ACS Medicinal Chemistry Letters

Letter

pubs.acs.org/acsmedchemlett

Synthesis of Aspartame by Thermolysin: An X-ray Structural Study

Gabriel Birrane,*[,†] Balaji Bhyravbhatla,[‡] and Manuel A. Navia*[,§,∥]

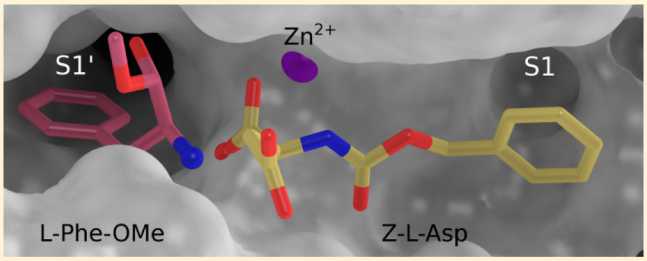
[†]Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, United States

[‡]Xtal BioStructures, 12 Michigan Drive, Natick, Massachusetts 01760, United States

[§]Altus Pharmaceuticals, Waltham, Massachusetts 02451, United States

Supporting Information

ABSTRACT: Protease mediated peptide synthesis (PMPS) was first described in the 1930s but remains underexploited today. In most PMPS, the reaction equilibrium is shifted toward synthesis by the aqueous insolubility of product generated. Substrates and proteases are selected by trial and error, yields are modest, and reaction times are slow. Once implemented, however, PMPS reactions can be simple, environmentally benign, and readily scalable to a commercial level. We examined the PMPS of a precursor of the artificial sweetener aspartame, a multiton peptide synthesis catalyzed by the enzyme thermolysin. X-ray structures of thermolysin in complex with aspartame substrates separately, and after PMPS in a crystal, rationalize the reaction's substrate preferences and reveal an unexpected form of substrate inhibition that explains its sluggishness. Structure guided optimization of this and other PMPS reactions could expand the economic viability of commercial peptides beyond current high-potency, low-volume therapeutics, with substantial green chemistry advantages.



KEYWORDS: *Protease mediated peptide synthesis, aspartame, crystallography, thermolysin*

Bergmann and Fraenkel−Conrat reported the first protease-mediated synthesis of a peptide bond in 1938.[1] Their elegantly simple procedure, catalyzed by the enzyme papain, led to a precipitate that provided a 22% yield of benzoyl-L-leucyl-L-leucine anilide but required a reaction time of 2 days. Protease-mediated peptide condensation reactions subsequently described in the literature[2] have been hindered similarly by excessive reaction times, trial and error manipulation of proteases and reaction conditions, and characteristically poor binding affinity of the unnatural, modified substrates used. As such, it has been difficult to exploit this approach. However, once a reaction is optimized,[3] its relative ease of scalability and benign environmental impact make PMPS commercially attractive in spite of its shortcomings, when compared to other large-scale peptide synthesis strategies.[4,5]

Aspartame (APM) is a protected dipeptide (L-aspartyl-L-phenylalanine methyl ester) that is widely used as a low-calorie sweetener.[6] More than 19 000 metric tons of APM are produced per year,[7] making it the most highly synthesized peptide in the world. Over 2000 metric tons of the annual output of APM is made enzymatically,[8] using the protease thermolysin (TLN) to catalyze the condensation of the chiral aspartame-precursor, carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZAPM), from the protected amino acid substrates carbobenzoxy-L-aspartic acid (ZA) and L-phenylalanine methyl ester (PM).[3,7−9] Deprotection of ZAPM by catalytic hydrogenation yields aspartame as the final product.[3,9] In the ZAPM precursor reaction, TLN is enantioselective for the desired L-phenylalanine methyl ester substrate from a racemic mixture of DL-phenylalanine methyl ester. In contrast, although both enantiomers of ZA can bind to TLN, only carbobenzoxy-L-aspartic acid is used in practice since carbobenzoxy-D-aspartic acid inhibits the enzyme.[10]

