# Research on the Proteolytic Enzymes in Fungi and Bacteria.

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THE presence of proteolytic enzymatic activity has been demonstrated in members of practically all the groups of the plant kingdom by several investigators, among them :—

Wurtz and Bochut, who discovered a trypsin-like ferment in *Carica papaya*.

Tommasoli and Dacommo, who found a proteolytic enzyme in *Anagallis arvensis*.

Markano, who isolated the ferment bromelin from *Ananas*.

Goroup Besanez, who found pepsin in the germinating seeds of *Vicia, Cannabis sativa, Linum usitatissimum*.

Green, who isolated from the germinating seeds of *Ricinus* a trypsin-like ferment, which had the power of converting albumin and globulin into peptone and asparagin.

For the first definite knowledge of proteolytic enzymatic activity in bacteria, however, we are indebted to Bitter, who in 1887 isolated from cultures of *Vibrio choleræ-asiaticæ* and *Vibrio Finkler-Prior*, a ferment which was capable of dissolving gelatine and fibrin.

The fundamental work in connection with the activities of proteolytic enzymes is that of Fermi, who, in his various experiments with members of the higher and lower forms of plant life, gives many instances of proteolysis.

These results and those obtained by other observers, combined with the general indications of the almost universal occurrence and activity of proteolytic enzymes throughout animal and vegetable life, would seem to render a systematic search for such enzymes amongst fungi and bacteria desirable, in order to ascertain, if their presence may be regarded as essential to fungoid and bacterial life, the functions they have in connection with such life, and the manner in which, as well as the conditions under which, these are carried out.

I have been fortunate enough to obtain a Carnegie Scholarship for the purpose of a research in this direction, and have been

[Notes R.B.G., Edin., No. XXI, August 1909.]

granted also the privilege of working in the Mycological Department of the Heriot-Watt College, Edinburgh, under its director, Dr. Westergaard. I wish to express here my sincere thanks for this.

In the two reports which follow will be found an account of the methods employed by me and the results which I have obtained in determining whether or not proteolytic enzymatic activity is generally present in fungi and bacteria.

## FIRST REPORT.

Up to the present I have confined my attention entirely to the higher fungi, as these can be most readily obtained in September and October growing in large numbers in woodland and meadow, and I have left the lower forms, which can be cultivated in the laboratory at any time for subsequent experiment.

Towards the end of September the following species of fungi were collected :—

*Agaricus melleus, A. sulphureus, A. squarrosus, Merulius lacrymans, Polyporus betulinus, P. sulphureus, P. squamosus, Lactarius insulsus, L. cilicioides, Russula Clusii, Cortinarius* sp., *Boletus* sp.

**Preparation of Enzyme.**—Each species of fungus was ground up separately in a mortar with sand and sufficient water to form a thick paste. After a homogeneous mixture had been obtained more water was added, and again the contents of the mortar were thoroughly mixed. The contents were then filtered, and to the filtrate was added twice its volume of absolute alcohol. On the addition of the alcohol a precipitate fell, which was filtered off, washed with alcohol and ether, and dried in a vacuum desiccator over concentrated sulphuric acid at room temperature.

My intention was, as stated in the account of the proposed work, to determine the proteolytic activity in each species of fungus by allowing the enzymatic preparation to act upon wheat protein, which is insoluble in water and soluble in 55 per cent. alcohol.

The method used by Kjeldahl and afterwards by Weise to obtain this protein—by extracting wheat flour with 55 per cent. alcohol and freezing the extract,—gives a very small yield, and is in addition rather long and troublesome, and the following modification suggested by Dr. Westergaard was therefore adopted :—

**Preparation of Protein.**—Gluten flour was extracted with three times its weight of 55 per cent. alcohol at room temperature

for twenty-four hours. To the filtered extract an equal volume of ether was added, which caused a heavy brown semi-liquid substance to separate out. This substance was, by treatment with cold absolute alcohol during several days, converted into a hard white brittle mass, which, on being suspended in cold water became soft and spongy, and again hardened, when placed in alcohol. By thus alternately washing with water and with alcohol, the preparation was purified as far as possible, and ultimately, after the last treatment with alcohol, it was washed with ether, dried in a vacuum at room temperature, and ground to powder.

**Estimation of Nitrogen in Protein.**—·1 gm. protein was boiled with 20 c.c. concentratd sulphuric acid (nitrogen free) and potassium sulphate till the liquid was quite clear, when potassium permanganate was added in excess and the ammonia estimated by distillation with caustic soda.

