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Cyclic peptides as proteases: A reevaluation

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ABSTRACT **A recent report [Atassi, M. Z. and Manshouri, T. (1993) *Proc. Natl. Acad. Sci. USA* 90, 8282–8286] described the design and synthesis of two 29-amino acid cyclic peptides that were reported to hydrolyze both ester and amide bonds with chymotrypsin-like or trypsin-like specificity. We have synthesized the trypsin-mimic peptide (TrPepz) and detect no activity toward either ester or peptide substrates. The same result was independently obtained by Wells *et al.* [Wells, J. A., Fairbrother, W. J., Otlewski, J., Laskowski, M., Jr., & Burnier, J. (1994) *Proc. Natl. Acad. Sci. USA* 91, 4110–4114.] Additionally, we found that Atassi and Manshouri failed to obtain accurate kinetic constants for trypsin- and chymotrypsin-catalyzed ester hydrolysis because the high concentrations of trypsin and chymotrypsin that they report using would have prevented evaluation of initial rates. These findings are incompatible with the claims, reported by Atassi and Manshouri, that TrPepz has trypsin-like activity.**

A recent report (1) has appeared describing two synthetic peptides of 29 amino acids, one designed to function like trypsin (TrPepz) and the other designed to function like chymotrypsin (ChPepz). These peptides were reported to hydrolyze esters with kinetic parameters that are remarkably similar to those obtained with native trypsin or chymotrypsin. TrPepz specifically hydrolyzed only trypsin substrates, ChPepz hydrolyzed only chymotrypsin substrates, and both cyclic peptides hydrolyzed the amide linkages of peptides and folded proteins. If true, these results would represent a major breakthrough in the field of protein design. However, several aspects of the reported experimental results and procedures led us to question the correctness of the findings. For example, the measured K_m values for ester hydrolysis by the cyclic peptides were virtually identical to the values Atassi and Manshouri (1) obtained for native trypsin or chymotrypsin. Such similarity is remarkable, as the cyclic peptides lack a defined S1 specificity pocket, subsite interactions, an oxyanion hole, and a rigid structure to spatially and electronically organize the catalytic triad (2). The lack of these structural features makes the observed hydrolysis of peptide amide linkages, which are much more stable than esters, an even more extraordinary result. We have attempted to reproduce the results described by Atassi and Manshouri (1) and have been unable to do so. We synthesized the TrPepz and found that it had no activity against either the ester or peptide substrates. Furthermore, the kinetic data reported by Atassi and Manshouri (1) for the hydrolysis of esters by trypsin and chymotrypsin differ strikingly from the values that we and others (3, 4) have obtained for these substrates.

MATERIALS AND METHODS

Peptide Synthesis and Analysis. Peptide TrPepz (CGYHFGGSDGQGSDGGVSWGLGGDGAAHC) was obtained from Rainin (Woburn, MA) and was prepared on a Symphony Multiplex peptide synthesizer using fluoren-9-ylmethoxycarbonyl chemistry. Mass spectral data were acquired by a VG Analytical (Manchester, England) 30-250 Quadrapole mass spectrometer using a standard VG Analytical electrospray source. Data were transformed using the manufacturer's standard algorithms. Automated Edman degradation was conducted by an Applied Biosystems model 477A amino acid sequencer with an online model 120A phenylthiohydantoin amino acid analyzer using standard manufacturer's programming and chemicals. The molecular weight of the peptide was confirmed by mass spectral analysis as 2711.38 ± 0.09 (Fig. 1*A*). A partial peptide sequence using 2 nmol of acyclic peptide confirmed the correct identity and location of every amino acid with the exception of the terminal cysteines and the tryptophan, which did not appear. Gln-11 was shown to be present at the correct position in >85% yield, indicating that deamination was not a significant problem. The crude peptide was analyzed by reverse-phase HPLC and was shown to be one peak with a broad tailing shoulder. HPLC was performed using a C_{18} Microsorb 5-μm 300-Å reverse-phase column (4.6 mm × 25 cm) (Rainin) using a 10–50% gradient of 0.1% trifluoroacetic acid in doubly distilled water (buffer A) and 0.08% trifluoroacetic acid in 95% (vol/vol) acetonitrile/5% doubly distilled water (buffer B). The presence of peptide was followed at 218 nm.