Isowa and collaborators discovered the synthesis of ZAPM by TLN after a systematic evaluation of the enzyme-mediated coupling of PM to a series of N-protected L-aspartic acid analogues.[3] Subsequently, a mechanism of action for the TLN-mediated synthesis of ZAPM in aqueous solvent was proposed by Oyama et al.;[10] this accounts for the rate saturation of the reaction observed with increasing concentration of the carboxy-donor substrate ZA and the linear rate increase of the reaction within the concentration range explored for the amino-donor substrate PM. These results were confirmed by Wayne and Fruton[11] and extended to biphasic aqueous−organic solvent mixtures[12] and organic solvents[13] by Nakanishi et al. The enzyme data obtained by these groups, summarized in Table 1, illustrate the poor binding of the substrates to thermolysin and the sluggishness with which the reaction proceeds. These characteristics make it difficult to study the reaction using conventional steady-state kinetic methods.

The TLN used in the synthesis of ZAPM is a heat stable extracellular Zn^{2+} metalloprotease (EC 3.4.24.4) that is readily isolated by direct crystallization from microbial extracts of *Bacillus thermoproteolyticus*.[14] As such, it is both plentiful and

Received: March 10, 2014
Accepted: April 10, 2014
Published: April 10, 2014

 © 2014 American Chemical Society

Table 1. Enzyme Parameters for the Synthesis of ZAPM by TLN As Obtained from the Literature

ref	solvent system	K_m (mM) ZA	K_m (mM) PM	k_{cat}/K_m (mM^{-1} min^{-1})
Oyama et al.[10]	aqueous[a]	n/d	10	0.160
Wayne and Fruton[11]	aqueous[a]	300	10	0.160
Nakanishi[12]	biphasic aq/org[b]	250	2.8	0.142

[a]The parameters reported by Oyama et al.[10] and Wayne and Fruton[11] were derived from a modified Michaelis–Menten model applied to data collected in aqueous solvent. [b]Nakanishi et al.[12] derived their enzyme parameters from data measured in a biphasic aqueous/organic solvent mixture. At the higher end of the ZA concentration range explored, a slight rate reduction vs ZA concentration was observed.[12]

inexpensive. High quality X-ray structures of TLN are available, in complex with both substrates[15−17] and inhibitors.[18−20] These provide a firm basis for understanding the mechanism of peptide hydrolysis used by the enzyme,[17,21] as well as the probable geometry of substrate binding.[20]

Here, we report the X-ray structures of TLN in complex with the amino- and carboxy-donor substrates PM and ZA individually, as well as with both substrates together. Earlier studies[22,23] with cross-linked microcrystalline TLN enzyme suspensions[24] showed that the latter conditions would lead to the synthesis of a ZAPM precipitate, whose chemical analysis has been described in detail.[23] On the basis of our new crystallographic data (Table S1 in the Supporting Information) and the existing literature (Table 1), we propose a mechanism of action for the synthesis of ZAPM by TLN that explains the sluggish reaction rate observed and provides a solid foundation to improve the efficiency of this commercially important process.

The crystal structures determined in this study (Table S1 in the Supporting Information) all show strong ligand electron density in the TLN active site (Figure 1) that is readily interpretable in chemical terms. In the structure of the amino-donor PM in complex with TLN, a PM molecule occupies the active site of TLN (Figure 1a) in a conformation that is consistent with that expected for an amino-donor substrate in a TLN-mediated peptide synthesis reaction. This productive conformation can be inferred, for example, from the structure of the amino-donor side (S_1'–S_2')[25] of the potent (K_i = 0.068 nM) phosphonamidate transition state analogue inhibitor carbobenzoxy-L-phenylalanine-phosphonamidate-L-leucyl-L-alanine (ZF^PLA)[26] in its complex with TLN.[20] The structures of ZF^PLA and PM in their respective complexes with TLN (Figure 2a), highlight the close correspondence between the position and directionality of the phosphonamidate nitrogen of ZF^PLA and the free amino group of PM. Given an appropriately configured carboxy-donor as a cosubstrate, it would be structurally reasonable for the amino-donor PM to participate in the formation of a tetrahedral intermediate within the ZAPM peptide coupling reaction (Scheme 1). In addition to the amino donor site, two molecules of PM occupy interpretable electron density adjacent to a crystal packing contact between TLN molecules. These molecules are 5.3 Å away from each other in exposed sites on the surface of the protein (Figure S1 in the Supporting Information).

