic and the recombinant OMTKY3 showed a broad front-tailing peak in analytical C18 reversed-phase HPLC (data not shown), although their corresponding reduced forms without exception gave a very sharp peak. Furthermore, when small fractions under the peak tip were collected and reinjected, a similarly shaped peak was observed. One possible explanation would be that, due to the high stability of OMTKY3, the protein was only partially denatured in the acidic HPLC solvent. This was later confirmed in the study of a backbone-engineered ester analog of OMTKY3.[31] However, the Mono-Q anion exchange FPLC (fast protein liquid chromatography) of the three different preparations gave a single superimposable peak, demonstrating chromatographic purity of the oxidized synthetic products (Figure 4).

Structural and Functional Assays. Is the synthetic protein correctly folded? The ultimate answer to that question undoubt-

(28) Jones, J. *The Chemical Synthesis of Peptides*; Clarendon Press: Oxford, 1991.

(29) Ryan, D. S.; Feeney, R. E. *J. Biol. Chem.* **1975**, *250*, 843−847.
(30) Bardwell, J. C. A.; Beckwith, J. *Cell* **1993**, *74*, 769−771.
(31) Lu, W.; Qasim, M. A.; Laskowski, M., Jr.; Kent, S. B. H. Submitted for publication to *Biochemistry*.

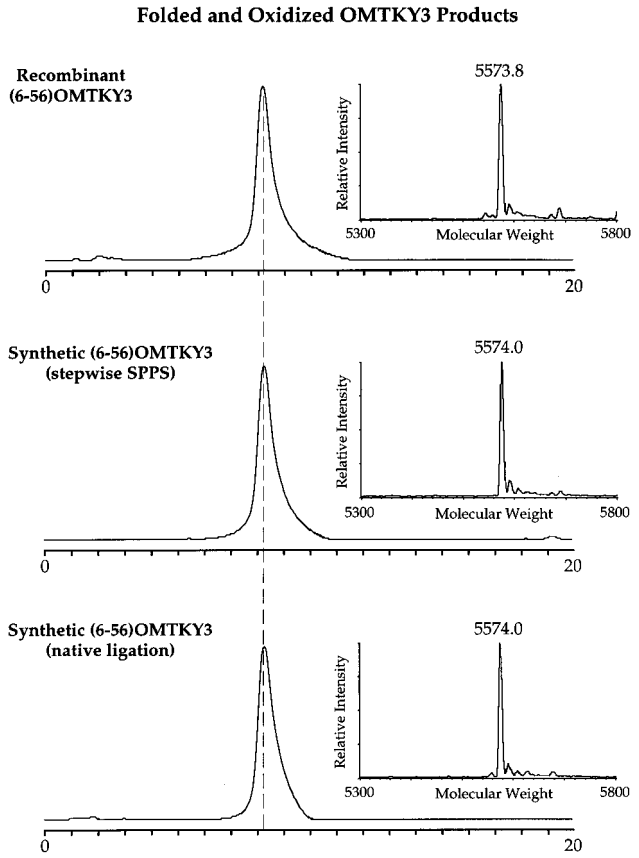


Figure 4. Chromatogram of recombinant and synthetic (6−56)-OMTKY3 analyzed with Mono-Q anion exchange FPLC at pH 9.1. A gradient of 0−50% B was run over 15 min. Observed masses (ES-MS) are shown. The calculated mass for folded (6−56)OMTKY3 was 5573.3 Da (average isotope composition). The observed mass of 5574.0 ± 0.8 Da after oxidization/folding is 6 Da lower than that of the reduced polypeptide chain (see Figures 2 and 3), as expected for the formation of three disulfides.

edly comes from structure determination by X-ray crystallography or NMR. However, stringent functional assays combined with analysis of characteristic chemical structures of the protein can be used to address the same issue. OMTKY3, with Leu at the P1 position, strongly inhibits most serine proteinases that have a neutral S1 pocket to accommodate hydrophobic P1 side chains. Association equilibrium constants (K_a) were measured at pH 8.3 for the two synthetic inhibitors interacting with six different serine proteinases: bovine α-chymotrypsin (CHYM), porcine pancreatic elastase (PPE), human leukocyte elastase (HLE), subtilisin Carlsberg (SUBT), and *Streptomyces griseus* proteinases A and B (SGPA and SGPB). The results are given in Table 1. All the K_a values for synthetic (6−56)OMTKY3 were in good agreement with those obtained for recombinant (6−56)OMTKY3, indicating that they were functionally identical. It should be emphasized that the integrity of the three-dimensional folded structure of a single-domain protein proteinase inhibitor is required for its function as well as for stability. Peptides comprising only the binding loop of the inhibitor (without the protein scaffold) exhibit a greatly weakened binding capability (typically 6 orders of magnitude), and are highly susceptible to proteolysis.[32]