RESULTS.—*Expt.* 1. 15·9 per cent. nitrogen.
*Expt.* 2. 16 „ „

These results are slightly lower than those obtained by Kjeldahl, whose protein contained 17·25 per cent. nitrogen.

This powder was then used for testing the proteolytic activity of the fungi or preparations of these in the following manner :—

In each case three flasks were employed, each containing accurately the same quantity of protein, enzymatic preparation, and water. To one of these flasks tannic acid and a trace of sodium acetate were added at once, whilst the other two were placed at 50° C. for twenty hours, when they each received the same quantity of tannic acid solution and sodium acetate as the first one.

The effect of the tannic acid is to precipitate the proteids, proteoses, and peptones present so that the filtrate will only contain nitrogenous compounds of a simpler constitution than these. The amount of nitrogen found in the filtrates from the flasks, which were kept at 50° C., compared with that contained in the filtrate from the flask that was precipitated at once, will be an indication of the degree of proteolytic activity, and the results obtained expressed in c.c.'s of deci-normal ammonia may be used for comparison if the conditions as time, temperature, concentration, etc., are kept uniform.

**Preliminary Experiments.**—In order to test the methods, the following three determinations were carried out, using the preparations obtained in the manner already described from :—*Polyporus sulphureus, P. squamosus, Merulius lacrymans.*

In each case ·5 gm. of the enzymatic preparation was dis-

solved in 20 c.c. water and filtered. Five c.c. of the filtrate were placed in each of three 100 c.c. flasks, along with ·2 gm. of the protein and 25 c.c. of water. The flasks were treated as described above, and after precipitation with tannic acid, made up to 100 c.c. with water, and the contents were filtered.

The nitrogen was in each case determined in 30 c.c. of the filtrate, with the following results :—

POLYPORUS SQUAMOSUS.

| | | | | | | |
|---|---|---|---|---|---|---|
| (*a*) | Flask with proteolytic activity gave | . | 3·5 c.c. | $\frac{N}{10}$ | $NH_3$ |
| (*b*) | ,, ,, ,, | . | 4·0 c.c. | ,, | ,, |
| (*c*) | ,, no ,, ,, | . | 1·0 c.c. | ,, | ,, |

POLYPORUS SULPHUREUS.

| | | | | | | |
|---|---|---|---|---|---|---|
| (*a*) | Flask with proteolytic activity gave | . | 4·4 c.c. | $\frac{N}{10}$ | $NH_3$ |
| (*b*) | ,, ,, ,, | . | 3·0 c.c. | ,, | ,, |
| (*c*) | ,, no ,, ,, | . | ·5 c.c. | ,, | ,, |

MERULIUS LACRYMANS.

| | | | | | | |
|---|---|---|---|---|---|---|
| (*a*) | Flask with proteolytic activity gave | . | 3·7 c.c. | $\frac{N}{10}$ | $NH_3$ |
| (*b*) | ,, ,, ,, | . | 4·5 c.c. | ,, | ,, |
| (*c*) | ,, no ,, ,, | . | ·6 c.c. | ,, | ,, |

These results show clearly that active proteolyses has been going on in all three cases.

The accuracy was not satisfactory, especially in the case of *P. sulphureus*, but this was found to be due, not to the method, but to minor errors in the manipulations ; in all the subsequent determinations the results of the duplicates have come out practically the same.

In order to test the effect of a slight trace of acid or alkali on the activity the following experiment was carried out with the enzymatic preparation obtained from *P. squamosus*.

·5 gm. of the preparation was dissolved in 30 c.c. water, filtered, and 3 c.c. of filtrate put into each of nine test tubes along with ·2 gm. of protein. To the first three tubes hydrochloric acid was added, so that in 6 c.c. of the resulting solution the acidity would be equal to ·1 per cent. To the second three tubes sodium carbonate was added, so that in 6 c.c. of resulting solution the degree of alkalinity would be equivalent to that of acidity in the first three. To the last three tubes 3 c.c. of water were added.

Each series of three tubes was then treated in the same manner as described in the case of *P. sulphureus*, etc., and the following results obtained :—

ACID SOLUTION.

(*a*) Tube with proteolytic activity gave . 2·4 c.c. $\frac{N}{10}$ $NH_3$
(*b*) ,, ,, ,, . 2·7 c.c. ,, ,,
(*c*) ,, no ,, ,, . 1·8 c.c. ,, ,,

ALKALINE SOLUTION.