In the structure of the carboxy-donor ZA in complex with TLN (Figure 1b), ZA unexpectedly occupies the same binding site as the amino-donor substrate PM (Figure 2b). The

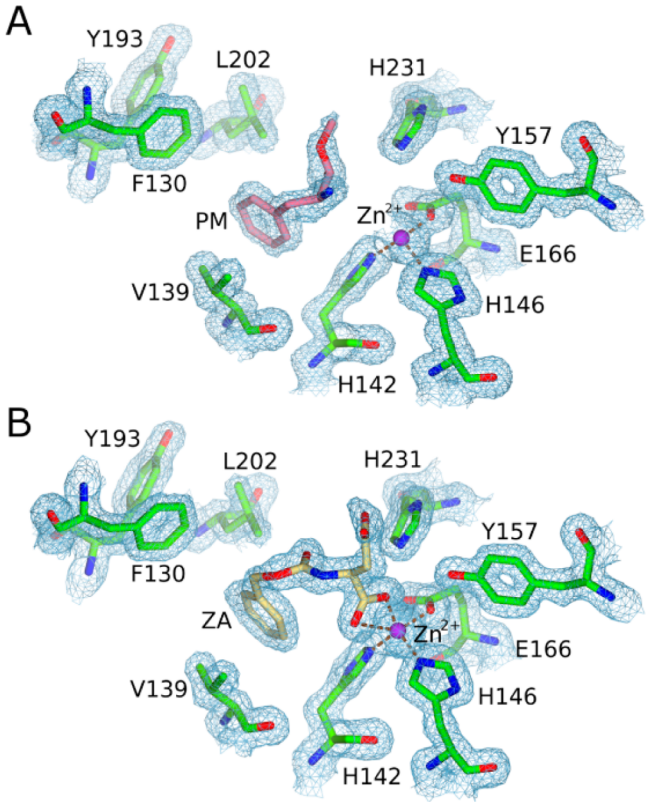


Figure 1. Structure of the active site region of thermolysin in complex with L-phenylalanine methyl ester (PM) and carbobenzoxy-L-aspartic acid (ZA). σ_A-weighted $2F_o - F_c$ electron density maps are contoured in blue at 1.5σ. Key residues in the active site of TLN[14−19] are identified (green). Nitrogen and oxygen atoms are shown in blue and red, respectively. The active site zinc atom is purple. (A) PM (pink) occupies the expected position for an amino-donor substrate in the peptide coupling reaction.[19−21,26] (B) Unexpectedly, the carboxy-donor ZA (khaki) occupies the same amino-donor site as does PM in panel a.

bidentate binding of the α-carboxyl of ZA to the TLN active site zinc closely resembles (Figure 2c) the interaction observed when TLN interacts with the potent (K_i = 50 nM) inhibitor *N*-(1-carboxy-3-phenyl-propyl)-L-leucyl-L-tryptophan (CLT).[19] The strong bond between CLT and TLN suggests that the intrinsic inhibitory potency of ZA vs TLN is likely to be substantial.

When PM and ZA substrates were both added to a diffraction quality crystal of TLN in stabilizing solution, a fine precipitate formed over time in the crystallization vessel, reminiscent of that formed in the synthesis of ZAPM by a slurry of cross-linked microcrystalline TLN.[23] The resulting structure (Table S2 in the Supporting Information) showed difference electron density (data not shown) equivalent to that observed when the carboxy-donor substrate ZA bound at the amino-donor site (Figures 2b,c), consistent with the greater relative binding affinity of ZA for the enzyme (Table 1). The absence of electron density corresponding to the product ZAPM was consistent with its aqueous insolubility and precipitation out of solution, which drives this reaction.[3,9−13,23] Additional electron density adjacent to a crystal packing contact between TLN molecules corresponds to the two PM molecules identified in the TLN–PM complex above (Figure S1 in the Supporting Information). The two PM molecules bind approximately 12

