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Dipeptide Seryl-Histidine and Related Oligopeptides Cleave DNA, Protein, and a Carboxyl Ester

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Abstract—The amino acids histidine (His) and serine (Ser), or amino acids similar to Ser, function together as key catalytic amino acids in the active sites of such diverse enzymes as the serine- and thiol-proteases, lipases, and esterases. Ser and His are also conserved in the intein–extein junctions of the phylogenetically widespread self-splicing proteins and at the N- and C-termini of the homing endonucleases spliced from them. Here we show that the dipeptide seryl-histidine (Ser-His) and related oligopeptides can themselves cleave DNA, protein, and the ester *p*-nitrophenyl acetate (*p*-NPA) over wide ranges of pH and temperature. Denaturing polyacrylamide gel electrophoresis (PAGE) of 5′-end labeled DNA samples incubated with Ser-His reveals a pattern of two bands per nucleotide position, consistent with the generation of both 3′-hydroxyl and 3′-phosphate DNA cleavage fragments, as would be expected of phosphodiester hydrolysis by Ser-His. To the best of our knowledge, Ser-His is the shortest peptide ever reported to show cleavage activity with multiple categories of natural substrates. The amenability of the dipeptide to variation through addition of amino acid residues, either internally or to the C-terminus while retaining its multiple cleavage activities, combined with its reactivity over wide ranges of pH and temperature, demonstrates the evolutionary capacity of the Ser/His dyad and evokes many questions about possible roles it may have played in molecular evolution and its potential role as a core for selection of oligopeptides with enhanced cleavage activities and target specificity.

Introduction

The amino acids serine (Ser) and histidine (His) function together in the active sites of various natural enzymes as direct participants in enzymatic reactions.[1–7] The side chain hydroxyl of serine often serves as a nucleophile, while histidine possesses an imidazole side chain that can serve as a proton donating or accepting group in enzymes. Ser and His are directly involved in the peptide bond and ester bond cleavage reactions of the serine proteases chymotrypsin, trypsin, and elastase.[1,8–10] Although a third amino acid residue, aspartate (Asp), forms a catalytic triad with Ser and His in the active sites of serine proteases,[1] lipases,[3–5] and esterases,[6,7] the Ser/His dyad has been shown to be sufficient for the cleavage reactions.[8–11] The roles of Ser (or related amino acid residues) and His in the peptide bond and ester bond cleavage reactions of these and other polypeptide enzymes are well documented. It was not previously known, however, that the dipeptide Ser-His could itself exhibit these activities or that Ser-His could cleave DNA.

Because the Ser/His dyad is highly conserved and performs essential catalytic roles in the active sites of many proteases, lipases, and esterases, coupled with the consideration that polypeptide enzymes likely evolved around oligopeptides that possessed in a primitive degree the fundamental functions of the active sites of modern protein enzymes, it could be hypothesized that the dipeptide Ser-His and related oligopeptides might

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exhibit rudimentary cleavage activities. It could be further hypothesized that if the Ser/His dyad is capable of ester bond cleavage, then the dipeptide Ser-His, unencumbered by the tertiary structure of a polypeptide active site, may also be capable of phosphodiester bond cleavage. To test these hypotheses, Ser-His and related oligopeptides were incubated with linear and circular DNAs, proteins, and the carboxyl ester *p*-NPA. Analysis of the incubation products indicated cleavage of all these substrates, and numerous control reactions unequivocally demonstrated the cleavage activities of Ser-His and the other oligopeptides.

Results

DNA cleavage

Linear bacteriophage λ-DNA at a final concentration of 20 ng/μL (60 μM in phosphodiester bonds), when incubated with 10 mM Ser-His in 40 mM Britton-Robinson (B–R) buffer, was gradually degraded into smears of progressively smaller fragments of heterogeneous sizes, as revealed by agarose gel electrophoresis after 72 h of incubation (Fig. 1(A)). In contrast, DNA samples incubated under the same conditions without Ser-His remained intact (Fig. 1(A)). The nucleolytic activity in samples incubated with Ser-His could be detected over wide ranges of pH (from 5 to 9, Fig. 1(A)), with a pH value near the p*K*a of imidazole (pH 6) being optimal for cleavage at 37 °C. The rate of cleavage was also temperature-dependent; incubation at 50 °C resulted in faster DNA cleavage than at 37 °C (Fig. 1(A)), and even higher rates of cleavage were observed at 60 °C (data not shown).