(*a*) Tube with proteolytic activity gave . 1·8 c.c. $\frac{N}{10}$ $NH_3$
(*b*) ,, ,, ,, . 1·9 c.c. ,, ,,
(*c*) ,, no ,, ,, . 1·6 c.c. ,, ,,

NORMAL SOLUTION.

(*a*) Tube with proteolytic activity gave . 1·2 c.c. $\frac{N}{10}$ $NH_3$
(*b*) ,, ,, ,, . 1·5 c.c. ,, ,,
(*c*) ,, no ,, ,, . ·5 c.c. ,, ,,

These results show that in this case acid aids while alkali retards proteolyses.

The several species of fungi mentioned at the beginning were examined in the following manner :—

1. Auto-digestions.
2. Preparation of enzyme.
3. Direct examination of the juice of the fungi.

1. The auto-digestions were made chiefly in order to have material to fall back upon for confirmation of results, and were carried out in the following way :—

In the case of each fungus two equal quantities were weighed out, one portion was boiled for a few minutes, both lots were then ground up separately in a mortar with sand and an equal volume of water till a homogeneous mixture was obtained ; they were then bottled and shaken up with a little toluol.

2. The preparation of the enzyme was carried out as already described.

3. Whilst the above-mentioned preparations were laid aside to be used afterwards for a detailed examination of the nature of each enzyme, the proteolytic activity of each of the fifteen

species was tested by using the juice pressed from the fresh fungi.

Fifty gm. of each of the species were ground up with sand and a few c.c.'s of distilled water, filtered, and the filtrate made up to 100 c.c. These liquids were then used for experiment on the lines described above in the case of *P. squamosus*.

In each case nine tubes were used, making 135 in all, which were divided into lots of three flasks each.

The first three flasks each received 8 c.c. of the fungus juice and 22 c.c. of distilled water.

The second three flasks each received the same amount of juice and so much sulphuric acid and water to bring the mixture to a total volume of 30 c.c. containing so much acid that the total acidity corresponded to ·1 per cent. sulphuric acid.

The third set of three flasks were made up as above, with the sole difference that sodium carbonate was used instead of sulphuric acid in quantities to make 30 c.c. of the liquid have a degree of alkalinity exactly equivalent to the acidity of the previous lot.

After the tubes were all ready, ·2 gm. of the protein was added and the digestions carried out at 50° C. for four hours, when the proteids, peptones, and proteoses, etc., were precipitated, leaving amino acids in solution.

## SECOND REPORT.

As mentioned in the first report extracts of fifteen fungi were brought into contact with protein.

In each case three sets of three tubes were used. In one set the extract was taken directly from the fungus; to the extract in the second set was added hydrochloric acid to such an extent that the total acidity was ·1 per cent.; to the extract in the third set sodium carbonate was added, so that the resulting alkalinity was equivalent to the degree of acidity in the second set.

The digestions of the protein were carried out at 50° C. for four hours, when the peptones, etc., were precipitated, leaving amino acids in solution.

The liquid was then filtered and evaporated down with a little concentrated sulphuric acid till fumes of the latter were observed to come off, when other 20 c.c. were added and the flask heated till the contents were clear. Potassium permanganate was then added in excess to oxidise any organic material present.

The flasks were then washed into larger ones, and concen-

trated caustic soda added when ammonia was distilled over and absorbed by deci-normal sulphuric acid.

The results obtained are found in the following table whose figures denote tenths of 1 c.c. deci-normal ammonia.

(Flasks (1) and (2) are duplicates, flask (3) is a blank.)

