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Evaluation of the Ser-His Dipeptide, a Putative Catalyst of Amide and Ester Hydrolysis

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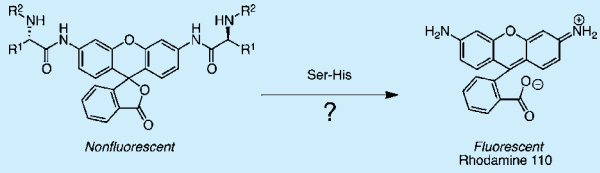
Supporting Information

ABSTRACT: Efficient hydrolysis of amide bonds has long been a reaction of interest for organic chemists. The rate constants of proteases are unmatched by those of any synthetic catalyst. It has been proposed that a dipeptide containing serine and histidine is an effective catalyst of amide hydrolysis, based on an apparent ability to degrade a protein. The capacity of the Ser-His dipeptide to catalyze the hydrolysis of several discrete ester and amide substrates is investigated using previously described conditions. This dipeptide does not catalyze the hydrolysis of amide or unactivated ester groups in any of the substrates under the conditions evaluated.



Enzymes constitute a source of ongoing fascination for chemists because of the catalytic efficiency observed for a wide array of reactions that cannot be comparably enhanced by synthetic catalysts. Amide hydrolysis is an instructive example. Many amide-cleaving enzymes are known (peptidases, proteases, deacetylases, etc.); these catalysts usually promote rapid amide hydrolysis under neutral or acidic conditions, at or near room temperature.[1] No synthetic catalyst has been convincingly shown to approach these capabilities, despite occasional claims to the contrary.[2] In the test tube, amide hydrolysis typically requires hours of heating in concentrated acid or base. The half-life of a typical amide bond in aqueous solution at room temperature, neutral pH, is 500 years.[3]

In 2000, Chen and co-workers reported that the dipeptide Ser-His (**1**, Figure 1A), a molecule selected because of the well-known roles of serine and histidine side chains in the serine hydrolase mechanism (Figure 1B), could catalyze amide bond hydrolysis (Figure 1).[4] This conclusion was drawn from experiments in which Ser-His was incubated with bovine serum albumin (BSA) in pH 6 buffer at 50 °C. The dipeptide–protein mixture was evaluated via polyacrylamide gel electrophoresis over time; within 6–12 h, the tight band arising from BSA was augmented by a "smear" with higher mobility that was attributed to BSA fragments generated via peptide bond hydrolysis. In addition, Ser-His was reported to promote the hydrolysis of *p*-nitrophenyl acetate (*p*-NPAc, **2**, Figure 1A) in pH 6 buffer, behavior that was interpreted to support the conclusion that Ser-His can catalyze amide hydrolysis.[4] This paper has been cited 81 times, and other researchers have sought to build on the Ser-His precedent in efforts to generate improved catalysts.[5]

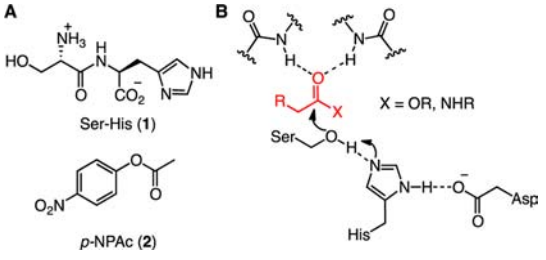


Figure 1. (A) Chemical structure of Ser-His dipeptide (**1**) and *p*-nitrophenyl acetate (*p*-NPAc, **2**); (B) catalytic triad and chemical mechanism for the hydrolysis of ester and amide bonds by serine hydrolases.

We were motivated to re-examine the activity of Ser-His because the original report lacked detailed product characterization for the BSA degradation studies, and to our knowledge there has been no subsequent study that provided incisive product characterization. Moreover, the conceptual and mechanistic links between amide bond hydrolysis and hydrolysis of an activated ester such as *p*-NPAc have been called into question.[6] We therefore evaluated Ser-His reactivity with a set of simple ester and amide substrates, under conditions reported by Chen and co-workers, as described below. No catalytic activity of Ser-His was detected for any amide or unactivated ester.

We first repeated the experiment of Chen and co-workers to evaluate Ser-His for the ability to catalyze ester hydrolysis using the chromogenic ester substrate *p*-NPAc (**2**). *p*-NPAc is a highly reactive ester because of the good leaving group (*p*-nitrophenolate; $pK_a \sim 7$ for the conjugate acid). This and other highly reactive esters such as 8-acetoxypyrene-1,3,6-trisulfonate have been historically very popular for efforts to discover

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biomimetic catalysts. These reactive substrates are still widely used for this purpose,[5] although some researchers have focused on more challenging substrates.[7] Many years ago, Menger and Ladika pointed out that enhancing reactive ester hydrolysis does not represent progress toward enzyme-like catalysis of more challenging reactions such as hydrolysis of amides or unactivated esters.[6]