To demonstrate that the nucleolytic activity was not restricted to linear DNA, a circular plasmid DNA, pBR322, was also used as a substrate for cleavage. In the presence of Ser-His, the DNA band corresponding to the supercoiled form disappeared first with a concomitant increase of the relaxed form (Fig. 1(B)). As the incubation continued, the relaxed form decreased and a new linear form appeared (Fig. 1(B)). At the final stages of incubation, the linear form was degraded into a heterogeneous smear similar to that seen with λ-DNA (Fig. 1(A)). These DNA cleavage results illustrate that the nucleolytic activity has no sequence specificity, which was further demonstrated in a cleavage experiment using a radio-labelled oligonucleotide substrate (Fig. 1(C)). A single-stranded (ss) oligonucleotide of 60 bases was labelled with ^{32}P at its 5′ end and incubated with Ser-His. Following denaturing PAGE (10%), a ladder of cleaved oligonucleotides with relatively uniform intensity and spacing of bands was revealed (Fig. 1(C), lane 2), indicating that cleavage occurs at all 4 nucleotide

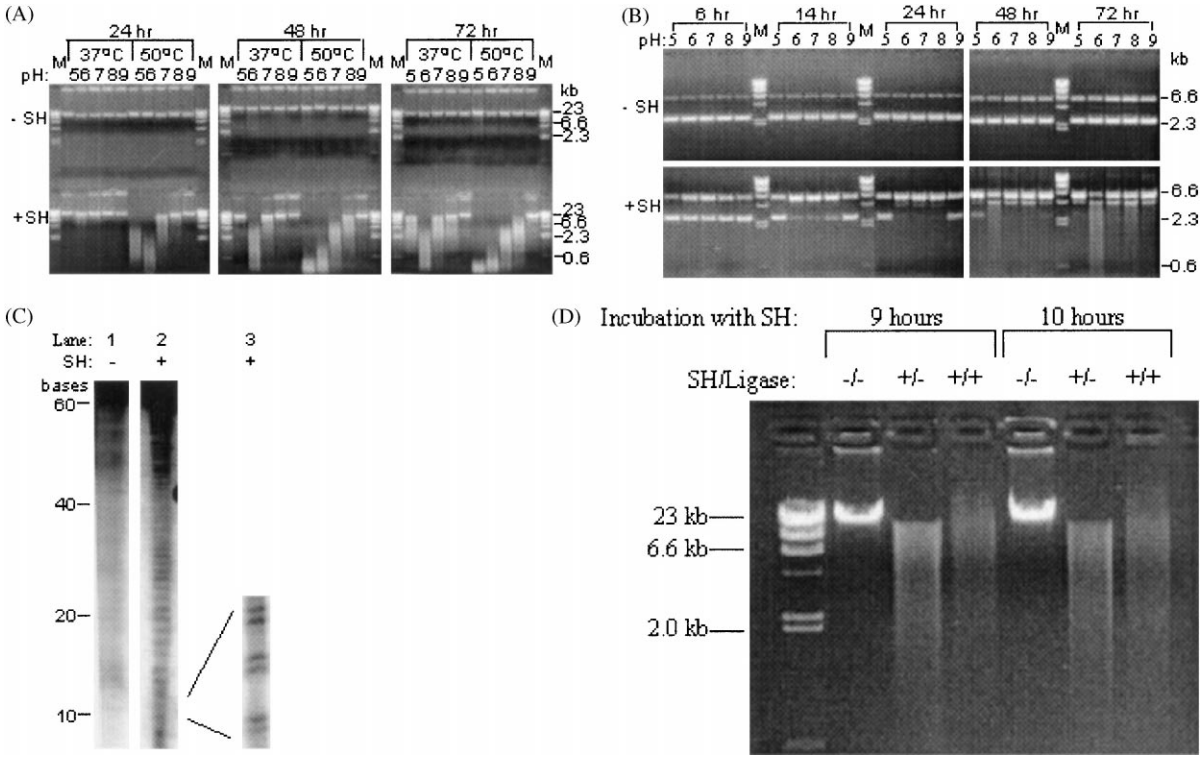

Figure 1(A–D). DNA cleavage by Ser-His. (A) Cleavage of λ-DNA. λ-DNA was incubated with (+SH) or without (−SH) dipeptide in B-R buffers of varying pH at either 37 or 50 °C for 24, 48, and 72 h. All samples were subjected to electrophoresis in a 1% agarose gel. M = λ-DNA/Hind III size marker. (B) Cleavage of plasmid DNA (pBR322). The same buffers were used as above. Incubation was at 37 °C only. (C) Cleavage of 5′ end-labeled ss DNA. An oligonucleotide (60 mer), radiolabeled at its 5′ end, was incubated with (lane 2) or without (lane 1) Ser-His in B-R buffer pH 6.5 at 50 °C for 48 h. Following incubation, the samples were subjected to 10% (lanes 1 and 2, one gel) or 15% (lane 3) PAGE. Lane 3 shows higher resolution of the bands corresponding to those in the indicated area of lane 2. (D) Ligation of λ-DNA cleavage fragments. λ-DNA was incubated with or without Ser-His at 50 °C for 9 and 10 h. Cleavage fragments produced from the incubations of λ-DNA with Ser-His were then incubated with or without T4 DNA ligase and ATP at 12 °C for 24 h. The incubation products were subsequently separated and visualized on a 1% agarose gel.

positions of the ssDNA with no pronounced base preference. When run for a longer time on a 15% denaturing PAGE gel, two bands were visible at each oligonucleotide position (Fig. 1(C), lane 3). This cleavage pattern is consistent with DNA hydrolysis by Ser-His, wherein the P–O bond on either side of a phosphodiester would be expected to be susceptible to hydrolysis, resulting in both 3′-hydroxyl (lower