ENZYMES OF *ASPERGILLUS ORYZAE*

III. THE SEQUENCE OF APPEARANCE AND SOME PROPERTIES OF THE ENZYMES LIBERATED DURING GROWTH

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Summary

When a strain of the mould *Aspergillus oryzae* is grown in surface culture on a protein-free sucrose-tartrate medium, enzymes are released into the culture medium in the following order: first, the carbohydrases sucrase and amylase together with phosphatase, second, the proteases and esterases, and third, catalase. Their liberation is accompanied by the appearance of protein nitrogen in the medium and loss in weight of the mycelium.

Evidence is presented for the presence of at least two proteases in addition to glycylglycine dipeptidase—one reduces the viscosity of gelatin very rapidly and is believed to act preferentially on the high-molecular-weight fractions of gelatin (the "viscometric protease") whereas the other degrades the lower molecular components of the gelatin more readily (the "gravimetric protease"). The pH activity curves for sucrase and amylase also indicate the presence of more than one component.

Inactivation of the various enzymes present during incubation at 40°C and at different pH values has been followed.

I. INTRODUCTION

The development of a simple liquid medium for the production of highly active proteolytic solutions by surface culture of *Aspergillus oryzae* (Maxwell 1952) has provided material for further investigations on the properties and purification of the proteases and associated enzymes. Previous research on fungal proteases, such as that on taka-diastase, has been concerned with the examination of aqueous extracts of bran cultures of moulds (for example, Lichenstein 1944) or of extracts of ground mould mycelium (Johnson 1934). Solutions prepared in this way contain more colloidal material than the culture filtrates used in the present investigations and they are therefore less amenable to fractionation and characterization studies.

This paper describes the range of enzymes detected, their sequence of appearance in the liquid medium, and some properties of individual enzymes as determined by examination of dialysed and concentrated culture filtrates.

II. ENZYMES DETECTED

Proteases which rapidly reduce the viscosity of gelatin solutions and cause rapid digestion of the lower-molecular-weight components of gelatin have been detected in the culture filtrate. Esterase, phosphatase, amylase, sucrase, and

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catalase were also liberated from the mycelium in sufficient quantity for convenient estimation. Enzymes catalysing the liberation of inorganic phosphate from phytic acid and lecithin, various dipeptidases, lactase, maltase, salicinase, nuclease, and urease were also detected and, judging by the loss of strength of cellophane during 3-4 hr dialysis of concentrated culture filtrates, a cellulase may also be present, but attempts to demonstrate the production of reducing sugar from cellulose during incubation for several days at 40°C were unsuccessful.

The following enzymes, which include several previously reported for *A. oryzae* (see, for example, Tauber 1949) were not detected: tannase, glucose dehydrogenase, glucose oxidase, tyrosinase, polygalacturonase. No significant hydrolysis of gum arabic, gum acacia, or agar agar could be detected.

III. Materials and Methods

(*a*) *Preparation of Mould Enzymes*

In experiments designed to measure the rate of enzyme production in the culture medium, the mould was grown at 22°C in 2-l bottles, each with 300 ml medium containing 4.0 per cent. sucrose, 3.0 per cent. sodium potassium tartrate, 1.1 per cent. NH_4Cl, 0.3 per cent. K_2HPO_4, 0.05 per cent. $MgSO_4.7H_2O$, 0.002 per cent. $FeSO_4.7H_2O$, and 0.1 p.p.m. Zn, adjusted to pH 6.2,* and giving a volume : surface ratio of 1.24. In all other experiments the volume of medium used was reduced to 225 ml and the ratio thereby to 0.93 in order to improve the yield of enzyme per unit volume of medium (Maxwell 1952).

The effect of pH on loss of activity of various enzymes during storage was studied using the filtrate from 10-day-old cultures. The culture filtrate (20 l) at 2°C was precipitated with 40 l ethanol at −20°C and the precipitate was suspended in 500 ml water and dialysed with agitation for 1 hr against tap water. The liquid was centrifuged to remove insoluble salts, dialysed for a further period of 2 hr in a rotating dialyser (Lennox 1949), and finally diluted to 2 l.

In the pH-activity studies, a mixed enzyme powder was prepared by adding acetate buffer at pH 5.6 to the above enzyme concentrate to give a final concentration of 0.1M, cooling to 2°C, precipitating with 4 l ethanol at −20°C, centrifuging, and vacuum-drying the precipitate in 3-5 hr. In determining the pH-activity curves for the action of proteases on some substrates a freeze-dried preparation of a dialysed enzyme concentrate was also used, and in some experiments protease-rich crystals were employed (Crewther and Lennox 1950).

