10-min heating on a steam bath. In all cases identical spectra resulted, indicative of III and IV being intermediates in the reaction of I and II with NBS. Tyrosinamide and tyrosine-N-methylamide gave similar results. Free tyrosine, 3,5-dibromotyrosine, tyramine, and N-acetyl-3,5-dibromotyrosine ethyl ester failed to produce the 320-mμ chromophore on treatment with NBS.

When the reaction was carried out on a preparative scale, starting with II, IV, or tyrosinamide in 40% aqueous acetic acid, crystalline products were isolated in 20% yield after chromatography on silica ($CHCl_3$–$CH_3OH$, 9:1). Infrared, ultraviolet, nmr, and mass spectra and elemental analyses suggest the structure of ethyl 5,7-dibromo-6-hydroxyindole-2-carboxylate (VI) or its amide for the 320-mμ product. The amide [mp 247° dec, $\lambda_{max}$ 315 mμ (EtOH)] exhibited a triplet of parent peaks at *m/e* 331.879, 333.874, and 335.873 in the mass spectrometer consistent with the formula $C_9H_6N_2O_2Br_2$ [calcd, 331.88 ($^{79}Br$–$^{79}Br$), 333.879 ($^{79}Br$–$^{81}Br$), and 335.878 ($^{81}Br$–$^{81}Br$)].

The formation of a side product, an unstable red aminochrome, $\lambda_{max}$ 480 and 320 mμ, is especially noticeable at pH values above 5. In addition, a colorless, crystalline, indolic compound [$\lambda_{max}$ 315 mμ (log ε 4.33), mp 152–155°, $C_{11}H_{10}NO_4Br$, *m/e* 298.979 and 300.979 (calcd 298.979 and 300.977 for $^{79}Br$ and $^{81}Br$)] is obtained from II or IV. The same product, ethyl 5,6-dihydroxy-7-bromoindole-2-carboxylate, was obtained from ethyl 3,4-dihydroxyphenylalanate on oxidation with NBS. NBS in this case converts tyrosine ester to products which as a rule arise only from DOPA.

The dibromoindole VI was reductively debrominated to VII with palladium on charcoal in buffered methanol [mp 169–175°; $\lambda\lambda_{max}$ (log ε) 320 mμ (4.32), 250 (3.95), 215 (4.28); parent *m/e* 205.078 (calcd for $C_{11}H_{11}NO_3$, 205.074)]. VII was methylated with dimethyl sulfate in anhydrous acetone containing fused potassium carbonate to give the known ethyl 6-methoxyindole-2-carboxylate (VIII). The ultraviolet spectrum [$\lambda_{max}^{EtOH}$ 320 mμ (log ε 4.28), 250 (3.93), 215 (4.28); $\lambda_{min}^{EtOH}$ 265 mμ (log ε 3.29)] and melting point (132–135°) agree with the reported data.[5]

The low-resolution mass spectrum reveals a parent peak (M) at *m/e* 219 and principal peaks at 204 (M − $CH_3$), 191 (M − $CH_2$=$CH_2$), 190 (M − $C_2H_5$), 173 (M − $C_2H_5OH$), 158 (M − $C_2H_5OH$, − $CH_3$), 145 (M − $C_2H_5OH$, − CO), 130 (M − $C_2H_5OH$, − $CH_3$, − CO), 119 (M − $C_2H_5OH$, − CO, − CN), 102 (M − $C_2H_5OH$, − $CH_3$, − 2CO); 76 (M − $C_2H_5OH$, − $CH_3$, − 2CO, − CN).

This transformation is now being used for the determination of amino-terminal tyrosine in proteins and for the synthesis of 6-hydroxyindole derivatives.