band) and 3′-phosphate (upper band) cleavage products at each nucleotide position on the gel. The distance between the upper and lower bands at each nucleotide position is approximately 1/4 of the distance between corresponding bands of consecutive nucleotide positions (Fig. 1(C), lane 3). This difference in distance between lower and upper bands coincides with the difference in average molecular mass of a nucleotide with a 3′-hydroxyl (~330 daltons) and a nucleotide with a 3′-phosphate (~330 + 80 daltons). This cleavage pattern is not consistent with the cleavage mechanisms of natural nucleases,[12] which generate single bands at each oligonucleotide position. To further eliminate the possibility of DNA cleavage resulting from contaminating nucleases or metal ions, Ser-His samples were either filter-sterilized or autoclaved, and incubated with DNA in the presence or absence of EDTA, followed by agarose gel analysis. The results of this study indicate that autoclaved Ser-His is as active as the filter-sterilized Ser-His in DNA cleavage (Table 1), whether in the presence or absence of EDTA, suggesting that the observed DNA cleavage activity is not due to polypeptide nuclease contamination. Furthermore, negative controls identical to the reaction solutions, except in that they did not contain Ser-His, showed no DNA cleavage activity at any of the tested pH and temperature conditions, even after 72 h of incubation (− SH samples in Fig. 1(A) and (B)), demonstrating that the observed cleavage activity is associated exclusively with addition of the Ser-His solution. Ser-His samples purchased from three different sources, including an HPLC purified Ser-His, all exhibit DNA cleavage activity (Table 1). Moreover, DNA samples incubated under the same conditions with varying concentrations of added Cu^{2+} or Fe^{2+} with or without EDTA and without Ser-His displayed no cleavage activity (Table 1). These findings, combined with the distinctive cleavage pattern (Fig. 1(C), lane 3), provide strong evidence of the DNA cleavage activity of Ser-His.

Table 1. Determination of the origin of DNA cleavage activity

Reaction conditions[a]	DNA Cleavage activity
Britton-Robinson buffer (B-R)	No
Ser-His[b] (filtered) + B-R	Yes
Ser-His[b] (autoclaved) + B-R	Yes
Ser-His[b] ± 1 μM EDTA + B-R	Yes
Ser-His[b] + B-R at 65 °C	Yes
$FeSO_4$[c] ± EDTA + B-R	No
$CuSO_4$[c] ± EDTA + B-R	No

[a]All reactions were performed at 37 °C (or 65 °C) in 40 mM Britton-Robinson buffer (pH 6.0) with 20 ng/μL λ-DNA in a total volume of 20 μL.
[b]Ser-His purchased from three different sources was used in this study (see Methods).
[c]The $FeSO_4$ and $CuSO_4$ solutions were each 1 and 10 μM.