(*b*) *Estimation of Enzyme Activity*

(i) *Protease.*—Estimations of the viscometric protease activity were carried out by the gelatin viscosity reduction method (Lennox and Ellis 1945) using an incubation period of 90 sec. Digestion of the lower-molecular-weight components of gelatin was determined by the gravimetric protease method (Crewther

* In some instances the concentrations of some of the constituents were varied slightly.

1952). The degradation of haemoglobin and ovalbumin in pH-activity experiments was measured by the method of Anson (1938), the recorded pH being that of the enzyme-substrate mixture.

(ii) *Peptidase.*—Samples (0.1 ml) were removed from a 0.1M solution of peptide containing the enzyme before and after 4 hr incubation at 40°C. After the addition of 1 ml of 90 per cent. acetone, containing 0.001 per cent. naphthyl red, the samples were titrated with 0.1N HCl in 90 per cent. ethanol from a 0.1 ml Rehberg microburette (Linderstrøm-Lang 1927).

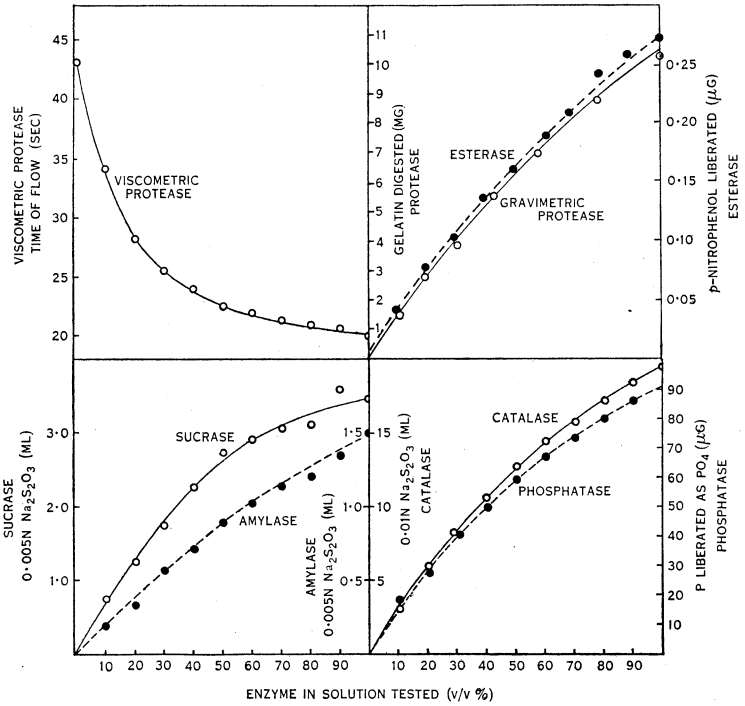

Fig. 1.—Dilution-activity curves of *A. oryzae* enzymes.

(iii) *Esterase.*—The method of Huggins and Lapides (1947) was used with *p*-nitrophenol acetate in phosphate buffer at pH 7.0 as substrate.

(iv) *Amylase.*—The reducing power of a mixture of 5 ml of buffer substrate (1.2 per cent. starch in 0.05M phosphate buffer at pH 7.5) and 1 ml of enzyme solution was estimated before and after incubation at 40°C for 1 hr using dilute Somogyi's reagent.*

(v) *Sucrase.*—As for amylase, but using a mixture of 1 ml of enzyme solution and 20 ml of 10 per cent. sucrose in 0.1N acetate buffer at pH 5.4, and incubating for 20 min at 40°C.

* Prepared by mixing 2 volumes of Somogyi's reagent (Somogyi 1945) with 2 volumes 0.025N KIO_3 and 5 volumes distilled water, and filtering immediately before use.

(vi) *Phosphatase.*—Inorganic phosphate in a mixture of 3 ml of buffer substrate (0.25 per cent. sodium β-glycerophosphate, 0.25 per cent. $MgCl_2$, in 0.1M phthalate at pH 5.0) and 1 ml of enzyme solution was estimated before and after incubation at 40°C for 1 hr, by the method of Fiske and Subbarow (1925).