dx.doi.org/10.1021/ml500101z | ACS Med. Chem. Lett. 2014, 5, 706−710

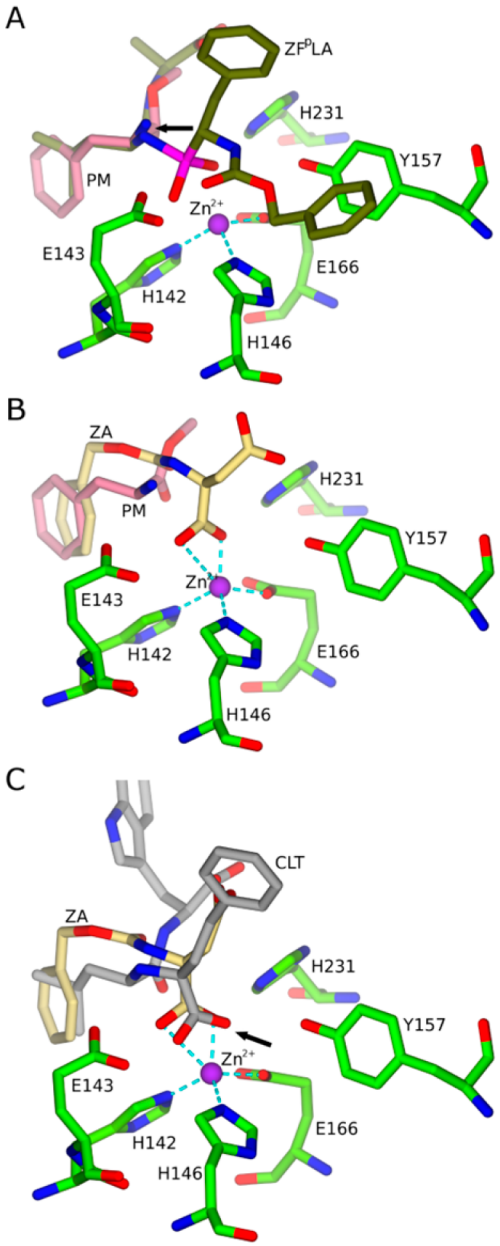
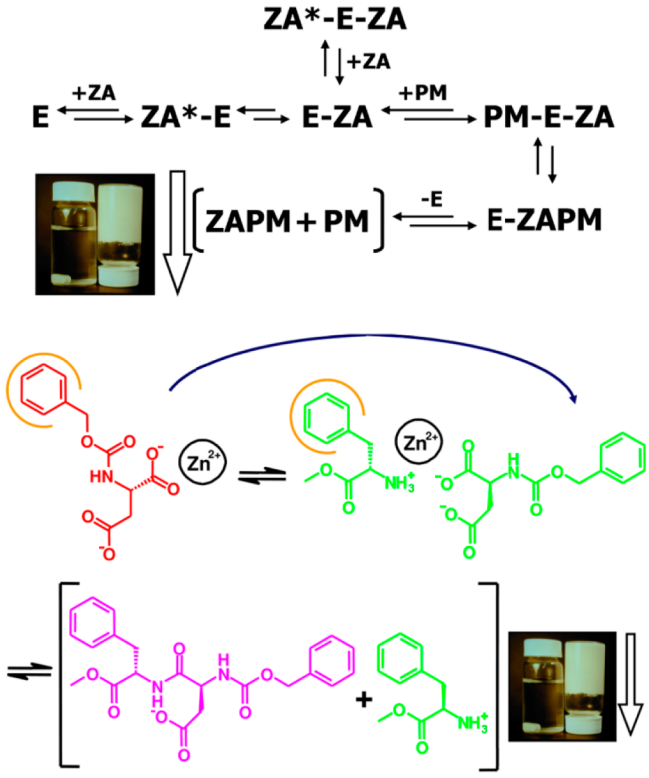