Ligation of DNA cleavage fragments

To investigate the DNA cleavage mechanism employed by Ser-His, cleavage fragments generated from incubation of λ-DNA with Ser-His were then incubated with T4 DNA ligase. The upward shift in fragment sizes on a 1% agarose gel (Fig. 1(D)) observed for the samples incubated with DNA ligase is evidence that fragments with 3′-hydroxyls and 5′-phosphates were among the DNA cleavage products generated by Ser-His, since DNA ligase requires free 3′-hydroxyls and 5′-phosphates for ligation. The generation of these terminal groups is consistent with hydrolysis of phosphodiesters by Ser-His, and would not be characteristic of cleavage of the DNA by a free-radical mechanism.

Protein cleavage

Since the serine proteases conserve Ser and His in their active sites, we speculated that Ser-His might also be able to cleave protein. BSA was used as a substrate to test this hypothesis. As revealed by PAGE, BSA is indeed cleaved by Ser-His into a smear of progressively smaller fragments (Fig. 2(A)), with optimal reaction conditions similar to those for DNA cleavage. The proteolytic activity of Ser-His was likewise apparent using lysozyme as a second protein substrate (data not shown). Various related oligopeptides were tested for proteolytic activity, and the results show a consistent pattern in modifications to Ser-His and corresponding changes in cleavage activity (Fig. 2(B)).

Roles of functional groups in DNA and protein cleavage

To study the roles of Ser and His in DNA and protein cleavage, different amino acid residues were used in substitution of Ser or His, or were added internally or to the N- or C-terminus of Ser-His. These oligopeptides were incubated individually with either DNA or BSA to test their respective cleavage activities. Solutions of the amino acids Ser and/or His do not exhibit DNA or protein cleavage activities (Table 2), indicating that Ser and His must be covalently linked to exhibit cleavage activities. His-Ser, a dipeptide identical to Ser-His in chemical composition but in reverse sequence, is also inactive (Table 2). The cleavage activities are also lost when Ser is replaced with any other amino acid except cysteine (Cys) or threonine (Thr), the latter of which retains only minimal activity. Like the hydroxyl of Ser, the sulfhydryl side chain of Cys can serve as a nucleophile, as is the case in the active sites of natural thiol-proteases.[2] The His residue cannot be replaced by any of the amino acids tested, including those with positively charged side chains (Table 2). The cleavage activities of Ser-His are reduced or lost when an amino acid is added to its N-terminus but are retained when one or more amino acids are added to the C-terminus. It is interesting to note that the cleavage activities of Ser-His-Asp, which contains the amino acids of the catalytic triad, are at least as efficient as those of Ser-His under conditions optimised for Ser-His (Fig. 2(B) and Table 2). It was also found that amino acids could be added between Ser and His without abolishing the cleavage

activities (Table 2). The effects of these modifications of Ser-His on DNA and protein cleavage were parallel (Table 2), showing that the same agent was cleaving both substrates and that its activity was related to the modifications of Ser-His in a highly predictable fashion. This provides strong evidence that the dipeptide and related oligopeptides are the cleaving agents, and implicates the hydroxyl (or sulfhydryl) functional groups of the N-terminal amino acid residues and the imidazole functional group of histidine as the requisite groups for cleavage.

Ester cleavage

Like the serine protease chymotrypsin, which cleaves proteins and carboxyl esters, Ser-His was found also to cleave the ester *p*-NPA. When incubated with Ser-His at room temperature, *p*-NPA showed a rapid linear increase in optical density (OD) at 400 nm over time, which is indicative of cleavage of the *p*-NPA to *p*-nitrophenol (Fig. 3). This change in OD was found to be dependent on the concentration of Ser-His (Fig. 3), as well as on pH and temperature (data not shown).