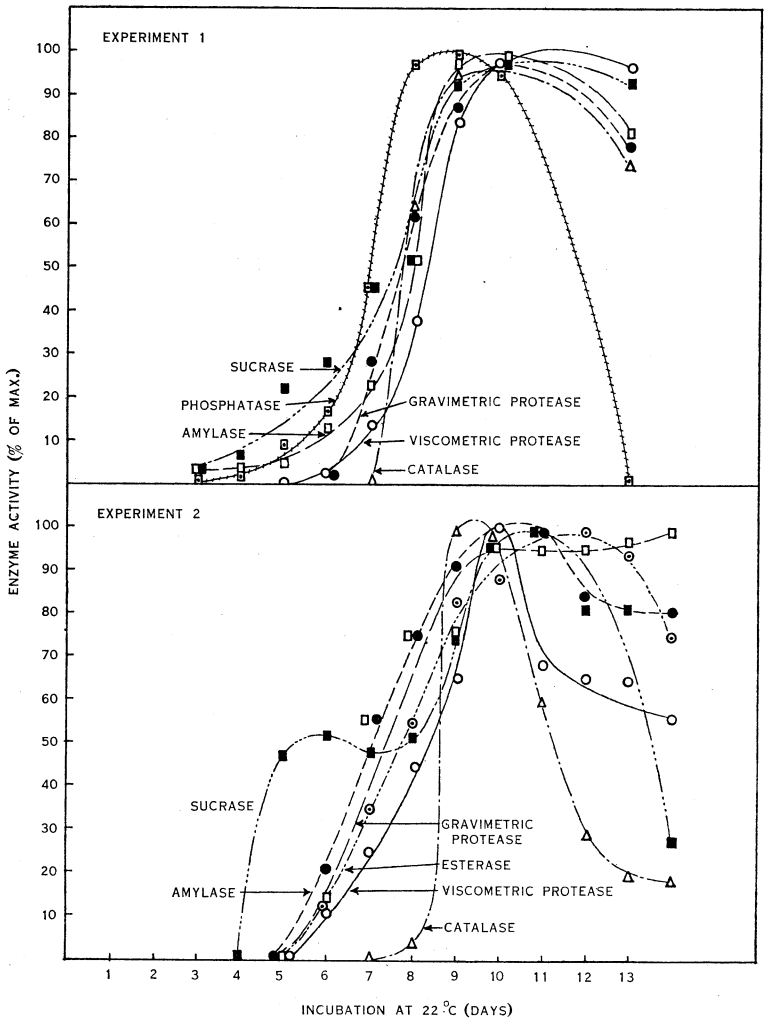

Fig. 2.—Enzyme liberation by *A. oryzae* at various stages of growth.

(vii) *Catalase.*—The buffer-substrate solution used contained 10 ml 100 volume H_2O_2 per l of pH 6.7, 0.01M phosphate buffer. After chilling to 0°C 0.5 ml enzyme was mixed with 2 ml substrate, the mixture was held for 30 min at 0°C, 2 ml 2N H_2SO_4 and 2 ml 10 per cent. KI were added, and the liberated I_2 was titrated with 0.01N $Na_2S_2O_3$.

A curve relating enzyme concentration to activity was obtained for each of the enzymes investigated (Fig. 1), and activity values were converted to rectilinear units of enzyme activity by reference to the appropriate curve.

(c) *Other Analytical Methods*

(i) *Weight of Mycelium.*—After filtration of the cultures, the tared filter paper and mycelium from each was dried for 7 days at 65°C and weighed.

(ii) *pH.*—pH was measured with a glass electrode assembly.

(iii) *Total Nitrogen.*—Estimated by H_2SO_4-H_2O_2 digestion of 1-ml portions of solution, containing 0.1-0.2 mg N, Nesslerization as directed by Koch and McMeekin (1924), and measurement of the light transmittance by these solutions at 412.5 mμ.