Peptide Cyclization. Cyclization was performed similarly to the procedure described by Atassi and Manshouri (1). A portion (40 mg) of the synthetic peptide was dissolved in 1.0 ml of 8.0 M urea containing 5% (vol/vol) 2-mercaptoethanol, preadjusted to pH 8.5 with triethylamine. The solution was agitated gently on a Vortex Genie for 3 h and then applied to a column (18 × 2.5 ml) of Sephadex G-15 (Pharmacia). Fractions (1 ml) were collected and the fractions that contained the peptide were identified by the appearance of an absorbance at 280 nm and by reactivity with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma) (5). A second more-intense DTNB-reactive peak occurred several fractions after the initial peak and contained 2-mercaptoethanol. To minimize the possibility that residual 2-mercaptoethanol would interfere with the subsequent oxidation only the first six peptide-containing fractions were retained. These initial fractions (which contained >50% of the UV-absorbent material) were pooled and diluted with 100 ml of 0.025 M acetic acid. This solution was adjusted to pH 8.0 with triethylamine. We chose to dilute the peptide in 100 ml rather than 3 liters because the smaller volume was more in line with published procedures (6) used previously to cyclize similar peptides by (D.R.C.). The solution was stirred in the dark for 3 days and the disappearance of free thiol was periodically assayed by titration with DTNB. When no more DTNB-reactive material remained, the material was lyophilized. Nineteen milligrams of peptide was recovered. A 500-μg portion was purified by

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Abbreviations: TrPepz, peptide designed to mimic the active site of trypsin; ChPepz, peptide designed to mimic the active site of chymotrypsin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TAME, *N*-tosyl-L-arginine methyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester.

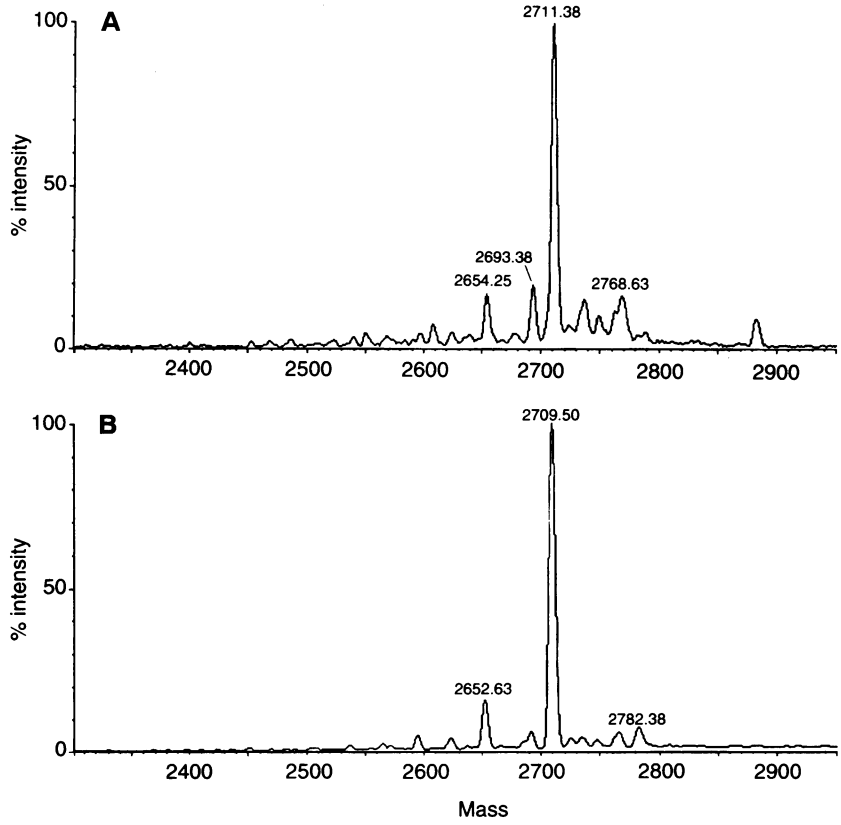

FIG. 1. Mass spectral analysis of HPLC-purified acyclic (*A*) and oxidized (*B*) TrPepz.