Figure 2. Binding of substrates and inhibitors to the active site of thermolysin. Residues E143, Y157, and H231 (green) represent the catalytic machinery of TLN, along with the active site zinc ion, which is held in place by residues H142, H146, and E166 (green). (A) Structure of PM (pink), superimposed on that of the transition state analogue inhibitor ZF^{P}LA[20,26] (PDB ID 4TMN, brown) after alignment of the TLN protein coordinates of the corresponding complex structures. The arrow highlights the close positional and orientational similarity between the amino group of PM and the phosphonamidate nitrogen of ZF^{P}LA. (B) Structure of PM (pink) superimposed on that of ZA (khaki) shows how both ligands occupy the same substrate amino-donor site on the TLN enzyme. (C) Structure of ZA (khaki) superimposed on that of the TLN inhibitor CLT[19,21] (PDB ID 1THL, gray). The α-carboxyl of ZA and the carboxyl of CLT both chelate the catalytic zinc ion of the enzyme in a bidentate inhibitory conformation (arrow).

and 16 Å away from the ZA molecules in the active site (Figure S2 in the Supporting Information).

Contrary to expectation, the carboxy-donor substrate ZA binds preferentially to the amino-donor site of enzyme (Figure

Scheme 1. Proposed Mechanism of Action for the Thermolysin-Catalyzed Synthesis of Aspartame Precursor Z-L-Asp-L-Phe-OMe (ZAPM)



2b) when it interacts with TLN (Figure 1b). Moreover, the structural similarity between ZA and CLT[19] (K_i = 50 nM) in their TLN complexes (Figure 2c) suggests that the binding affinity of ZA for the amino-donor site of TLN is likely to be substantial. This atypical form of substrate inhibition would not be detectable by steady-state enzyme kinetics alone. Absent the structural insights presented here, the cryptic competitive inhibition of PM substrate binding by ZA, would only show itself as a shift to higher apparent K_m and a slower overall reaction rate (k_{cat}). This profile is consistent with the poor binding and sluggish performance reported[10−12] (Table 1) for the TLN-mediated synthesis of ZAPM.

A further complication in this reaction is possible if, after bound ZA transfers to the carboxy-donor site of TLN (as in Figure 3), a second ZA molecule (ZA*) were to bind to the amino-donor site in competition with PM. This form of substrate inhibition[27] would manifest as a decrease in the steady-state rate of reaction at higher concentrations of ZA, which is consistent with the reaction inhibition profile reported by Nakanishi et al.[12]

In its conventional hydrolysis of peptides and proteins, TLN is modestly specific for residues like Ile, Leu, and Phe on the amino-donor (S1′) side of the scissile bond but will accept a broad spectrum of residues on the carboxy-donor (S1−S2) side.[19,21,28] This is consistent with the depth and texture of the S1′ specificity pocket (into which both PM and ZA bind preferentially; Figure 2b) and the contrasting lack of distinctive structural features on the enzyme surface corresponding to S1 and S2 on the carboxy-donor side (Figure 3). Tight, specific binding to the S1′ site alone is probably sufficient to properly orient a natural protein or extended peptide substrate for cleavage by TLN. However, in an unnatural peptide synthesis