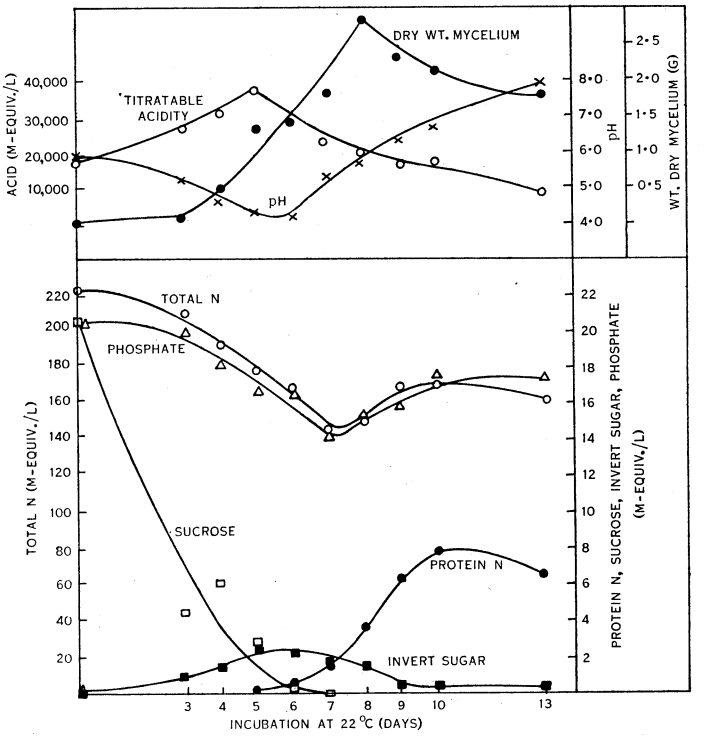

Fig. 3.—Changes in composition of medium during growth of *A. oryzae.*

(iv) *Precipitable Protein Nitrogen.*—After dialysis with continuous agitation for 4 hr, protein was precipitated from 1 ml culture filtrate by the addition of 2 ml 10 per cent. trichloroacetic acid, allowed to stand overnight to flocculate, centrifuged, washed with 10 per cent. trichloroacetic acid, and N in the precipitate estimated as described above.

(v) *Reducing Power.*—The apparent reducing sugar was estimated in 1-ml portions of the culture filtrate by the copper reduction method described above for amylase.

(vi) *Sucrose.*—The sucrose in 1 ml culture filtrate was hydrolysed by incubation for 24 hr at 40°C with 0.5 ml invertase (Pfanstiehl) and diluted to 20 ml for estimation, by the copper reduction method, of the increase in the content of reducing sugar due to the enzymic hydrolysis.

(vii) *Phosphate.*—The method used was that described for estimating the phosphate liberated by phosphatase.

IV. Results

(*a*) *Order of Appearance of Enzymes and Associated Changes in Composition of the Culture Medium*

As will be seen from Figure 2, no enzymes were detected in the medium for several days after inoculation. Sucrase, phosphatase, and amylase were the first to appear after 4-5 days, followed by the proteases and esterase. Owing to the high sensitivity of the method of estimating catalase, traces of this enzyme were detected after only 3 days' incubation, but it was not until the eighth day that any significant fraction of the maximum catalase activity appeared. Other changes in the composition of the medium are shown in Figure 3. Of chief interest is the disappearance of most of the sucrose by the sixth day, the highest concentration of invert sugar occurring on the fifth day. This coincides with the appearance of significant quantities of enzymes and precipitable protein in the medium, both of which are maximal at the 10th day. The mycelial weight, on the other hand, is maximal at the eighth day with a rapid decrease to the 10th day corresponding with the rapid increase in enzyme activity.

Table 1

DISTRIBUTION OF VISCOMETRIC PROTEASE BETWEEN THE CULTURE MEDIUM AND THE MYCELIUM

Age of Culture (days)	Culture Filtrate (viscometric units)	Water Washings (viscometric units)	Water Extract (viscometric units)	0·1M $NaHCO_3$ Extract (viscometric units)
4	Nil	Nil	Nil	Nil
5	0·13	0·15	0·40	0·26
6	2·45	0·62	1·12	1·00

The amount of protease in the mycelium was determined by washing the mycelium with a volume of water equal to that of the culture medium (225 ml), the residuum then being extracted in the Waring Blendor with 225 ml of water followed by 225 ml of 0.1M $NaHCO_3$. The results for 5-day and 6-day mycelial mats are shown in Table 1.

(*b*) *Proteases of* A. oryzae

Considerable evidence has accumulated for the presence of more than one protease in the culture filtrates. For example, the ratio of the gravimetric protease activity to the viscometric activity, after surface culture of *A. oryzae* on a medium containing a mixture of acetate and formate in place of the usual tartrate, was found to increase from 5.8 to greater than 78 as the ammonium chloride content of the medium was reduced from 1 to 0.2 per cent. (Table 2). Citrate medium provided similar results.