HPLC as described above to yield 280 μg of peptide. The molecular weight as determined by mass spectral analysis was 2709.50 ± 0.10, consistent with an intramolecular cyclization having formed the disulfide (Fig. 1*B*). The material was stored dry in a desiccated chamber at −20°C or was stored dissolved in 0.025 M acetic acid at 4°C.

Ester Hydrolysis. Spectrophotometric assays were performed on a Beckman DU 650 using quartz cuvettes with the standard 1-cm path length. All assays were done in 50 mM Tris·HCl, pH 8.0/10 mM $CaCl_2$ at 25°C. *N*-Tosyl-L-arginine methyl ester (TAME) hydrolysis by trypsin or TrPepz was followed by monitoring the increase in absorbance at 244 nm and chymotrypsin hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) was followed by monitoring the increase in absorbance at 256 nm. Extinction coefficients of 0.00059 $\mu M^{-1} \cdot cm^{-1}$ and 0.00081 $\mu M^{-1} \cdot cm^{-1}$ for TAME and BTEE, respectively, were used to calculate k_{cat}. The reaction rates were linearly dependent on the concentration of enzyme for trypsin or chymotrypsin between 2 and 40 nM. For K_m determination, a range of TAME concentrations from 5 to 200 μM and a range of BTEE concentrations between 25 and 200 μM were tested for hydrolysis by trypsin or chymotrypsin, respectively. The data were collected at several enzyme concentrations. The peptide was measured for activity at TAME concentrations between 100 and 1000 μM. The peptide concentration range tested for activity was 0.03–35 μM. Substrates were purchased from Aldrich, and bovine trypsin and chymotrypsin were purchased from Sigma. Kinetic constants were calculated using the k_{cat} program (BioMetallics, Princeton, NJ).

Peptide Hydrolysis. Peptide hydrolysis was performed as described (7). Peptides YLVGPKGFFYDA (390 μM) or YLVGPRHFFYDA (148 μM) were treated with TrPepz (33 μM) in 50 mM Tris·HCl, pH 8.0/20 mM $CaCl_2$/100 mM NaCl (TN100 buffer). The mixtures were incubated at 37°C for 48 h and then analyzed by C_{18} reverse-phase HPLC as described above.

Active-Site Titration. A stock (5 mg/ml) solution of 4-methylumbelliferyl *p*-guanidinobenzoate (8) was prepared in water. The stock was diluted 1:5 in TN100 buffer and 10 μl of the dilution was added to 990 μl of TN100 buffer in a fluorometer cuvette. Fluorescence was measured on a Perkin–Elmer LS 50b with an excitation wavelength of 330 nm and an emission wavelength of 445 nm. A background rate was observed for 1 min, after which peptide was added and the monitoring was continued for an additional 9 min. Trypsin was titrated during separate runs to ensure that the assay was functioning properly.

RESULTS

We obtained K_m values for the hydrolysis of TAME (K_m = 9.4 μM) by trypsin and for the hydrolysis of BTEE (K_m = 29 μM) by chymotrypsin, which were 280- and 40-fold lower, respectively, than the values reported by Atassi and Manshouri (1) (Table 1). While 2- to 3-fold differences in the measured K_m for an enzyme from one publication to the next are common, this discrepancy is too large to be explained by simple experimental variation. Our results (Table 1) are in close agreement with previously reported values for trypsin-catalyzed hydrolysis of TAME (3) and for chymotrypsin-catalyzed BTEE hydrolysis (4). We found that the rates for the hydrolysis of TAME or BTEE by trypsin or chymotrypsin, respectively, were linear over a range of enzyme concentrations from 2 to 40 nM, in agreement with previous reports (9). Enzyme concentrations above this range exhaust the substrate in less than a minute, precluding measurement of the initial rate of the reaction (Fig. 2) and, therefore, these concentrations cannot be used to determine accurate kinetic constants. This problem is exacerbated at substrate concentrations below or near the K_m. For those substrate concen-

