benzyloxycarbonylglutamyltyrosine, which is a good substrate for Penicillium janthinellum. This enzyme appears to be very similar to pepsin.*

The acid proteinase, peptidase A, of Penicillium janthinellum shows many similarities to pepsin, is competitive inhibitor of peptidase A activity. Some work on the specificity of peptidase A has been carried out, with the S-sulfo B chain of insulin as substrate. The acid proteinase, peptidase A, of Penicillium janthinellum is com-

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**A Pepsin-like Enzyme from Penicillium janthinellum**

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**SUMMARY**

The acid proteinase, peptidase A, of Penicillium janthinellum which shows many similarities to pepsin is completely inactivated by diazoacetyl-DL-norleucine methyl ester. One equivalent of inhibitor becomes covalently bound to 1 molecule of the enzyme. The binding site is probably an activated carboxyl group although another group cannot be excluded at present. p-Bromophenacyl bromide, another inhibitor of pepsin, does not inhibit peptidase A.

In 1964, Hofmann and Shaw (1) described an extracellular acid proteinase, peptidase A, which is secreted by the mold, Penicillium janthinellum. This enzyme appears to be very similar to pepsin in many of its properties. The amino acid composition (1) is comparable to that of pepsin in that the number of hydrophobic, hydrophobic, basic, and acidic residues, respectively, can be closely correlated in the two enzymes; the major difference is the absence of methionine, half-cystine, and arginine in the mold enzyme. The molecular weight of 32,000 (1) is close to that of pepsin. Some work on the specificity of peptidase A has been carried out, with the S-sulfo B chain of insulin as substrate. There are indications that peptidase A hydrolyses the same major bonds as does pepsin (2) but no further activity could be detected because of the inability of the enzyme to hydrolyze small peptides (3). However, it is interesting to note that benzoylcarbonylglutamyltyrosine, which is a good substrate for pepsin, is a competitive inhibitor of peptidase A activity. Thus binding of small peptides without hydrolysis probably takes place.

In view of these similarities it was of considerable interest to determine whether inhibitors which react covalently with pepsin would also inhibit peptidase A, and thereby provide evidence for a similarity in the structure of the active site and in the mechanism of action.

One such inhibitor, diazoacetyl-DL-norleucine methyl ester, was recently described by Rajagopalan, Stein, and Moore (3). In the presence of cupric ions it reacts specifically with an activated carboxyl group at the active site. This communication describes the effect of this reagent on P. janthinellum peptidase A.

Peptidase A was isolated from the culture medium of the organism and purified by a modification of the method described by Hofmann and Shaw (1). The final specific activity was higher than that of the original preparations although no change in the amino acid composition was found. The preparations ran as single bands on acrylamide gel at pH 8.5.

Reactions with diazoacetyl-DL-norleucine methyl ester, prepared as described previously (3), were carried out in the presence of cupric ions at pH 3.4, 4.5, and 5.0 in 0.1 M sodium acetate buffer. The reaction mixture was incubated for 20 min at 36° and the resultant activity was determined with the trypsinogen activation assay (1) or with bovine serum albumin as substrate. It was found that, under the conditions employed, peptidase A was inhibited and that the inhibition was optimal at pH 5.0. At higher pH the enzyme becomes unstable. With 80 mols of diazoacetyl-norleucine methyl ester, and of cupric ions, per mole of enzyme at pH 5.0, complete loss of activity could be achieved. However, in the absence of cupric ions, even with a 500-fold excess of reagent, no significant loss of activity could be detected after incubation for 1 hour at 36°.

To determine whether the reaction was occurring in a similar manner to that described for pepsin, a sample of inactivated peptidase A was analyzed for its norleucine content. Cupric acetate (0.2 M, 50 μl) followed by diazoacetyl-norleucine methyl ester (0.2 M in methanol, 50 μl) was added to a solution of 2 mg of peptidase A in 5 ml of 0.1 M acetate buffer, pH 5.0, to give a 160-fold molar excess of the reagents over the enzyme. The reaction mixture was then incubated for 20 min at 36°, after which it was exhaustively dialyzed. The protein was precipitated with an ethanol-acetone-ether mixture (10:10:1, by volume), and washed twice with the same solvent to remove excess reagents before it was subjected to amino acid analysis on a Spinco, model 120 B, automatic analyzer (4).

The results shown in Table I indicate that only 1 eq of norleucine was incorporated per mole of enzyme after subtracting a small value for a nonspecific incorporation, which was determined on a sample that had been denatured beforehand by incubation at pH 8.0 and 36° for 15 min. Treatment of the inactivated enzyme with 2 M hydroxylamine for 12 hours at pH 9.5 removed the norleucine, suggesting that the reaction involves the formation of an ester bond between diazoacetyl-norleucine methyl ester and a carboxyl group of the enzyme, as postulated by Rajagopalan et al. (3) for pepsin.

The fact that, as in pepsin, only one group (Group A) reacts and produces complete inactivation suggests that this group is

**Table I**

<table>
<thead>
<tr>
<th>Peptidase A</th>
<th>Residues of norleucine incorporated per mole of enzyme</th>
<th>Residual activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>1.1–1.34</td>
<td>0</td>
</tr>
<tr>
<td>Denatured by exposure to pH 8.0</td>
<td>0.57</td>
<td>0</td>
</tr>
<tr>
<td>Treated with inhibitor followed by 2 M hydroxylamine.</td>
<td>0.075</td>
<td>0</td>
</tr>
</tbody>
</table>
activated and is at the active site. The presence of a similar or identical residue at the active site of the two enzymes is strong indication of a similarity in the mechanism of action, and raises the possibility of an evolutionary link. The results also bear upon the classification of proteinases. Hartley (5) defined a group of “acid proteinases” which are characterized by a low pH optimum. Our findings indicate that another common feature of acid proteinases may be the presence of a carboxyl group at the active site. If this is used as a criterion for placing enzymes in the same class, then the basis of all four groups of Hartley’s classification will become uniform and will be the chemical nature of residues at the active site.

There is a notable difference between the two enzymes. Another reagent, p-bromophenacyl bromide (6, 7), which also inhibits pepsin activity by reacting with a carboxyl group, has no effect on peptidase A. That this carboxyl (Group B), a β-carboxyl group of an aspartic acid residue (7), is not the same as the activated Group A which reacts with diazoacetyl-norleucine methyl ester has been recently shown by Erlanger et al. (8). Furthermore, reaction at Group B does not give complete inhibition and, therefore, this group does not play an essential role in catalysis, whereas Group A does (8). Erlanger et al. (8) suggest that steric factors are responsible for inhibition at Group B. Peptidase A appears to lack an analogous group.

Work is now in progress to determine whether the similarities between pepsin and peptidase A extend beyond those already observed. It would be particularly interesting to find out whether the two enzymes display any homology in their amino acid sequence. This would provide more conclusive evidence of an evolutionary link between these enzymes from two widely divergent organisms.

REFERENCES
A Pepsin-like Enzyme from *Penicillium janthinellum*
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