TABLE 2

EFFECT OF AMMONIUM CHLORIDE CONCENTRATION IN THE MEDIUM ON THE RELATIVE AMOUNTS OF VISCOMETRIC AND GRAVIMETRIC PROTEASE PRODUCED

A. oryzae was grown on a medium containing 4 per cent. sucrose, 1 per cent. acetic acid, 0·5 per cent. formic acid, and salts as in the standard medium, and adjusted to pH 6·2

NH_4Cl in the Medium (%)	Protease Activity		Ratio
	Viscometric (arbitrary units)	Gravimetric (arbitrary units)	Gravimetric Activity / Viscometric Activity
0·2	1·6	125	78
0·5	1·1	33	30
1·0	1·3	7·5	6

The pH-activity curves obtained by the gravimetric and viscometric techniques also diverge to a considerable extent. The gravimetric technique (Fig. 4) suggests the presence of at least three enzymes with optima at pH about

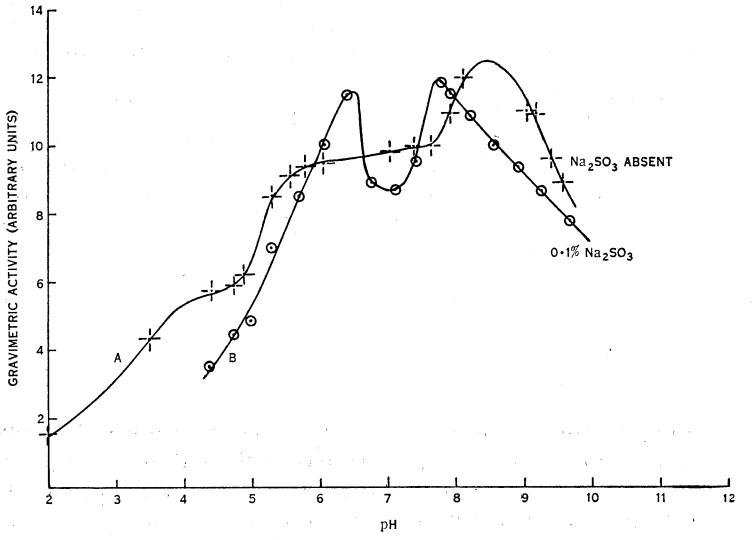

Fig. 4.—Effect of pH on the gravimetric protease activity.

4.5, 6.0, and 8.5, whereas the viscometric method, which also provides an atypical curve, suggests the presence of two components with optima at pH 7.0-7.5, and 10.0 (Fig. 5). Several enzymes also may be involved in the digestion of ovalbumin and haemoglobin.

TABLE 3

EFFECT OF INHIBITORS ON THE PROTEOLYTIC ACTION OF PARTLY PURIFIED MOULD PROTEASE

Inhibitor	Final Concentration (M)	Inhibition (%)	
		Viscometric Method	Gravimetric Method
Sodium *p*-aminophenyl arsenate	$1{\cdot}5\times10^{-1}$	27	Slight activation
Mercuric acetate	1×10^{-3}	67	Slight activation
Cupferron	1×10^{-3}	Slight activation	Slight activation
8-Hydroxyquinoline	1×10^{-3}	91	56
Iodoacetamide	5×10^{-1}	1	Nil
Sodium iodoacetate	5×10^{-1}	Nil	Nil

TABLE 4

EFFECT OF OXIDIZING AND REDUCING AGENTS ON THE PROTEOLYTIC ACTIVITY OF FILTRATES FROM TWO CULTURES OF *A. ORYZAE*

Enzyme Preparation	Reagent	Final Concentration of Reagent (M)	Protease Activity (% of original)	
			Viscometric	Gravimetric
Enzyme 1				
Undialysed	Water		100	100
	$KMnO_4$	$1{\cdot}5\times10^{-3}$	33	47
	I_2	$1{\cdot}5\times10^{-3}$	14	7
	H_2O_2	$3{\cdot}0\times10^{-2}$	65	245
Dialysed	Water		70	20
	Na_2SO_3	$1{\cdot}0\times10^{-1}$	40	95
	Cysteine	$2{\cdot}0\times10^{-2}$	80	40
	Cystine	$1{\cdot}0\times10^{-3}$	45	15
Enzyme 2				
Undialysed	Water		100	100
Dialysed	Water		90	73
	Na_2SO_3	$1{\cdot}0\times10^{-1}$	86	30
	H_2O_2	$2{\cdot}0\times10^{-1}$	94	0·3
	Na_2SO_3	1×10^{-1} (30 min after 2×10^{-1} H_2O_2)	88	16

The effects of sulphydryl-reacting compounds, metal chelating agents, oxidizing and reducing agents, and dialysis of the enzyme preparation have also been found to vary with the method used for estimating the protease activity (Tables 3 and 4). The presence of sulphite in the buffer substrate used for the gravimetric protease method also affects the shape of the pH-activity curve obtained by the gravimetric method (Fig. 4), the sulphite activating or inhibiting according to the pH. The effects of sulphite on the activity of the mould proteases also depend on the concentration of the enzyme used (Fig. 6).