Table 1. Comparison of kinetic constants for ester hydrolysis

	Atassi and Manshouri (1)		Literature values (3, 4)		This work	
	K_m, μM	k_{cat}, sec^{-1}	K_m, μM	k_{cat}, sec^{-1}	K_m, μM	k_{cat}, sec^{-1}
Trypsin	2560 ± 160	221 ± 9.7	12.5	60	9.4 ± 1.4	39 ± 1.1
Chymotrypsin	1070 ± 160	185 ± 10.3	22	86	29 ± 5.4	21 ± 1.0
TrPepz	2420 ± 90.0	85 ± 2.6	—	—	ND	ND
ChPepz	1111 ± 150	147 ± 8.5	—	—	—	—

Data represent reported hydrolysis of TAME by trypsin and TrPepz and reported hydrolysis of BTEE by chymotrypsin and ChPepz. ND, not detectable under conditions where we could have detected a rate 10^5-fold less than that of trypsin. —, Not available.

trations, we had to use the low end of our enzyme concentration range to prevent substrate depletion in the time frame of the assay.

Atassi and Manshouri (1) collected data at 800 nM trypsin or chymotrypsin, far above the range where accurate detection of initial rates is possible. For example, at a substrate concentration of 5 μM (50% of K_m for trypsin-catalyzed hydrolysis of TAME), 800 nM enzyme represents 16% of the available substrate. Thus, after a single turnover, taking 25 msec, the substrate concentration will be decreased by 16%. This assay condition is clearly not compatible with a major assumption used in the derivation of the Michaelis–Menten equation (10) (e.g., that the enzyme concentration is much less than the substrate concentration). Additionally, 100% substrate depletion will occur after only 156 msec, a time scale that could only be measured by stopped-flow techniques.

The trypsin analog peptide TrPepz was synthesized. Peptide sequencing in conjunction with mass spectral analysis (Fig. 1*A*) indicated that the linear peptide was identical to that described by Atassi and Manshouri (1). The peptide was cyclized similarly to the method described by Atassi and Manshouri (1). Mass spectral analysis indicated a molecular weight of 2709.50 ± 0.10 (Fig. 1*B*), consistent with the loss of two protons through intramolecular oxidation of the cysteines. No multimeric forms were observed, although a species with a molecular weight of 2652.63 ± 0.19 is indicative of the presence of a minor product lacking one glycine.

We assayed cyclized TrPepz both before and after HPLC purification and found no detectable activity above the background rate for a matrix of substrate concentrations ranging from 0.1 to 1.0 mM TAME and peptide concentrations ranging from 0.03 to 35 μM. We observed a significant rate of background hydrolysis at concentrations >0.5 mM TAME, including an initial rapid rise in absorbance of ≈0.01 unit in the first 30 sec followed by a slow increase in absorbance ranging from 0.0005 to 0.004 unit/min at 0.5 mM TAME. We typically followed the reactions for 1–5 min but waited as long as 80 min without seeing any change in absorbance above background for TrPepz. During this experiment we could have readily detected an activity that was 10^5-fold lower than that displayed by trypsin. Additionally, cyclized TrPepz failed to react with 4-methylumbelliferyl *p*-guanidinobenzoate, a standard titrant that rapidly reacts with the active site serine of wild-type trypsin (<5 sec) and will even titrate trypsin lacking both His-57 and Asp-102[8].