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## Amino Acid Sequence around the Histidine Residue of the α-Lytic Protease of *Sorangium sp.*, a Bacterial Homolog of the Pancreatic Serine Proteases

*Sir:*

The elucidation of the amino acid sequence of chymotrypsinogen A,[1] chymotrypsinogen B,[2] trypsinogen,[3,4] and, in part, elastase[5] has established that the pancreatic serine proteases are homologous with respect to a subsequence in which two histidines and a cystine residue are components.[6] In chymotrypsin, for example, these components are histidine-40 and -57 and the cystine residue comprised of half-cystine-42 and -58. The discovery of this common structure, in which a disulfide bond could be considered to have the function of bringing the two histidines close together, has prompted several proposals of reaction mechanisms[7–9] which assign a catalytic function to both histidine residues. However, the kinetic evidence implicates only one.[10]

The α-lytic protease of *Sorangium sp.* is a bacterial protease which is unusual in two respects: it has the same active serine sequence as the pancreatic proteases, *i.e.*, Asp-Ser-Gly-Gly, and it has only one histidine residue.[11–13] The sequences at its three disulfide bridges are under investigation and, in this communication, we report the sequence of a histidylcystine structure which establishes a further homology with the pancreatic enzymes.

The α-lytic protease, prepared by methods previously described,[14] was digested with pepsin and submitted to the pH 6.5 diagonal procedure of Brown and Hartley.[15] The resulting peptide map is shown in Figure 1. A single peptide (B1) staining for histidine was observed off the diagonal and was paired with a more acidic peptide B2. Peptide B1 stained red and B2 yellow with the cadmium–ninhydrin reagent of Heilmann, *et al.*[16] Peptide B1, after isolation by preparative electrophoresis at pH 6.5, was found to be separable into two histidine peptides, B1A and B1B, by electrophoresis at pH 1.8 (60 v/cm for 60 min). Peptides B1A, B1B, and B2 were subjected to amino acid and sequential analysis from the N-terminal by the "dansyl–Edman" procedure.[17,18] Peptide B1A

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Table I. Amino Acid Composition (Ratios) and Sequence Analysis of Peptides Isolated from Peptic Digests of α-Lytic Protease of *Sorangium*[a]

| Peptide | Sequence |
|---|---|
| B1A | Val – Thr – Ala – Gly – His – $CySO_3H$ – Gly – Thr – Val – Asn – Ala |
| | → → → ← |
| | 0.97 0.97 0.97 1.00 0.97 1.06 1.00 0.97 1.00 1.03 1.00 |
| | L1b L1a L4 L2a |
| | → → → → → → |
| | 1.08 0.92 1.00 1.00 0.93 0.80 1.33 1.00 0.93 0.93 1.07 |
| | L1c L3 |
| | → → → → ← |
| | 1.00 0.93 1.07 0.98 1.00 0.97 0.99 1.00 1.07 |
| | L2c |
| | → |
| | 1.10 1.06 0.95 0.79 1.06 1.02 1.02 |
| B1B | Phe – Val–Thr – Ala – Gly – His – $CySO_3H$ – Gly – Thr – Val – Asn – Ala |
| | → → → |
| | 0.60 1.02 0.97 1.12 1.07 0.87 1.07 1.07 0.97 1.02 1.07 1.12 |
| B2 | $CySO_3H$ – Ser – Val – Gly – Phe |
| | → → → → → |
| | 0.96 1.02 1.02 1.04 0.96 |

[a] The symbols → → indicate N-terminal analyses by the "dansyl-Edman" procedure; ← represents C-terminal analyses with carboxypeptidase.

Table II. Histidine Disulfide Structures in α-Lytic Protease, Chymotrypsins A and B, Trypsin, and Elastase[a]

| | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chymotrypsin A | Phe | His | Phe | Cys | Gly | Gly | Ser | Leu | Ile | Asn | Glu | Asn | Try | Val | Val | Thr | Ala | Ala | His | Cys | Gly | Val | Thr | Thr | Ser |
| Chymotrypsin B | Phe | His | Phe | Cys | Gly | Gly | Ser | Leu | Ile | Ser | Glu | Asp | Try | Val | Val | Thr | Ala | Ala | His | Cys | Gly | Val | Thr | Thr | Ser |
| Trypsin | Tyr | His | Phe | Cys | Gly | Gly | Ser | Leu | Ile | Asn | Ser | Gln | Try | Val | Val | Ser | Ala | Ala | His | Cys | Tyr | Lys | Ser | Gly | Ile |
| Elastase | Ala | His | Thr | Cys | Gly | Gly | Thr | Leu | | | | | | | | Thr | Ala | Ala | His | Cys | Val | Asp | Arg | Glx | |
| α-Lytic protase | | | | Cys | Ser | Val | Gly | Phe | | | | | | Phe | Val | Thr | Ala | Gly | His | Cys | Gly | Thr | –Val | Asn | Ala |