It is worth noting that a panel of eight serine proteinases, including *S. griseus* trypsin (SGT) and *S. griseus* Glu-specific proteinase (SGPE), were originally used in the study of 20 coded recombinant P1 variants of OMTKY3.[9] As expected, SGT and SGPE, which prefer a basic and an acidic residue at the P1 position of the inhibitor, respectively, were extremely weakly inhibited by the wild type inhibitor ($K_a \approx 10^2-10^3$ M^{-1}) and therefore were not used in this study.

(32) Kelly, C. A.; Qasim, M. A.; Otlewski, J.; Laskowski, M., Jr. Unpublished results.

Table 1. Association Equilibrium Constants for Recombinant and Two Synthetic (6−56)OMTKY3's with Six Different Serine Proteinases[a]

	$K_a \times 10^{-9}$ (M^{-1})		
enzyme	recombinant	stepwise SPPS	native chemical ligation
CHYM	190	150	160
PPE	42	34	38
SUBT	34	41	34
SGPA	300	250	290
SGPB	56	53	57
HLE	6.1	4.6	5.3

[a] The K_a values were measured at 21 ± 1 °C, pH 8.3, with an accuracy of ±20%. CHYM = bovine α-chymotrypsin; PPE = porcine pancreatic elastase; SUBT = subtilisin Carlsberg; HLE = human leukocyte elastase; SGPA and SGPB = *S. griseus* proteinase A and B.

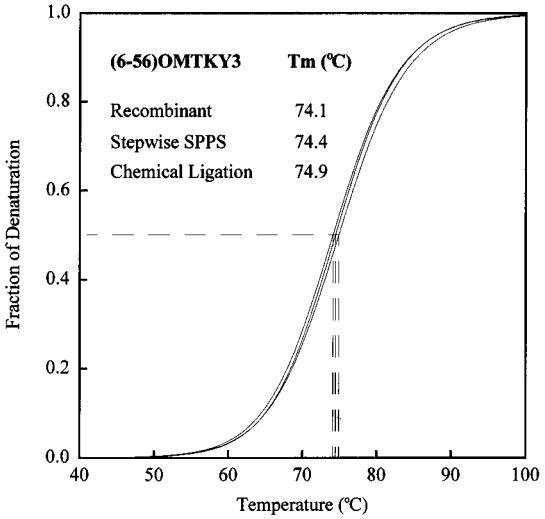


Figure 5. Thermal denaturation of recombinant and synthetic (6−56)OMTKY3 at pH 4.3 (see the text for details).

Correct pairing of disulfide bonds is a prerequisite for a native conformation. Unfortunately, OMTKY3, like many other protein proteinase inhibitors, is resistant to proteolysis by most enzymes under normal conditions (Michael Laskowski, Jr., personal communication). Thus, disulfide mapping for the synthetic inhibitors was not vigorously pursued (it has been, however, demonstrated in the study of a backbone ester analog of OMTKY3,[31] that the three disulfides in the inhibitor are indeed correctly paired). Instead, thermal denaturation studies of the synthetic products were conducted in order to establish a structural similarity between synthetic and recombinant (6−56)OMTKY3. A strong hydrogen bond between $Asp^{27}O^{\delta 2}$ and $Tyr^{31}O^{\eta}$ (2.45 Å) is totally conserved among 157 of the 159 natural ovomucoid third domain variants isolated from egg whites of 153 avian species (Michael Laskowski, Jr., personal communication). The rupture of this hydrogen bond upon heating gives rise to a characteristic fluorescence signal which can be used for monitoring the melting transition and determining the melting temperature (T_m). The melting behavior of recombinant OMTKY3 and the two synthetic samples, after normalization as fraction denaturation curves, were virtually identical with regard to their melting transition curves and T_m values (Figure 5). The T_m values of the three preparations were within ±1 °C of each other. The structural integrity of synthetic inhibitors shown by thermal stability studies strongly suggested that the synthetic OMTKY3 had folded correctly.