Evaluation of Ser-His as a catalyst for *p*-NPAc hydrolysis at pH 6, as reported in ref 4, is complicated by the small extinction coefficient of *p*-nitrophenol (**3**, Figure 2A), which is

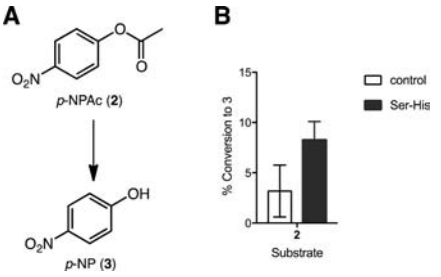

Figure 2. (A) Hydrolysis of *p*-NPAc (**2**) to *p*-NP (**3**); (B) extent of *p*-NPAc hydrolysis, as judged by optical absorbance, upon incubation with 10 mM Ser-His for 16 min at room temperature, 2 mM substrate, 40 mM Britton–Robinson buffer (40 mM phosphoric acid, 40 mM acetic acid, and 40 mM boric acid) titrated to pH 6 with NaOH. Control was the same reaction in the absence of the dipeptide. Reactions were monitored via absorbance at 400 nm in a M1000 PRO Microplate reader.

the dominant form of the leaving group under these conditions. A relatively high concentration of **2** (2 mM) was employed to obtain sufficient signal over background. We studied the reaction over 16 min, as reported in ref 4, and observed <10% conversion to product in the presence or absence of Ser-His (Figure 2B). This observation is in contrast to the published finding of much more extensive hydrolysis of **2** in the presence of 5 mM Ser-His under these conditions.[4] Our data showed no sign of an initial burst phase (see Supporting Information, Figure S6), which might have indicated rapid formation of an acylated Ser-His intermediate.

The original report stressed the importance of pH for the observation of catalysis with Ser-His.[4] We therefore investigated the hydrolysis of *p*-NPAc (**2**) to *p*-nitrophenol (**3**)/*p*-nitrophenolate in Britton–Robinson buffer at both pH 4 and pH 8, to complement our measurements at pH 6.[4] At pH 4 the reaction displayed less than 1% conversion to product in both the absence and presence of Ser-His (Figure S8). This result is not surprising because the histidine moiety should be fully protonated at pH 4. At pH 8, the data indicated a substantial difference in extent of hydrolysis of **2** in the presence versus the absence of Ser-His (Figure S8). This result is consistent with the well-known ability of imidazole to catalyze the hydrolysis of *p*-NPAc at alkaline pH (Figure S8).[8] Indeed, we observed that hydrolysis of **2** at pH 8 in the presence of 10 mM imidazole was even faster than in the presence of 10 mM Ser-His (Figure S8). Given the significant difference in reaction rate we observed between pH 6 and pH 8, and the difference between our observations at pH 6 and those reported in ref 4, it seems possible that the conditions employed for *p*-NPAc hydrolysis by the authors of ref 4 involved a pH somewhat higher than was described. However, our data indicate that Ser-His is less catalytically active than imidazole at elevated pH.

Given the limited sensitivity of the chromogenic substrate *p*-NPAc at pH 6, we probed the ability of Ser-His to catalyze ester hydrolysis using two fluorogenic substrates. The first was the classic esterase substrate fluorescein diacetate (FDA, **4**, Figure 3A). The second was fluorescein di(3-methoxy-propanoxy-

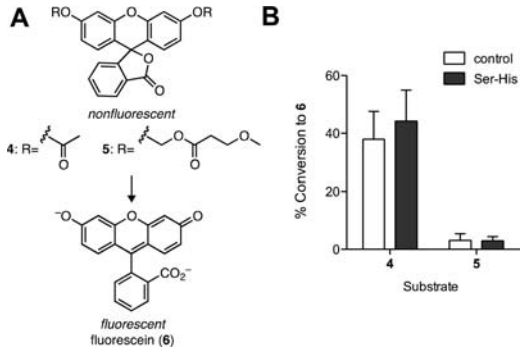

Figure 3. Extent of ester substrate reaction, as judged by development of fluorescence, after incubation with 10 mM Ser-His for 48 h, 50 °C, 1.8 μg/mL substrate, 40 mM Britton–Robinson buffer (40 mM phosphoric acid, 40 mM acetic acid and 40 mM boric acid) titrated to pH 6 with NaOH. Control was the same reaction in the absence of the dipeptide. Reactions were monitored in a M1000 PRO Microplate reader, via fluorescence intensity measurements with λ_{ex} = 490 nm and λ_{em} = 512 nm.

methyl ether) (FD-MOP-DE, **5**, Figure 3A). Both generate the fluorophore fluorescein (**6**) after two rounds of hydrolysis. Similar to *p*-NPAc (**2**), fluorescein diacetate (**4**) can be considered an activated ester as the pK_a of fluorescein (conjugate acid of the ultimate leaving group) is 6.3. Replacement of the acetyl units with acyloxymethyl ether units substantially decreases hydrolytic reactivity to a level characteristic of unactivated esters. The 3-methoxypropionate-containing substrate (**5**) was chosen because this molecule demonstrated high activity and solubility in enzymatic assays.[9] We assessed the increase in fluorescence in the same buffer conditions as for *p*-NPAc (**2**) but increased the temperature and incubation time to mirror those used for the reported BSA studies (48 h at 50 °C).[4] Evidence of considerable hydrolysis of FDA (**4**) was observed under these conditions, but the presence of Ser-His did not lead to a significant enhancement in the extent of reaction. The acyloxymethyl ether substrate was substantially more stable but also showed no evidence for catalysis of hydrolysis by Ser-His (Figure 3B). Overall, these observations indicate that Ser-His is not an effective catalyst of ester hydrolysis at pH 6.