| | Flasks. | | |
|---|---|---|---|
| AGARICUS AGGREGATUS. | (1) | (2) | (3) |
| Digestion of protein by enzyme in HCl soln. . | 30 | — | 11 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 26 | 24 | 15 |
| ,, ,, ,, $H_2O$ ,, . | 22 | 23 | 15 |
| PEZIZA SP. | | | |
| Digestion of protein by enzyme in HCl soln. . | 40 | 40 | 25 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 35 | 36 | 30 |
| ,, ,, ,, $H_2O$ ,, . | 36 | — | 30 |
| A. AGARICUS (SPECIES NOT DETERMINED). | | | |
| Digestion of protein by enzyme in HCl soln. . | 26 | 25 | 20 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 23 | 22 | 20 |
| ,, ,, ,, $H_2O$ ,, . | 38 | 36 | 17 |
| AGARICUS ARVENSIS. | | | |
| Digestion of protein by enzyme in HCl soln. . | 58 | 57 | 50 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 57 | 55 | 50 |
| ,, ,, ,, $H_2O$ ,, . | — | — | — |
| AMANITA VAGINATUS. | | | |
| Digestion of protein by enzyme in HCl soln. . | 30 | 29 | 25 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 24 | 25 | 20 |
| ,, ,, ,, $H_2O$ ,, . | 30 | 30 | 20 |
| NO. 27 (NAME NOT DETERMINED). | | | |
| Digestion of protein by enzyme in HCl soln. . | 30 | 30 | 20 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 25 | 24 | 22 |
| ,, ,, ,, $H_2O$ ,, . | 25 | 24 | 20 |
| AGARICUS MELLEUS. | | | |
| Digestion of protein by enzyme in HCl soln. . | 10 | — | 5 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 15 | 14 | 10 |
| ,, ,, ,, $H_2O$ ,, . | 15 | 16 | 7 |

| | Flasks. | | |
|---|---|---|---|
| | (1) | (2) | (3) |
| **Lactarius insulsus.** | | | |
| Digestion of protein by enzyme in HCl soln. . | 14 | 15 | 10 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 10 | 9 | 7 |
| ,, ,, ,, $H_2O$ ,, . | 10 | 10 | 2 |
| **Russula cyanoxantha.** | | | |
| Digestion of protein by enzyme in HCl soln. . | 15 | 14 | 10 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 17 | 18 | 15 |
| ,, ,, ,, $H_2O$ ,, . | 20 | 19 | 5 |
| **Agaricus sp. B.** | | | |
| Digestion of protein by enzyme in HCl soln. . | 20 | 19 | 14 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 15 | 15 | 10 |
| ,, ,, ,, $H_2O$ ,, . | 15 | 14 | 10 |
| **Lactarius cilicioides.** | | | |
| Digestion of protein by enzyme in HCl soln. . | 25 | — | 17 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 19 | 18 | 12 |
| ,, ,, ,, $H_2O$ ,, . | — | — | — |
| **Agaricus sp. C.** | | | |
| Digestion of protein by enzyme in HCl soln. . | 18 | 18 | 15 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 14 | 14 | 12 |
| ,, ,, ,, $H_2O$ ,, . | — | — | — |
| **Polyporus betulinus.** | | | |
| Digestion of protein by enzyme in HCl soln. . | 25 | 26 | 20 |
| ,, ,, ,, $Na_2Co_3$ ,, . | — | 16 | 13 |
| ,, ,, ,, $H_2O$ ,, . | 22 | 21 | 15 |
| **Russula Clusii.** | | | |
| Digestion of protein by enzyme in HCl soln. . | 16 | 15 | 10 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 15 | 14 | 10 |
| ,, ,, ,, $H_2O$ ,, . | 13 | 12 | 8 |
| **Agaricus squarrosus.** | | | |
| Digestion of protein by enzyme in HCl soln. . | 18 | 17 | 13 |
| ,, ,, ,, $Na_2Co_3$ ,, . | 20 | 19 | 11 |
| ,, ,, ,, $H_2O$ ,, . | 14 | 13 | 9 |

On analysing the above table, subtracting the figures in the third column from those in columns (1) and (2) and taking the average, the figures set out in the following table are obtained :—

| Fungus | HCl | $Na_2Co_3$ | $H_2O$ |
|---|---|---|---|
| *Agaricus aggregatus* | 19·0 | 10·0 | 7·5 |
| *Peziza* sp. | 15·0 | 5·5 | 5·5 |
| *Agaricus* sp. A. | 5·5 | 2·5 | 20·0 |
| *Agaricus arvensis* | 7·5 | 6·0 | — |
| *Amanita vaginatus* | 4·5 | 2·5 | 10·0 |
| No. 27 | 10·0 | 2·5 | 4·5 |
| *Agaricus melleus* | 5·0 | 4·5 | 8·5 |
| *Lactarius insulsus* | 4·5 | 2·5 | 8·0 |
| *Russula cyanoxantha* | 4·5 | 2·5 | 15·5 |
| *Agaricus* sp. B. | 5·5 | 5·0 | 4·5 |
| *Lactarius cilicioides* | 8·0 | 6·5 | — |
| *Agaricus* sp. C. | 3·0 | 2·0 | — |
| *Polyporus betulinus* | 5·5 | 3·0 | 6·5 |
| *Russula Clusii* | 5·5 | 4·5 | 4·5 |
| *Agaricus squarrosus* | 4·5 | 8·5 | 4·5 |

In studying the above table one observes—

1. That proteolytic activity is present in every case.

2. That the proteolytic activity in the acid medium is distinctly highest in three, viz. :—*Agaricus aggregatus, Peziza,* and No. 27.