dx.doi.org/10.1021/ml500101z | ACS Med. Chem. Lett. 2014, 5, 706−710

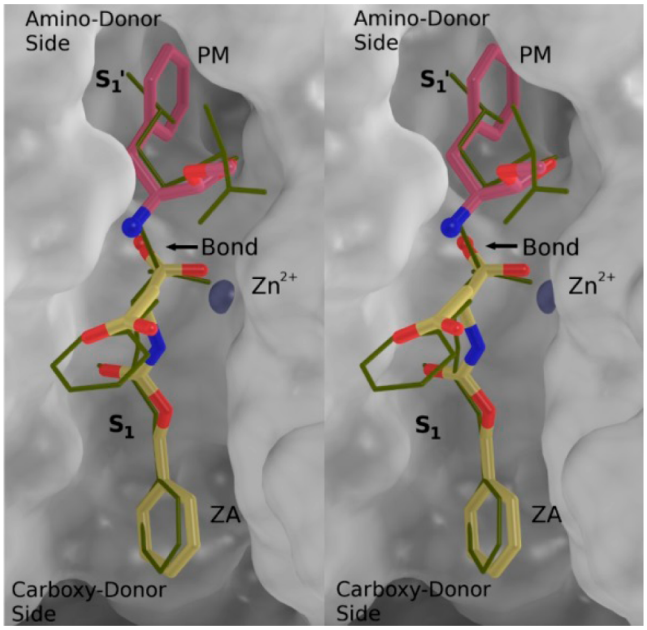


Figure 3. Stereo view of the active site of thermolysin. PM and ZA are shown bound to the amino-donor S_1' and carboxy-donor S_1–S_2 sites of the enzyme, respectively, in a hypothetical productive conformation for the TLN-catalyzed condensation of the ZAPM dipeptide product. PM binding is as shown in Figure 2a. In turn, the productive conformation of ZA is modeled using the atom-for-atom topological (though not chemical) equivalence between ZA and the C-terminal half of the transition state analogue inhibitor ZF^{P}LA. The structure of ZF^{P}LA in the active site of TLN[20] is shown in thin bonds colored brown and immediately suggests a structure for the transition state leading to the synthesis of the ZAPM product. All figures were made using POVSCRIPT[29] and ray-traced with POV-Ray (www.povray.org).

reaction such as TLN-mediated synthesis of ZAPM, the suboptimal carboxy-donor S1 and S2 substrate-binding site present a problem since both substrates must be accommodated and properly oriented in a catalytically productive configuration (as in Figure 3) for the reaction to proceed. This problem may be resolved in the reaction by channeling the initial binding of both substrates to TLN through the S1′ site on the amino-donor side of the active site.

On the basis of the enzyme–substrate complex structures reported here and the enzyme kinetics data from the literature summarized in Table 1, we propose a chemically reasonable mechanism of action for the TLN-catalyzed synthesis of the aspartame precursor ZAPM, as shown in Scheme 1. We hypothesize that the reaction is initiated by the preferential binding of the carboxy-donor substrate ZA to the amino-donor (S1′) side of the enzyme active site. While still tethered to the enzyme through its interaction with the catalytic zinc ion, ZA then rotates out of the amino-donor site to accommodate PM binding to the enzyme. No steric clashes are evident that would prevent ZA from sampling a productive carboxy-donor conformation (Figure 3) as it reorients within the TLN active site. Conversely, there do not appear to be any compelling interactions in the poorly defined carboxy-donor site that would appropriately orient ZA absent its initial binding to zinc. Once both substrates are productively bound to TLN, the enzyme can catalyze the formation of a tetrahedral intermediate, analogous in structure to the inhibitor ZF^{P}LA (Figure 3). Collapse of the intermediate would then lead to the formation of the product ZAPM, whose precipitation as the water-insoluble Phe-OMe salt drives the overall reaction in the direction of peptide synthesis.

In spite of their commercial importance, potentially broad applicability and technical simplicity, protease-catalyzed peptide synthesis reactions have not been adequately characterized, optimized, or broadly accepted in the practice of modern, large-scale, solution enzymology. The crystal structures described here highlight the degree of complexity that can accompany such unnatural reactions in which experimental conditions are artificially manipulated to achieve an economically viable, albeit enzymatically inefficient, result. These structures may also help to improve the aspartame precursor synthesis reaction directly, either by guiding the rational selection of alternate substrates, or by inferring modifications of the TLN enzyme (or other closely related enzymes)[30] for increased reaction efficiency. More broadly, these results lead one to consider the extent to which the inefficiency (and unpredictability) associated with this approach to peptide synthesis may be attributed to similarly encrypted substrate inhibition that might be correctable, once identified by more powerful methods like X-ray crystallography. Fortunately, many of the enzymes that have been used in the protease-mediated peptide syntheses documented in the literature[2] have themselves been the subject of extensive crystallographic study,[31] and these data may help predict how the enzymes will interact with various substrates. To prioritize the reactions that would benefit most from additional analysis by X-ray crystallography, equilibrium dialysis, or other direct ligand binding studies could be used to screen suboptimal peptide synthesis approaches, in the hope of improving the efficiency, and expanding the commercial applicability of protease-mediated peptide synthesis reactions.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, supplementary Table S1 and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The atomic coordinates and structure factors have been deposited in the Protein Data Bank as entries 3QGO, 3QH1, and 3QH5.

■ AUTHOR INFORMATION

Corresponding Authors

*(G.B.) Tel: (617) 667 0025. Fax: (617) 975 5243. E-mail: gbirrane@bidmc.harvard.edu.