Conclusion. Total chemical synthesis of (6−56)OMTKY3 was performed using both the stepwise SPPS and the native chemical ligation techniques (Scheme 1). The synthetic inhibitors were extensively characterized and were found to be functionally as well as structurally identical to the recombinant counterpart. The two chemical synthesis approaches gave comparable yields for the 51-residue protein inhibitor. Other work has suggested that native chemical ligation is superior to stepwise chain assembly SPPS in synthesis of larger proteins.[18,20] Moreover, since the ligation strategy effectively eliminates the redundancy of repeated synthesis of unaltered parts in a protein, native chemical ligation will be ultimately much more efficient in generating a large number of protein analogs.

Experimental Section

Materials. Boc-L-amino acids and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Novabiochem; Boc-Cys-(4-CH_3Bzl)OCH_2-Pam-resin and *N*,*N*-diisopropylethylamine (DIEA) were obtained from Applied Biosystems. Aminomethyl-resin was prepared in this lab by Dr. Lynne Canne. Dichloromethane (DCM) and acetonitrile (ACN) were purchased from Fisher Scientific, dimethylformamide (DMF) was purchased from J. T. Baker, trifluoroacetic acid (TFA) was purchased from Halocarbon Products Co., and HF was purchased from Matheson Gas Products. Benzyl bromide, benzyl mercaptan, and thiophenol were purchased from Aldrich Chemical Co. Bovine α-chymotrypsin was purchased from Worthington Biochemical Co., human leukocyte elastase was purchased from Elastin Products Co., Inc., porcine pancreatic elastase was a generous gift from the late Dr. M. Laskowski, Sr., and subtilisin Carlsberg was obtained from Sigma Chemical Co. *Streptomyces griseus* proteinases A and B were purified from pronase (Sigma). All the chromogenic substrates were purchased from Bachem Bioscience Inc.

Methylchymotrypsin was prepared according to Ryan and Feeney,[29] and was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the coupling procedures provided by the manufacturer. Analytical reversed-phase HPLC was performed on an Applied Biosystems 140B solvent delivery system equipped with an Hewlett-Packard Series 1050 detector, using a Vydac C-18 narrowbore column (5 μm, 2.1 × 150 mm). Preparative reversed-phase HPLC was carried out on a Waters Delta Prep 4000 system using a Vydac C-18 column (15−20 μm, 50 × 250 mm). Solvent A for HPLC was water containing 0.1% TFA; solvent B was 90% ACN containing 0.09% TFA. Analytical anion-exchange chromatography was performed on a Pharmacia FPLC system using a Mono-Q HR5/5 column. Buffer A was 20 mM NH_4-Cl, pH 9.1; buffer B was A plus 100 mM NaCl. Mass spectrometry analysis was carried out on a PE Sciex API-III quadrupole electrospray ionization mass spectrometer.

Stepwise Synthesis of (6−56)OMTKY3. (6−56)OMTKY3 was synthesized in stepwise fashion on Boc-Cys-(4-CH_3Bzl)OCH_2-Pam-resin on a custom-modified ABI 430A peptide synthesizer, using the published *in situ* neutralization/HBTU activation protocol for Boc chemistry.[24] Boc-amino acids were used with the following side chain protection: Arg(Tosyl), Asn(Xanthyl), Asp(OcHxl), Cys(4MeBzl), Glu(OcHxl), His(DNP), Lys(2ClZ), Ser(Bzl), Thr(Bzl), Tyr(BrZ). Coupling yields were quantitated by the standard ninhydrin assay.[33] After removal of (His)-DNP (in DMF containing 10% DIEA and 20% β-mercaptoethanol) and *N*-Boc groups (in 100% TFA), peptides were fully deprotected and simultaneously cleaved from the resin by treatment with HF containing 5% *p*-cresol/*p*-thiocresol (1:1) for 1 h at 0 °C. The crude peptide was purified by preparative reversed-phase HPLC using a gradient of 25−40% B over 60 min at 30 mL/min. The fractions of correct mass were pooled and lyophilized for protein folding.

