The action of the enzyme tyrosinase on proteins has recently been made the subject of a study by Sizer (1). He has claimed that among a variety of proteins tested those which contained tyrosine could be oxidized by tyrosinase either directly or after pretreatment with trypsin. The proteins most susceptible to the action of tyrosinase were said to be the proteolytic enzymes chymotrypsin, trypsin, and pepsin. Furthermore it was stated that the proteolytic activities of these enzymes were not decreased after the oxidation by tyrosinase of a certain part of the tyrosine residues, and the inference was drawn that the intactness of at least a part of the tyrosine residues was not essential for the action of the enzymes. This question has been reinvestigated in this laboratory as it seemed to us that for several reasons the experimental conditions used by Sizer did not permit an unequivocal interpretation.

Methods and Results

We have in our experiments tried to keep the experimental conditions as close as possible to those used by Sizer.

The crystalline proteolytic enzymes were all prepared in this laboratory (2–4). Stock solutions of the enzymes were made up in dilute hydrochloric acid at pH 3 to 4 and at a concentration of 50 mg. per ml. calculated on dry substance. The chymotrypsin and trypsin preparations contained about 50 per cent magnesium sulfate. The protein concentration was calculated from the Kjeldahl nitrogen.

The tyrosinase preparation used was prepared from common mushrooms and had an activity of 500 Miller and Dawson catecholase units (5) and 16 Adams and Nelson p-cresolase units per ml. (6).

The oxygen uptake was followed in the Warburg apparatus at 37.0° and at a shaking rate of 120 complete oscillations per minute. All experiments were performed in the following way, with one exception stated below. To the reaction flask which had a volume of about 5 ml. were added 1.6 ml. of m/15 phosphate buffer, pH 7.3, 0.2 ml. of the neutralized

* Fellow of the Medical Research Council of Sweden.

1 Kindly supplied by Professor John M. Nelson of the Department of Chemistry, Columbia University, New York.
proteolytic enzyme solution, and 1 drop of toluene. Into the side arm was introduced 0.4 ml. of the tyrosinase solution, together with 1 drop of toluene. It was found necessary to let the vessels shake in the constant temperature bath for 2 to 3 hours in order to attain gas equilibrium. After that time the experiment was started by mixing the solutions. When, at the end of the experiment, the oxygen uptake had ceased, tyrosine was added in order to test the activity of the tyrosinase. In all the experiments the tyrosinase was found still to be active. Controls were also run, differing only in that boiled tyrosinase solution was substituted for the active tyrosinase.

In order to find the equivalency of tyrosine to oxygen in the oxidation by tyrosinase, runs have been made with various amounts of tyrosine oxidized by tyrosinase to completion. On the average 1 mg. of tyrosine took up 205 microliters of oxygen. An equivalency of 3 atoms of oxygen per 1 mole of tyrosine would correspond to 186 microliters of oxygen.

Non-protein tyrosine and tryptophane were determined in the trichloroacetic acid filtrate by the method of Folin and Ciocalteau with Anson's modifications (7). These determinations were made both on the stock enzyme preparation prior to the incubation and on the control with boiled tyrosinase at the end of the experiment. This figure cannot be compared directly with the tyrosine equivalent of the oxygen absorbed, since the former represents both tyrosine and tryptophane.

The reactions were also followed by measurements of the proteolytic activity. In addition to the measurements of protease activity after incubation with boiled or active tyrosinase, the activity was also determined before incubation to determine the effect of the experimental conditions on the proteases. No difference in activity was ever observed between the samples incubated with active tyrosinase and those incubated with the inactive preparation. Chymotrypsin activity was determined by the milk clotting test in the same way as that described by Herriott for pepsin (8), trypsin activity by a new spectrophotometric method, worked out by Kunitz (9), and pepsin activity as described by Anson (7), with the modification that tyrosine and tryptophane were measured spectrophotometrically.

To make a comparison with Sizer's results more convenient our results have been recalculated to a common basis of 25 mg. of protease preparation. This recalculcation involves only the multiplication by the ratio of the amount of proteolytic enzyme preparation used by Sizer and that used by us.

The results are presented in Table I and Fig. 1 but require some comments.

In the case of chymotrypsin there was a slow uptake of oxygen which
TABLE I
Incubation of 25 Mg. of Chymotrypsin, Trypsin, and Pepsin Preparations at pH 7.3 and 37°, with and without Tyrosinase

<table>
<thead>
<tr>
<th>Protease preparation</th>
<th>Reaction time*</th>
<th>Total oxygen uptake</th>
<th>Non-protein tyrosine and tryptophane in absence of tyrosinase, in mg. tyrosine</th>
<th>Per cent loss in proteolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hrs.</td>
<td>microliters</td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Chymotrypsin.........</td>
<td>4½</td>
<td>72</td>
<td>0.28</td>
<td>0.47</td>
</tr>
<tr>
<td>Trypsin I...........</td>
<td>9</td>
<td>320</td>
<td>0.42</td>
<td>3.05</td>
</tr>
<tr>
<td>Pepsin I.............</td>
<td>6½</td>
<td>202</td>
<td>0.95</td>
<td>1.00</td>
</tr>
<tr>
<td>&quot; II..................</td>
<td>8</td>
<td>60</td>
<td>0.11</td>
<td>0.23</td>
</tr>
</tbody>
</table>

* In the reaction time is included the equilibration time prior to the addition of the tyrosinase.