Importantly, Atassi and Manshouri (1) reported no evidence that they had an activity that was dependent on peptide concentration; e.g., they did not demonstrate that the rate of the reaction was linearly dependent on the concentration of cyclic peptide. Without this data we cannot conclude that they measured anything other than background hydrolysis or artefactual hydrolysis by contaminating hydrolases.

Atassi and Manshouri (1) report that TrPepz hydrolyzes both peptides and folded proteins, although they again fail to show a dependence of hydrolysis on the concentration of TrPepz. We assayed cyclized TrPepz with short arginine- or lysine-containing peptides (see *Materials and Methods* for sequence information) and were unable to detect any hydrolysis over a 48-h period. Trypsin is able to process these same peptides with turnover rates of 100–140 min^{-1}. We also note that if the cyclic peptides were peptidases, it would be surprising that the ChPepz peptide, which contains two phenylalanines, would be stable during the extended incubation at pH 8.0 required to form the disulfide or during the extended peptide or protein digestion reported by Atassi and Manshouri (1) since chymotrypsin rapidly cleaves TrPepz, which possesses similar sites for hydrolysis.

DISCUSSION

If the data and conclusions of Atassi and Manshouri (1) are correct, our understanding of proteolysis and the importance

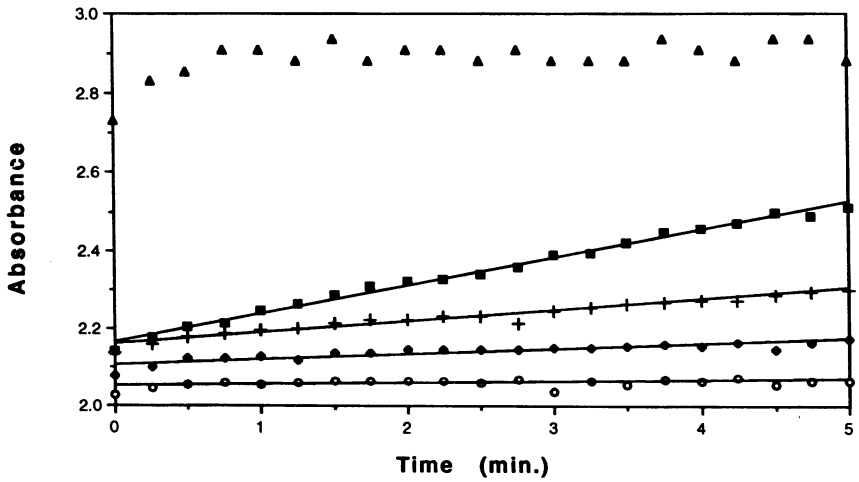

FIG. 2. Dependence of the reaction rate for TAME hydrolysis (1 mM TAME) by trypsin on trypsin concentration. ○, No trypsin; ●, 8 nM trypsin; +, 16 nM trypsin; ■, 32 nM trypsin; ▲, 800 nM trypsin.

and evolution of enzyme structure would be forced to undergo a revolutionary change. We have attempted to repeat their experiments and have been unable to detect the catalysis of either ester or amide bonds by a cyclic peptide. In striking contrast to the high activity of TrPepz reported by Atassi and Manshouri (1) (Table 1), we estimate that if the TrPepz displays any activity it would be at least 10^5-fold lower than that of trypsin. Moreover, our results indicate that the assays performed by Atassi and Manshouri (1) for trypsin and chymotrypsin toward ester substrates could not have yielded accurate data because the concentration of protease was much too high to allow the determination of initial rates. Failure of this relatively straightforward experiment must inevitably cast doubt on the reproducibility of their other results. Moreover, belief in the reported catalysis by TrPepz and ChPepz requires reconciliation of contradictory results (e.g., high reactivity toward chemically stable peptides and folded proteins, yet no reactivity toward reactive titrants and no reactivity for ChPepz toward itself). The evidence presented by Atassi and Manshouri (1), coupled with the negative findings from our laboratory and others (11), does not appear to justify this. As a result, we must conclude that hydrolysis of ester and amide linkages by cyclic peptides is unproven.

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