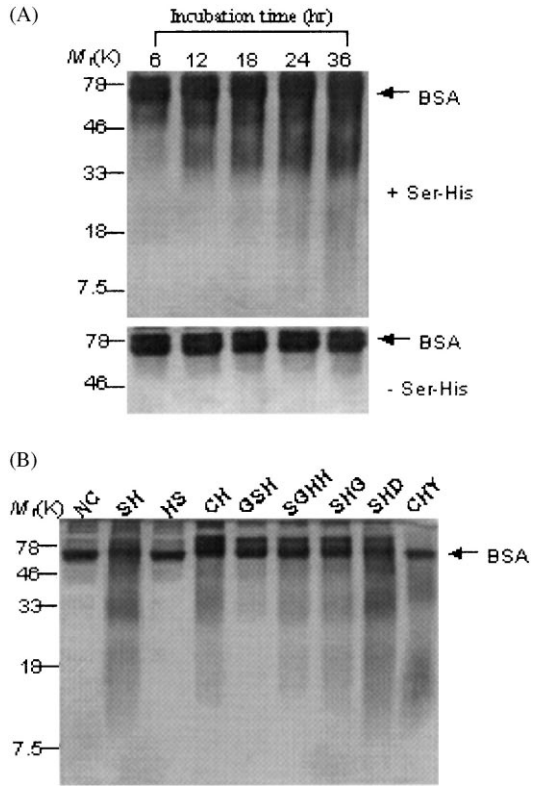

Figure 2. BSA cleavage by Ser-His and related oligopeptides. (A) Silver-stained 10% reducing PAGE showing progressive cleavage of BSA (5 μg) after incubation with Ser-His (10 mM) and B-R buffer (pH 6) at 50 °C for various times. The lower gel (−Ser-His) shows samples incubated under identical conditions but without dipeptide. (B) 10% reducing PAGE of BSA incubated with various oligopeptides (10 mM) with B-R buffer (pH 6) at 50 °C for 48 h. Chymotrypsin (100 nM) incubated with BSA (5 μg) and B-R buffer (pH 7.8) at 37 °C for 40 min. NC, negative control (no oligopeptide); SH, Ser-His; HS, His-Ser; CH, Cys-His; GSH, Gly-Ser-His; SGHH, Ser-Gly-His-His; SHG, Ser-His-Gly; SHD, Ser-His-Asp; CHY, chymotrypsin.

Discussion

Although the finding that a dipeptide can cleave protein, ester, and DNA is surprising, the results of extensive experimentation controvert the possibility that these activities arise from contaminants, such as nucleases or transition metals, known to cleave DNA. Cleavage of the substrates is only observed after addition of the Ser-His and does not occur with the B-R buffer alone (Table 1). Autoclaved and/or filtered Ser-His samples retain cleavage activities and still cleave DNA in the presence or absence of 1 mM EDTA even at 65 °C (Table 1),

Table 2. DNA and protein cleavage activities of Ser-His and related oligopeptides

Oligopeptide	DNA cleavage[a] activity	Protein cleavage[a] activity
Ser	–[b]	–
His	–	–
Ser + His	–	–
His-Ser	–	–
Ser-His	+ + + + +[b]	+ + + + +
Cys-His	+ + +	+ + +
Thr-His	±	+
Asp-His	–	–
Ser-Arg	–	–
Ser-Lys	–	–
Gly-Ser-His	–	+
His-Ser-His	–	–
Ser-His-Gly	+ + +	+ + +
Ser-His-His	+ + +	+ + +
Ser-His-Asp	+ + + + +	+ + + + +
Ser-Gly-His-His	+ +	+ +
Ser-Gly-Gly-His-His	+ +	+ +

[a]The cleavage assays were performed at the conditions optimal for Ser-His cleavage activities: in B-R buffer (pH 6.0); 10 mM oligopeptide, 20 ng/μL of DNA (or 250 ng/μL of BSA), incubated at 50 °C for 48 h and the cleavage products were separated and visualized by agarose or acrylamide gel electrophoresis.
[b]'–' stands for no detectable cleavage activity; each '+' stands for approximately 20% of Ser-His cleavage activity. '±' stands for marginally detectable cleavage activity.