![Diagram](http://www.jbc.org/)

Fig. 1. Oxygen uptake catalyzed by tyrosinase of preparations of crystalline chymotrypsin, trypsin, pepsin, and tyrosine. Each curve represents the oxygen uptake of 25 mg. of enzyme preparation in the presence of about 1 ml. of tyrosinase solution. The tyrosine curve represents 2.5 mg. of tyrosine oxidized by 0.1 ml. of tyrosinase solution.
ceased after about 1 hour. In the starting material there was present a small amount of non-protein tyrosine and tryptophane, which showed a slight increase during the experiment. No decrease could be observed in the proteolytic activity as compared with the starting material.

The results with trypsin (Trypsin I, Table I and Fig. 1) were quite different. A considerable oxygen uptake was observed. There was also a formation of large amounts of non-protein tyrosine and tryptophane during the experiment. Further, no proteolytic activity was left after the 9 hours duration of the experiment. A certain amount of this non-protein tyrosine and tryptophane must have been formed during the equilibration time of 3 hours prior to the addition of the tyrosinase. In order to escape this preliminary decomposition of trypsin the experiment was modified in that, during the equilibration time, the acidified trypsin solution was kept in the side arm instead of the tyrosinase solution. As may be seen (Trypsin II, Fig. 1) the initial rate of oxygen uptake was markedly slower in this case.

The pepsin preparation also took up large amounts of oxygen when treated with tyrosinase (Pepsin I, Table I and Fig. 1). The starting material contained considerable amounts of non-protein tyrosine and tryptophane. No proteolytic activity was left at the end of the experiment. It was found, however, that this loss in activity occurred almost instantaneously after the neutralization of the acidified stock solution of pepsin. This was to be expected, since swine pepsin is known to be rapidly destroyed under the conditions of the experiment (10).

Denaturated pepsin was prepared from native pepsin by adjusting the pH of its solution to 7.3. The denaturated pepsin was then freed from the main part of the contaminating non-protein tyrosine by reprecipitating the protein several times at pH 4, at which the denaturated protein has a minimum of solubility. This preparation was then subjected to the treatment with tyrosinase. As may be seen (Pepsin II, Table I and Fig. 1), the preparation had then lost by far most of its ability to be oxidized by tyrosinase. In spite of the purification the denaturated pepsin still contained a small amount of non-protein tyrosine and tryptophane and an additional small amount was formed during the incubation.

DISCUSSION

From the results obtained certain facts appear, many of which have been known for a long time. In the first place it is shown that all the enzyme preparations tested contain a greater or lesser amount of protein split-products. This is generally the case when no special precautions are taken to avoid it. It is also demonstrated in the case of pepsin that when the main part of the non-protein tyrosine is removed the capacity of the
enzyme preparation to be oxidized by tyrosinase is also mainly lost. Consequently the inference must be drawn that the main substrate for the tyrosinase is the protein split-products present. Actually we have not been able to obtain unequivocal evidence of the action of tyrosinase on any of the proteases investigated.

In the case of trypsin the situation is more complicated in that this enzyme is unstable at pH 7.3 (11) and is rapidly autolyzed during the incubation, with the formation of non-protein tyrosine. When, however, trypsin is incubated for several hours at pH 7.3 prior to the addition of tyrosinase, the initial rate of oxidation by tyrosinase is much faster, which also strongly indicates that it is the split-products containing tyrosine which are oxidized.

The behavior of chymotrypsin is not in conflict with the explanation proposed. The small oxygen uptake might well be explained by amounts of non-protein tyrosine present.

It has been claimed by Sizer that the proteolytic activities of trypsin and pepsin were not decreased during the incubation with tyrosinase at pH 7.3. This statement seems difficult to reconcile with the well established fact that crystalline trypsin is very unstable at this pH in the absence of its substrate (11). Further, it has been known for a long time that pepsin is irreversibly, completely, and almost instantaneously inactivated at a neutral reaction (10, 12, 13). These circumstances were confirmed in our experiments in which we found a complete loss in proteolytic activity of these enzymes after the incubation. The main cause of these divergent results must be that Sizer in his activity measurements used only the enzyme solutions incubated with inactive tyrosinase as controls, whereas we in addition used the original, untreated enzyme solution for the same purpose. The enzyme solutions incubated with boiled tyrosinase, however, are insufficient controls, as they have undergone a profound decomposition which is quite independent of the presence or absence of tyrosinase.

**SUMMARY**

The action of tyrosinase on chymotrypsin, trypsin, and pepsin has been studied. The start of the reaction depends on the purity of the protease preparation used. Preparations which contain non-protein tyrosine absorb considerable quantities of oxygen, whereas in those which have been freed from most of the non-protein tyrosine there is much less reaction. Some decomposition occurs during the course of the reaction itself, especially in the case of trypsin; so that it is difficult to say with certainty that no reaction occurs with the protein, although such a reaction seems unlikely.

Pepsin is instantly inactivated at the pH of the reaction mixture; so
that no conclusion as to the effect of tyrosinase on the activity of this enzyme is possible.

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BIBLIOGRAPHY

Pehr Edman

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