*(M.A.N.) Tel: (781) 389 0686. Fax: (617) 357 7476. E-mail: mnavia@oxbio.com.

Present Address

‖(M.A.N.) Oxford Bioscience Partners, 535 Boylston Street, Suite 402, Boston, Massachusetts 02116, United States.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

Data for this study were measured at beamline X29 of the National Synchrotron Light Source. Financial support comes principally from the Offices of Biological and Environmental Research and of Basic Energy Sciences of the US Department of Energy, and from the National Center for Research

Resources (P41RR012408) and the National Institute of General Medical Sciences (P41GM103473) of the National Institutes of Health. Data were also collected at the Cornell High Energy Synchrotron Source (CHESS), Ithaca, NY, which is supported by the National Science Foundation and the National Institutes of Health/National Institute of General Medical Sciences under NSF award DMR-0936384.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Anne-Laure Dassier for her assistance during data collection and Dr. Paula Kuzontkoski (Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Boston, MA) for her editing recommendations.

■ ABBREVIATIONS

PMPS, protease mediated peptide synthesis; TLN, thermolysin; ZA, carbobenzoxy-L-aspartic acid; ZAPM, carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester; PM, L-phenylalanine methyl ester; APM, aspartame

■ REFERENCES

(1) Bergmann, M.; Fraenkel-Conrat, H. The enzymatic synthesis of peptide bonds. *J. Biol. Chem.* **1938**, *124*, 1−6.
(2) Fruton, J. S. Proteinase-catalyzed synthesis of peptide bonds. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1982**, *53*, 239−306.
(3) Isowa, Y.; Ohmori, M.; Ichikawa, T.; Mori, K.; Nonaka, Y.; Kihara, K.-I.; Oyama, K.; Satoh, H.; Nishimura, S. The thermolysin-catalyzed condensation reactions of N-substituted aspartic and glutamic acids with phenylalanine alkyl esters. *Tetrahedron Lett.* **1979**, *28*, 2611−2.
(4) Andersson, L.; Blomberg, L.; Flegel, M.; Lepsa, L.; Nilsson, B.; Verlander, M. Large-scale synthesis of peptides. *Biopolymers* **2000**, *55*, 227−50.
(5) Kelley, W. S. Therapeutic peptides: the devil is in the details. *Bio/Technology* **1996**, *14*, 28−31.
(6) Mazur, R. H.; Schlatter, J. M.; Goldkamp, A. H. Structure−taste relationships of some dipeptides. *J. Am. Chem. Soc.* **1969**, *91*, 2684−91.
(7) Yagasaki, M.; Hashimoto, S.-I. Synthesis and application of dipeptides; current status and perspectives. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 13−22.
(8) Broxterman, Q. B.; Boesten, W. J.; Schoemaker, H. E.; Schulze, B. DSM gets the biocatalysis bug. *Spec. Chem.* **1997**, *17*, 186−93.
(9) Meijer, E. M.; Boesten, W. J. H.; Schoemaker, H. E.; van Balken, J. In *Biocatalysis in Organic Synthesis*; Tramper, J. A. M., van der Plas, H. G., Linko, P., Eds.; Elsevier Science Publishers B. V.: Amsterdam, The Netherlands, 1985, 135−56.
(10) Oyama, K.; Kihara, K.; Nonaka, Y. On the mechanism of the action of thermolysin: kinetic study of the thermolysin-catalysed condensation reaction of *N*-benzyloxycarbonyl-L-aspartic acid with L-phenylalanine methyl ester. *J. Chem. Soc., Perkin Trans. 2* **1981**, *2*, 356−60.
(11) Wayne, S. I.; Fruton, J. S. Thermolysin-catalyzed peptide bond synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3241−4.
(12) Nakanishi, K.; Kimura, Y.; Matsuno, R. Kinetics and equilibrium of enzymatic synthesis of peptides in aqueous/organic biphasic systems. Thermolysin-catalyzed synthesis of *N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester. *Eur. J. Biochem.* **1986**, *161*, 541−9.