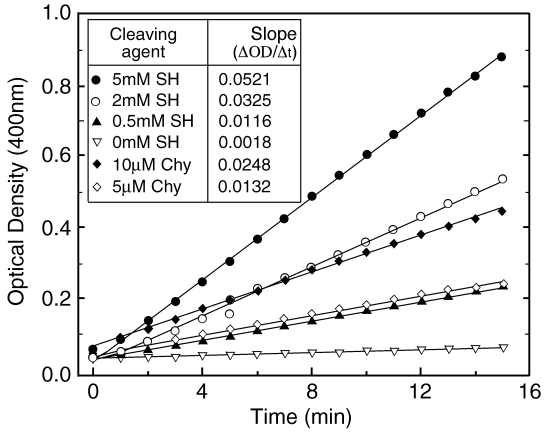

Figure 3. *p*-NPA cleavage by Ser-His. *p*-NPA (2 mM) was incubated with various concentrations of Ser-His in B-R buffer (40 mM, pH 6) at room temperature. Rates of *p*-NPA cleavage were measured by monitoring changes in the optical density at 400 nm of each sample over time. *p*-NPA incubated without Ser-His served as a baseline reference, and *p*-NPA samples incubated with chymotrypsin (pH 7.8) were included to compare relative cleavage efficiencies (inserted table).

effectively eliminating the possibility of protein nuclease contamination. Transition metals, particularly Fe^{2+} and Cu^{2+}, are known to cleave DNA in the presence of EDTA and other reducing agents,[13–15] but failed to cleave DNA under reaction conditions optimised for Ser-His (Table 1). Furthermore, the presence of two 3′ cleavage products at each nucleotide position generated from DNA cleavage by Ser-His (Fig. 1(C)) and the successful ligation of the DNA cleavage fragments (Fig. 1(D)) are strong evidence against metal-assisted cleavage of the DNA[16] but are indicative of the 3′-hydroxyls and 5′-phosphates that support a hydrolysis mechanism. Combined with the observation of a consistent and predictable pattern of parallel cleavage of DNA and protein by the various oligopeptides tested (Table 2), these facts provide overwhelming evidence that Ser-His and the related oligopeptides are themselves the cleaving agents.

There are many examples in nature of polypeptide enzymes that use an amino acid residue with a hydroxyl group (Ser) or a thiol group (Cys) and a His residue in their active sites to perform peptide or ester bond cleavage. The serine- and thiol-proteases, for example, form two of the four known families of modern proteases[1,2] and can cleave both peptides and esters. The use of Ser (or Cys) and His as a catalytic dyad in these protease active sites is a recurring theme apparent from the evolution of these enzymes. For example, subtilisin is a bacterial serine protease that has very low amino acid sequence homology to chymotrypsin; yet through convergent evolution, it also utilizes the Ser/His combination in its active site.[17] Ser and His are likewise conserved in the active sites of lipases and esterases.[3–7] A Ser/His dyad was also discovered in the active site of a catalytic antibody that catalyzes the hydrolysis of norleucine and methionine phenyl esters,[18] indicating that antibodies can converge on the active site structures that have been selected by natural enzyme evolution. Protein self-splicing provides another example of the Ser/His catalytic dyad. This peptide bond cleaving process, recently found in organisms of all three kingdoms, invariably uses Ser or Cys at the N-terminus and His (plus an asparagine (Asn)) at the C-terminus of an internal protein sequence (intein) to enable cleavage at the splice junctions and the rejoining of the external protein sequences (exteins).[19–23] More interestingly, the spliced intein always has Ser or Cys at its N-terminus and His-Asn at its C-terminus, and functions as a homing endonuclease to cleave chromosomal DNA.[20–23] It seems reasonable, given our findings, to speculate that these Ser and His residues may function not only in the intein splicing reaction, but in the subsequent DNA cleavage as well. The common feature of these various enzyme active sites is embodied in the dipeptide Ser-His, which can itself cleave DNA, proteins, and at least one ester.

Computer modelling has predicted a low energy conformation of Ser-His that closely matches the relative orientations of the Ser and His residues in the chymotrypsin active site (data not shown). Though the mechanisms by which Ser-His cleaves the various substrates have not been fully elucidated, the dipeptide is suspected to function similarly to the chymotrypsin active site by employing hydrolysis to cleave protein, ester, and even phosphodiester substrates. The requisite N-terminal position of the Ser may be an indication that Ser uses its own α-amino group as a general base for improving the nucleophilicity of the hydroxyl group, as appears to be the case in the hydrolysis of amide bonds by penicillin acylase.[11] The requirement of the His and the optimal cleavage activities near its pK_a suggest a possible role for the imidazole group as a general acid in protonating the leaving groups in the cleavage reactions. Because Ser-His and the related oligopeptides are not constrained in fixed conformations, the possible interactions of the functional groups are varied and highly complex. In addition to the λ-DNA cleavage fragment ligation experiment discussed previously, the DNA cleavage mechanism is currently being investigated by labelling the DNA cleavage fragments to identify the different termini produced by cleavage. Combined with a study of the reaction kinetics currently underway, these experiments will resolve whether or not Ser-His functions enzymatically in these reactions and will help clarify the exact roles of the functional groups involved and the mechanisms employed. Answers to these fundamental questions may allow biochemical mimicry, through in vitro selection systems,[24–26] of early bio-molecular evolution of peptide enzymes.