Synthesis of (6−23)αCOSH and (24−56)OMTKY3. The peptide (6−23)αCOSH was synthesized manually by stepwise SPPS methods using the same protocol as used for Boc chemistry discussed above. The synthesis was carried out on Boc-Leu-(thioester linker)-aminomethyl-resin, where the Boc-Leu-(thioester linker) was prepared according to Canne et al.,[27] and was subsequently coupled to the aminomethyl-resin. Peptides were deprotected and cleaved in HF containing 5% anisole for 1 h at 0 °C. After removal of HF, the crude peptide was precipitated in cold anhydrous Et_2O, and dissolved in 40% B HPLC solvent and then lyophilized.

The C-terminal segment (24−56)OMTKY3 was also made by manual stepwise solid-phase synthesis on Boc-Cys-(4-CH_3Bzl)OCH_2-Pam-resin using the same *in situ* neutralization/HBTU protocol.[24] After deprotection and cleavage by HF plus 5% *p*-cresol/*p*-thiocresol (1:1) for 1 h at 0 °C, the crude peptide was purified on the preparative C18 reversed-phase HPLC column running a gradient of 25−40% B over 60 min at 30 mL/min. The fractions of correct mass were collected and lyophilized for ligation.

Native Chemical Ligation. (6−23)αCOSH was dissolved, at 6−7 mg/mL, in 0.1 M acetate and 6 M GuHCl, pH 4.0, followed by the addition of benzyl bromide (4 μL/mL). The reaction was allowed to proceed with stirring for 30 min, and the reaction mixture was then loaded onto the preparative C18 reversed-phase HPLC column (30−55% B over 60 min at 30 mL/min). Fractions containing the desired benzyl thioester peptide, (6−23)αCOSBzl, were pooled and lyophilized. The ligation of (6−23)αCOSBzl and (24−56)OMTKY3 was carried out as described.[18,20] In brief, equal molar amounts of (6−23)αCOSBzl and (24−56)OMTKY3 were dissolved, at a total concentration of about 8 mg/mL, in 0.1 M phosphate buffer containing 6 M GuHCl, pH 7.5, to which 1% benzyl mercaptan and 3% thiophenol (v/v) had been added. The ligation reaction was allowed to proceed to completion with stirring for 36 h. Excess DTT was added to the reaction solution to reduce partially oxidized ligated product before it was loaded onto the preparative C18 reversed-phase HPLC using 25−40% B over 60 min at 30 mL/min. The desired fractions were pooled and lyophilized for protein folding.

Protein Folding and Purification. (6−56)OMTKY3 in the reduced form was dissolved at 1.2 mg/mL in 0.6 M Tris/HCl, 6 M GuHCl, and 6 mM EDTA, pH 8.7. Protein refolding was initiated by a rapid 6-fold dilution with water. The solution was gently stirred in an open-air container for 8 h before being loaded onto a methylchymotrypsin-Sepharose affinity column. After washing with 0.2 M NaCl, active (6−56)OMTKY3 was eluted with 0.01 M HCl and 0.2 M NaCl. The bound fraction was then desalted on a Sephadex G-25 column and lyophilized for further characterization.

Thermal Denaturation. Thermal denaturation of OMTKY3 was carried out in 50 mM KOAc, pH 4.3, on a Perkin-Elmer LS50 spectrofluorimeter equipped with an automated temperature control unit. Fluorescence change induced by the rupture of the $Tyr^{31}\cdots Asp^{27}$ hydrogen bond upon heating was monitored at an excitation wavelength of 275 nm and an emission wavelength of 305 nm, with constant stirring of about 100 μg of sample in a 3-mL stoppered cuvette. Heating, integrating, data sampling, and processing were fully automated. Melting temperatures (T_m) were obtained by nonlinear least squares analysis using Enzfitter with an accuracy of ±1 °C, and data were then normalized on the basis of the fraction of denaturation.

Inhibitory Activity Assays. The inhibitor−enzyme association equilibrium constants were measured on a Hewlett-Packard HP8450A spectrophotometer by a modified version of the Green and Work method.[3,34] The experiment was carried out at 21 ± 1 °C at pH 8.3 in 0.1 M Tris/HCl buffer containing 0.02 M $CaCl_2$ and 0.005% Triton X-100. The dynamic range of K_a values that can be measured is from 10^3 to 10^{12} with an accuracy of ±20%.

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(33) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147−157.

(34) Green, N. M.; Work, E. *Biochem. J.* **1953**, *54*, 347−352.