[a] The disulfide bridge is between residues corresponding to half-cystines-42 and -58 of chymotrypsin in each case.

was further degraded by digestion with α-lytic protease (molar ratio 100:1 in 0.05 *M* N-ethylmorpholine buffer, pH 8.0, for 16 hr at 37°). The resulting peptides (designated as L) were separated by high-voltage electrophoresis at pH 6.5 and 1.8 and subjected to amino acid and sequential analysis. Peptides B1A and B1AL3 were also digested with carboxypeptidase to determine C-terminal residues. The results of these analyses are presented in Table I.

The above results assign sequences to peptides B1B and B2 which are compared in Table II with the sequences around histidine-40 and histidine-57 of the pancreatic serine proteases. Within the limits of the comparison, the histidine sequence of the bacterial enzyme shows as much homology with the histidine-57 sequences of the pancreatic enzymes as the latter do with one another; *e.g.*, like trypsin, the bacterial enzyme has six direct matches with the chymotrypsin sequence from residues 52 to 63; in addition, it has a simple reversal of the chymotrypsin sequence at residues 60 and 61. As histidine-57 is the histidine which is alkylated when chymotrypsin[19,20] and trypsin[21] react with their chloromethyl ketone inhibitors, the homology is with sequences around a histidine of proven significance. The existence of a "histidine loop" in the bacterial enzyme is unproven at present, and peptide B2 is placed on the N-terminal side of peptide B1B merely for comparison with the C-terminal sequences of histidine-40. Peptide B2 shows no homology with these sequences.

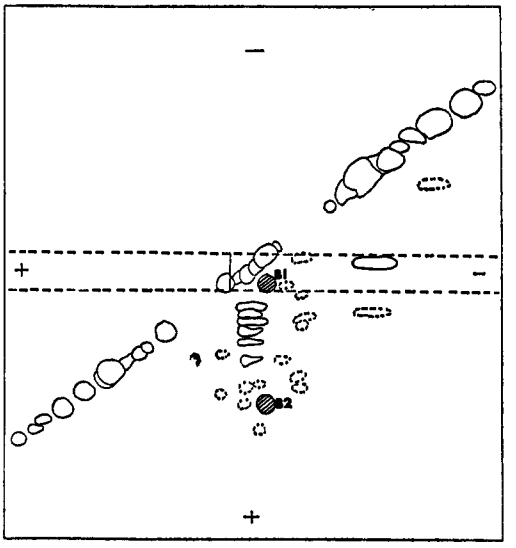


Figure 1.

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The sequence of subtilisin has been reported recently.[22] Its histidine and active serine sequences are completely different from those of the pancreatic enzymes. The present evidence indicates therefore that bacterial serine proteases have evolved along at least two independent pathways.

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## Kinetic Properties of the α-Lytic Protease of *Sorangium sp.*

*Sir:*

An accompanying communication[1] gives evidence that the amino acid sequence around the only histidine residue of a bacterial protease is homologous with the sequence around histidine-57 of chymotrypsin. This evidence, coupled with previous evidence[2] of homology

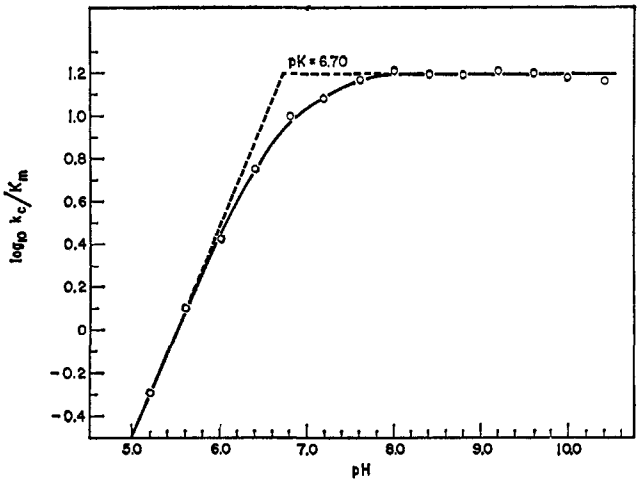


Figure 1. Hydrolysis of N-acetyl-L-valine methyl ester in 0.10 *M* KCl at 25.0°.

around the active serine residue, raises the question as to whether the reaction mechanism of the *Sorangium* enzyme differs in any way from that of chymotrypsin for, if it does not, reaction mechanisms which require chymotrypsin to have two catalytically functional histidines are clearly in need of reappraisal.