3. That the proteolytic activity in the alkaline medium is distinctly highest in one, viz. :—*Agaricus squarrosus.*

4. That the proteolytic activity in the media to which neither acid nor alkali were added is distinctly highest in six, viz. :—*Agaricus* A., *Amanita vaginatus, Agaricus melleus, Lactarius insulsus, Russula cyanoxantha,* and *Polyporus betulinus.*

5. That proteolytic activity is greater in acid than in alkaline media in three, viz. :—*Lactarius cilicioides, Agaricus* C., and *Agaricus arvensis.*

6. That proteolytic activity is increased by both acid and alkali in *Agaricus aggregatus.*

7. That the proteolytic activity is practically the same in all three media in the case of *Agaricus* B.

Having finished this experiment with the above members of the higher fungi, and made enzymatic preparations from several others which have yet to be examined, pure cultures were made of the following lower fungi and bacteria :—

*Mucor Mucedo, Mucor spinosus, Mucor racemosus, Aspergillus niger, Aspergillus glaucus, Aspergillus oryzæ, Monilia*

*candidia, Chalara mycoderma, Oidium lactis, Cladosporium herbarium, Bacillus subtilis, Tyrothrix tenuis, Bacillus prodigiosus, Streptothrix lactici acidi.*

After getting pure cultures of the above fungi, ten flasks containing sterile malt extract were inoculated with the ten forms.

The pure cultures of the bacteria were introduced separately to flasks containing sterile glucose peptone (1 per cent. peptone, 4 per cent. glucose). When the development had proceeded sufficiently far the fungi or bacteria were separated from the nutrient medium, and both organisms and media tested for proteolytic activity.

In the case of the fungi the malt extract was filtered off and the mass of fungus mycelium washed a few times with distilled water. The fungus was then ground into a fine state with sand and water, and the extract filtered off. Twenty-five c.c. of the filtrate were put into each of three flasks containing ·2 gm. protein. To one 5 c.c. of a 20 per cent. solution of tannic acid was at once added, and all three flasks incubated at 47° C. for five hours.

After this time the other two flasks received 5 c.c. of tannic acid ; all three made up to 50 c.c. and filtered.

Twenty-five c.c. of the filtrate were evaporated down with a little concentrated sulphuric acid till fumes of the latter came off, when other 20 c.c. were added, and the flasks heated till the contents were clear, when distillation was carried out in the way already mentioned.

The malt extract was tested in the same manner as the mycelium extract, 25 c.c. being used.

So far the experiments with *Aspergillus niger* and *Cladosporium herbarium* have been completed with the following results :—

(Figures represent tenths of 1 c.c. deci-normal ammonia.)

Result of digestion with—

| | Medium. | | | Extract from Fungus. | | |
|---|---|---|---|---|---|---|
| | (1) | (2) | (3) | (1) | (2) | (3) |
| *Aspergillus niger* . . | 127 | 127 | 30 | 20 | 20 | 10 |
| *Cladosporium herbarium* . . | 42 | 41 | 21 | 18 | 17 | 10 |

from which we get the following figures, representing average proteolytic activity in the medium and mycelium :—

| | Medium. | Extract from Mycelium. |
|---|---|---|
| *Aspergillus niger* . . . | 97 | 10 |
| *Cladosporium herbarium* . . | 20·5 | 7·5 |

It will be noticed that proteolytic activity is present in both extracts in both fungi, but it is extremely marked in the medium in the case of *Aspergillus niger*.

This indicates that the fungus during its growth excretes into the medium, in which it is situated, a proteolytic enzyme, which brings the complex proteids into a fit state for assimilation by the organisms.

In experimenting with *Bacillus prodigiosus*, the bacteria were separated from the medium by means of a centrifugal machine, and ground in a mortar with solid carbon dioxide and ether. Ten c.c. of distilled water were then added, and 3 c.c. of the mixture put into each of three flasks with ·2 gm. protein, and the experiment carried out as in the other cases. The medium was filtered through a Chamberland filter in order to get rid of the remaining bacteria, and then used for the digestions in the usual manner.