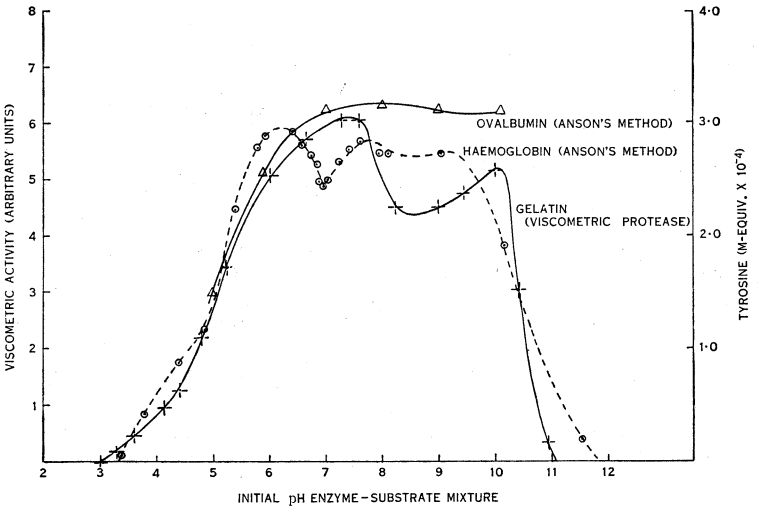

Fig. 5.—Effect of pH on the digestion of ovalbumin, haemoglobin, and gelatin.

The chemical evidence for the individuality of the proteases estimated by the two methods has been confirmed by physical experiments. A concentrate of the mould culture filtrate was fractionated with ethanol in 0.1M acetate buffer, and equal amounts of the fraction containing most of the proteolytic components were dissolved in aliquots of 0.01M pyridine acetate at various pH values. Cold ethanol was then added to 5-ml aliquots of the solutions in varying amount and the precipitates were dissolved in 50 ml water and proteolytic activity determined by both methods. As Table 5 shows, at pH 7.1 and 7.9 and in the presence of high ethanol concentrations, the precipitate obtained was rich in the gravimetric enzyme but contained little, if any, viscometric protease. It would be difficult to determine whether these results indicate that the two enzymes were separated or that one enzyme was inactivated by the procedures used, because the supernatant liquid from these experiments resisted attempts to recover the dissolved proteins by further addition of precipitating agents.

Filter paper electrophoresis of a freeze-dried, dialysed, mould culture filtrate was carried out. The paper was soaked in 0.02M glycine and about 0.12 ml of a 20 per cent. enzyme solution applied on a line across the paper from the edge of a well-cleaned razor blade. Electrophoresis was done at 1°C with a

potential gradient of 9 V/cm for 6 hr, using the apparatus described by Woods and Gillespie (1953). Transverse strips of the paper, 1 cm wide, were then extracted with 4 ml of 0.05M $CaCl_2$, to prevent possible thermal inactivation of the enzyme due to the removal of cations (Gorini 1951), and the activities of the filtered extracts estimated by the viscometric and gravimetric techniques.

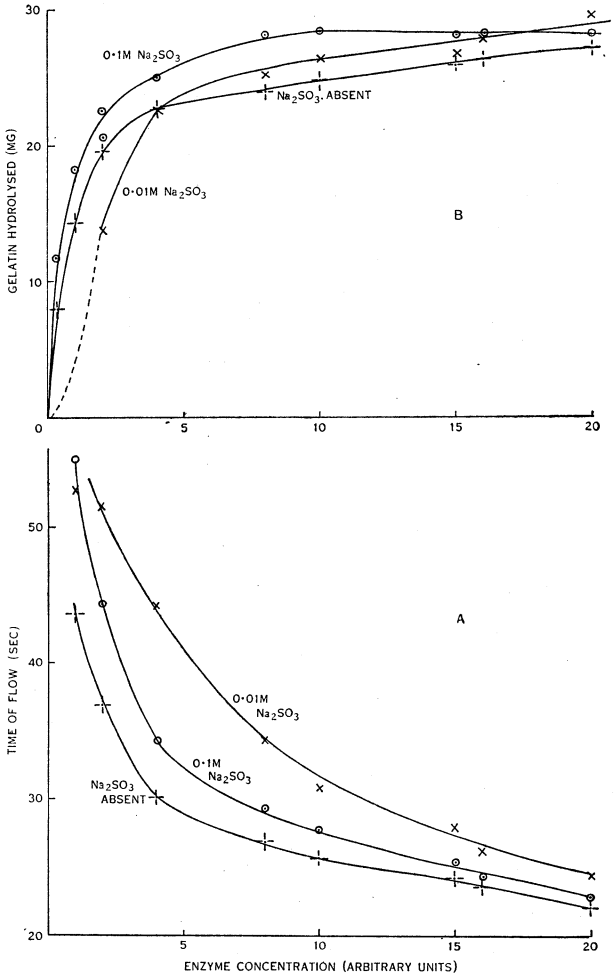

Fig. 6.—Inhibition and activation of protease by sulphite. *A*, inhibition of viscometric protease; *B*, inhibition and activation of gravimetric protease.