Conclusion

To the best of our knowledge, the dipeptide Ser-His is the shortest peptide ever reported to have multiple cleavage activities. Results of preliminary experiments indicate that in addition to DNA, protein, and ester cleavage, Ser-His is also capable of cleaving RNA (data not shown). Because of its ability to interact with multiple classes of biological molecules over such wide ranges of physical and chemical conditions, Ser-His and related oligopeptides may have played important roles, either independently or as cofactors[27] to RNA, in the hypothetical 'RNA world'[28] from which the modern 'protein world' emerged. The ability of Ser-His to retain its multiple cleavage activities when amino acids are added internally or to its C-terminus demonstrates the extraordinary evolutionary capacity of the dipeptide Ser-His. Since the number of catalytically active combinations available for the evolution of polypeptide enzymes is limited by the relatively small number of functional groups provided by the naturally occurring amino acids, it is not surprising that the successful combination of Ser and His is repeatedly selected in the evolution of diverse groups of enzymes.

Experimental

Oligopeptides and cleavage substrates

Ser-His was purchased as an acetate salt from Sigma and Bachem (HPLC purified), or as the dipeptide from Research Genetics. Other oligopeptides were purchased from either Sigma or Research Genetics. The powdered oligopeptides were dissolved in double deionized and

sterilized (dds) H_2O, and were then either filter- or autoclave-sterilized. Plasmid DNA pBR322 and λ-DNA were purchased from Life Science Technology. A 60-mer single-stranded oligonucleotide, 5′-CGGATTACCA GGG ATTTCAG TCGATGTACA CGTTCGTCAC ATCT-CATCTA CCTCCCGGTT-3′, was purchased from Integrated DNA Technologies. The 5′ end of the oligonucleotide was labelled with [γ-^{32}P] ATP (Amercham) by T4 polynucleotide kinase. Bovine serum albumin (BSA) and lysozyme were purchased from Sigma and were dissolved in ddsH_2O. The carboxyl ester *p*-NPA was purchased from Sigma and was dissolved in isopropanol.

Cleavage reactions and cleavage product analyses

Oligopeptides were individually mixed with a cleavage substrate in B-R buffer (equal amounts of phosphate, borate, and acetate), to buffer reactions in the pH range 5–9, and ddsH_2O to a final volume of 20 μL in PCR reaction tubes, sealed, and incubated in a GeneAmp PCR System (Perkin–Elmer 9600) at designated temperatures for pre-determined periods of time. After incubation, a portion of the incubated solution was removed and analysed by 1% agarose gel electrophoresis (for pBR322 and λ-DNA) or 10% and 15% denaturing PAGE (for the single-stranded 5′-end-labeled oligonucleotide), or by 10% reducing PAGE (for proteins). The cleavage reaction of *p*-NPA with Ser-His or chymotrypsin was carried out in triplicate in a 96-well microtiter plate at a designated temperature in a volume of 100 μL, and was monitored, recorded, and analysed using a SPECTRAmax 250 microtiter plate reader system at a wavelength of 400 nm.

Ligation reaction of λ-DNA cleaved by Ser-His

λ-DNA (20 ng/μL) was incubated with Ser-His (10 mM) in B-R buffer (40 mM, pH 6.5) in a reaction volume of 20 μL for either 9 or 10 h at 50 °C to generate λ-DNA cleavage fragments, which were then incubated for 24 h at 12 °C with T4 DNA ligase in a ligation buffer containing ATP. The ligation reaction samples were subsequently subjected to electrophoresis on a 1% agarose gel (Fig. 1(D)) alongside negative control samples for the cleavage and ligation reactions, which were incubated without Ser-His and T4 DNA ligase, respectively.

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