We evaluated Ser-His as a catalyst for amide hydrolysis using four fluorogenic amide substrates (**7**–**10**, Figure 4A). Each is a diacylated derivative of rhodamine 110 (**11**), which is highly fluorescent. Monoacylated derivatives, which would presumably be intermediates in any amide hydrolysis process, display ∼10% of the fluorescence intensity of rhodamine 110 itself.[10] Three of our amide substrates have a single amino acid as the acyl fragment, derived from Asp (acidic side chain, **7**), Ala (neutral side chain, **8**), or Arg (basic side chain, **9**). The fourth substrate (**10**) has a tetrapeptide, Asp-Val-Glu-Asp, as the acyl fragment; this substrate has been used to detect caspase-3 activity.[11] In each case, 1.8 μg/mL substrate was combined with 10 mM Ser-His in 40 mM Britton–Robinson buffer[12] containing 40 mM phosphate, pH 6, and heated to 50 °C for 48 h. These conditions are based on those used in the reported experiments

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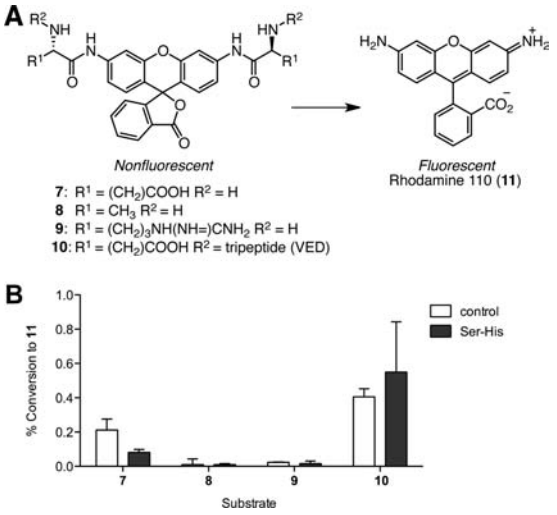

Figure 4. Extent of amide substrate reaction, as judged by development of fluorescence after incubation with 10 mM Ser-His for 48 h, 50 °C, 1.8 μg/mL substrates **7**−**10**, 40 mM Britton−Robinson buffer (40 mM phosphoric acid, 40 mM acetic acid and 40 mM boric acid) titrated to pH 6 with NaOH. Control was the same reaction in the absence of the dipeptide. Reactions were monitored in a M1000 PRO Microplate reader, via fluorescence intensity measurements with λ_{ex} = 485 nm and λ_{em} = 530 nm. Percent conversion was calculated based on the assumption that both amide bonds involving fluorophore amines were hydrolyzed to generate rhodamine 110.

with BSA.[4] For each substrate, a control solution lacking Ser-His was prepared and analyzed alongside the solution containing Ser-His. Figure 4B shows the fluorescence increase for each of the substrates with or without Ser-His, represented as percent conversion, which was calculated based on the assumption that both amide groups have been hydrolyzed to release rhodamine 110. We observed only small increases in fluorescence intensity after 48 h (<1% in each case). There was no significant difference between samples containing Ser-His relative to analogous samples lacking Ser-His. Experiments were run with amide substrate **7** also at pH 8 with 10 mM Ser-His or 10 mM imidazole (Figure S3). Background hydrolysis was more substantial at this elevated pH after 48 h, but neither Ser-His nor imidazole caused an increase in the extent of this hydrolysis. We therefore conclude that Ser-His does not function as a general catalyst of amide hydrolysis.

Developing enzyme-mimetic catalysts that significantly enhance the rates of challenging reactions near neutral pH and room temperature remains a profound challenge in molecular engineering. Progress has been made in terms of de novo enzyme design for a few reactions,[13] but chemical transformations as demanding as amide hydrolysis have yet to be addressed in this arena. It is noteworthy that DNA-based catalysis of aromatic amide hydrolysis has been achieved.[7] In light of the difficulties historically encountered in this field of design, it would be remarkable if a molecule as simple as Ser-His displayed significant catalytic prowess. The findings described here suggest that previously reported results[4] cannot be interpreted as evidence that Ser-His or any other short His-containing peptide is competent to catalyze amide hydrolysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01279.

Calibration curves, detailed procedures, additional experiments, and characterization of Ser-His (PDF)

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Notes

The authors declare no competing financial interest.

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