Care was taken that the enzyme solutions were not subjected to a long period at 40°C prior to estimation. The distribution of protease along the paper is shown in Figure 7, the gravimetric activities being plotted on a scale 1/1450 that of the viscometric figures. Within experimental error the distribution as shown by the two methods is identical, but whereas the ratio of activity as determined by the viscometric method to that determined by the gravimetric

method was 1 : 1450 the ratio of the activities of the original material was 1 : 41. Paper electrophoresis experiments conducted in the above manner for periods of 0, 1, 2, 5, and 6 hr at 1°C each produced a single peak of activity but the total viscometric protease activity decreased progressively, the gravimetric activity remaining practically constant. That this inactivation was not due to removal of essential metal ions from the enzyme was shown by deionization of an enzyme preparation with a mixture of the "Amberlite" exchange resins IR, 4B, and IR

TABLE 5

PRECIPITATION OF PROTEASE IN THE PRESENCE OF PYRIDINE

Solution of protease in 0·01M pyridine acetate precipitated with cold ethanol

pH	Ethanol (%)	Protease Activity of Solution of Precipitate	
		Viscometric Method (arbitrary units)	Gravimetric Method (arbitrary units)
4·0	44	Nil	Nil
	55	Nil	Nil
	62	Nil	Nil
	67	Nil	Nil
	71	Nil	4
6·0	44	Nil	Nil
	55	Nil	11
	62	0·4	91
	67	0·9	116
	71	0·6	75
7·1	44	Nil	2
	55	Nil	2
	62	0·4	71
	67	0·9	113
	71	Nil	119
7·9	44	Nil	1
	55	0·4	2
	62	1·0	72
	67	2·0	112
	71	0·5	120

120, the final pH of the solution being 6.5. There was no change in the activity of the solutions. Similarly, electrodialysis of the enzyme solution did not appreciably affect the viscometric activity. The possibility that inactivation of the viscometric enzyme might result from separation of two enzymes essential for reduction of the viscosity of gelatin was eliminated by determining the activity of mixtures of the solutions obtained by extraction of the paper strips after electrophoresis. It could be further shown that, when enzyme solution was allowed to remain on paper containing buffer at pH 5.0 or 9.0 and the

whole of the enzyme recovered by extraction, there was a progressive loss in activity with time. Chromatographic experiments which separated the viscometric protease from the gravimetric protease in the enzyme concentrate were carried out in conjunction with Dr. H. Lindley. A suspension of the enzymes

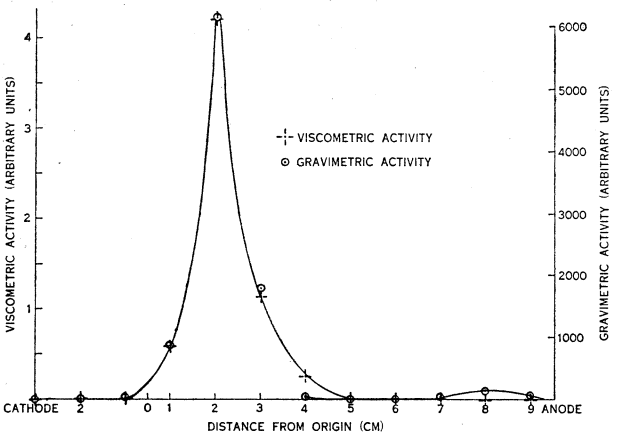

Fig. 7.—Distribution of proteases following paper electrophoresis of enzyme mixture.