(13) Nakanishi, K.; Kamikubo, T.; Matsuno, R. Continuous synthesis of *N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester with immobilized thermolysin in an organic solvent. *Bio/Technology* **1985**, *3*, 459−64.
(14) Endo, S. The protease produced by thermophilic bacteria. *J. Ferment. Technol.* **1962**, *40*, 3346−3353.
(15) Colman, P. M.; Jansonius, J. N.; Matthews, B. W. The structure of thermolysin: an electron density map at 2.3 A resolution. *J. Mol. Biol.* **1972**, *70*, 701−24.
(16) Holmes, M. A.; Matthews, B. W. Structure of thermolysin refined at 1.6 A resolution. *J. Mol. Biol.* **1982**, *160*, 623−39.
(17) Holland, D. R.; Tronrud, D. E.; Pley, H. W.; Flaherty, K. M.; Stark, W.; Jansonius, J. N.; McKay, D. B.; Matthews, B. W. Structural comparison suggests that thermolysin and related neutral proteases undergo hinge-bending motion during catalysis. *Biochemistry* **1992**, *31*, 11310−6.
(18) Holland, D. R.; Hausrath, A. C.; Juers, D.; Matthews, B. W. Structural analysis of zinc substitutions in the active site of thermolysin. *Protein Sci.* **1995**, *4*, 1955−65.
(19) Monzingo, A. F.; Matthews, B. W. Binding of *N*-carboxymethyl dipeptide inhibitors to thermolysin determined by X-ray crystallography: a novel class of transition-state analogues for zinc peptidases. *Biochemistry* **1984**, *23*, 5724−9.
(20) Holden, H. M.; Tronrud, D. E.; Monzingo, A. F.; Weaver, L. H.; Matthews, B. W. Slow- and fast-binding inhibitors of thermolysin display different modes of binding: crystallographic analysis of extended phosphonamidate transition-state analogues. *Biochemistry* **1987**, *26*, 8542−53.
(21) Hangauer, D. G.; Monzingo, A. F.; Matthews, B. W. An interactive computer graphics study of thermolysin-catalyzed peptide cleavage and inhibition by *N*-carboxymethyl dipeptides. *Biochemistry* **1984**, *23*, 5730−41.
(22) Margolin, A. L.; Navia, M. A. Protein crystals as novel catalytic materials. *Angew. Chem., Int. Ed.* **2001**, *40*, 2204−22.
(23) Persichetti, R. A.; St. Clair, N. L.; Griffith, J. P.; Navia, M. A.; Margolin, A. L. Cross-linked enzyme crystals (CLECs) of thermolysin in the synthesis of peptides. *J. Am. Chem. Soc.* **1995**, *117*, 2732−7.
(24) St. Clair, N. L.; Navia, M. A. Cross-linked enzyme crystals as robust biocatalysts. *J. Am. Chem. Soc.* **1992**, *114*, 7314−6.
(25) Schechter, I.; Berger, A. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157−62.
(26) Bartlett, P. A.; Marlowe, C. K. Possible role for water dissociation in the slow binding of phosphorus-containing transition-state-analogue inhibitors of thermolysin. *Biochemistry* **1987**, *26*, 8553−61.
(27) Copeland, R. A. *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*; VCH Publishers: New York, 1996.
(28) Hersh, L. B.; Morihara, K. Comparison of the subsite specificity of the mammalian neutral endopeptidase 24.11 (enkephalinase) to the bacterial neutral endopeptidase thermolysin. *J. Biol. Chem.* **1986**, *261*, 6433−7.
(29) Fenn, T. D.; Ringe, D.; Petsko, G. A. POVScript+: a program for model and data visualization using persistence of vision ray-tracing. *J. Appl. Crystallogr.* **2003**, *36*, 944−7.
(30) Ogino, H.; Tsuchiyama, S.; Yasuda, M.; Doukyu, N. Enhancement of the aspartame precursor synthetic activity of an organic solvent-stable protease. *Protein Eng. Des. Sel.* **2010**, *23*, 147−52.
(31) Howard, A. J.; Gilliland, G. L.; Finzel, B. C.; Poulos, T. L.; Ohlendorf, D. H.; Salemme, F. R. Use of an imaging proportional counter in macromolecular crystallography. *J. Appl. Crystallogr.* **1987**, *20*, 383−7.