The kinetic data reported in this communication are based on measurements of esterase activity in a pH-Stat. The enzyme was prepared by a simplified version[3] of the original procedure. Enzyme concentrations were determined from amino acid analyses and the known amino acid composition per mole of enzyme.[4]

Table I gives a comparison of esterase activities toward various N-benzoyl- and N-acetylamino acid esters under reaction conditions which gave initial rates in direct proportion to both the enzyme and the substrate concentration. As the over-all kinetics are Michaelis–Menten kinetics, the second-order velocity constants are designated as $k_c/K_m$ ratios where $k_c$ is the catalytic rate constant ($k_{cat}$).[5] It is evident from the range of substrate concentration which gave second-order kinetics that all values of $K_m$ are greater than 10 m*M*. An earlier comparison[6] of the enzyme's action pattern on the A and B chains of oxidized insulin had indicated a pancreatic elastase-like specificity for linkages involving the carbonyl groups of neutral, aliphatic amino acids; the specificity shown by Table I is in accordance with these findings.

**Table I.** Esterase Activities of the α-Protease at pH 8.0 in 0.10 *M* KCl at 25.0°[a]

| Substrate | $[S]_0$, m*M* | $k_c/K_m$, $M^{-1}$ sec$^{-1}$ |
|---|---|---|
| Bz-Arg-OEt | 11 | 0.00 |
| Ac-Tyr-OEt | 6.3 | 0.00 |
| Ac-Trp-OMe | 2.3 | 0.00 |
| Bz-Gly-OMe | 2.6–10.5 | 8.07 |
| Ac-Val-OMe | 0.60–6.0 | 16.3 |
| Ac-Ala-OMe | 1.0–3.0 | 26.3 |
| Bz-Ala-OMe | 1.02–10.2 | 723 |
| Bz-D-Ala-OMe | 11.0 | 0.00 |

[a] The enzyme concentration was $8.0 \times 10^{-8}$ *M* for Bz-Ala-OMe and $5.0 \times 10^{-6}$ *M* for the other esters.

Figure 1 shows the pH dependence of the hydrolysis of N-acetyl-L-valine methyl ester. The values of $k_c$ and $K_m$ for this substrate (Table II) were estimated from Eadie plots at higher substrate concentrations. When

**Table II.** $K_m$ and $k_c$ for Hydrolysis of N-Acetyl-L-valine Methyl Ester in 0.10 *M* KCl at 25.0°

| pH | $K_m$, m*M* | $k_c$, sec$^{-1}$ |
|---|---|---|
| 6.30 | 61 ± 8 | 0.28 ± 0.01 |
| 7.00 | 72 ± 6 | 0.94 ± 0.04 |
| 8.00 | 65 ± 4 | 1.1 ± 0.1 |

water is replaced by $D_2O$, the p*K* shifts from 6.7 (Figure 1) to 7.35, and $k_c/K_m$ at the plateau is reduced 2.03-fold. The pH dependence of the hydrolysis of N-benzoyl-L-alanine methyl ester is essentially the same as that shown in Figure 1 (p*K* = 6.55) although, as indicated in Table I, the value of $k_c/K_m$ at the plateau is much higher. Activity toward this substrate over the same pH range is unaffected by acetylation of the enzyme with acetic anhydride at pH 6.8 and 0°. The N-terminal alanine residue and the two ε-amino groups of the enzyme no longer react with 1-fluoro-2,4-dinitrobenzene or with cyanate[7] after this treatment; the electrophoretic mobility of the enzyme at pH 5.0 is reduced by about one-third.

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