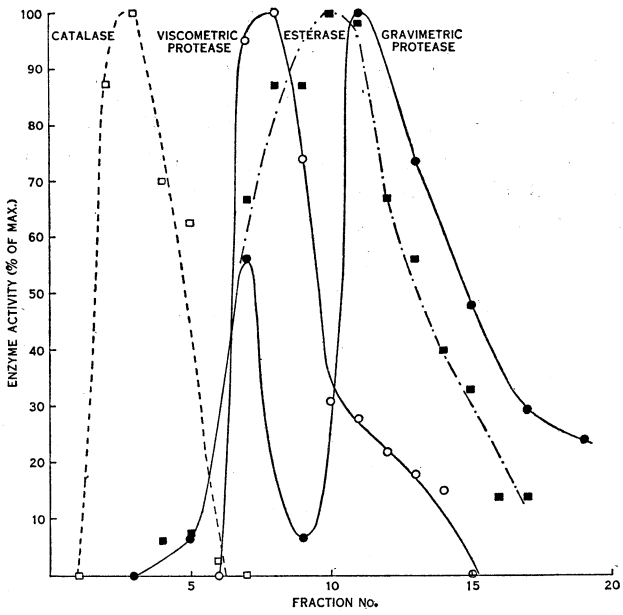

Fig. 8.—Chromatographic separation of some *A. oryzae* enzymes in ammonium sulphate solution on a silica column.

in 3M $(NH_4)_2SO_4$ (8 ml) was applied to a 3-g silica gel column and a solution of 2.5M $(NH_4)_2SO_4$ was used to develop the chromatogram. Successive 3.5-ml

fractions were collected and the activity of various enzyme constituents was estimated. The distribution of activities is shown in Figure 8.

(*c*) *Hydrolysis of Glycylglycine and Other Peptides*

Of the available peptides which were soluble at 0.1M concentration, glycylglycine, L-leucylglycine, and DL-leucylglycylglycine were hydrolysed, the rate decreasing in that order (cf. the dipeptidase of *A. parasiticus* (Johnson and Peterson 1935)). Activation of glycylglycine hydrolysis by cobaltous ions in prefer-

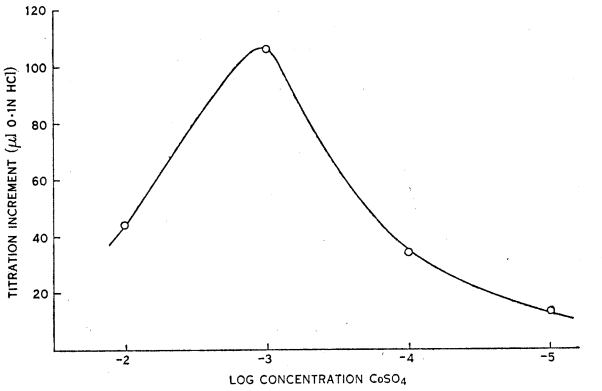

Fig. 9.—Effect of concentration of cobalt on activity of glycylglycine dipeptidase.

ence to other divalent cations, as shown for dipeptidases from various sources by Berger and Johnson (1939), has also been demonstrated for the glycylglycine dipeptidase of *A. oryzae*, the optimum concentration of cobalt ions being 0.001M (Figure 9). A shift of the optimum pH from 6.3 to 7.5 on addition of 0.001M cobalt sulphate (Fig. 10) is similar to that reported for peptidases by Berger and Johnson.

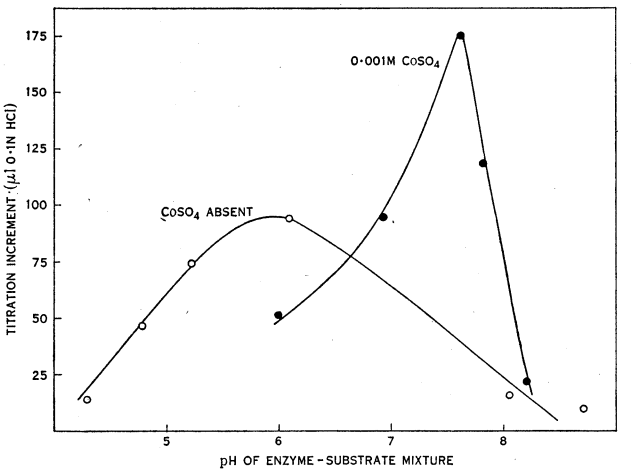

Fig. 10.—pH-activity curves for glycylglycine dipeptidase in the presence and in the absence of cobalt.

The glycylglycine dipeptidase is unstable both in concentrated solutions of the mould enzyme and in the protease-rich crystals under conditions which do not appreciably affect the activity of the other two proteases.

(d) Effect of pH on Activity and Thermal Stability of Enzymes

The pH curves in Figure 11 were determined using a solution of the mixed enzyme powder. The pH values are those of the enzyme-substrate mixtures measured immediately after incubation.

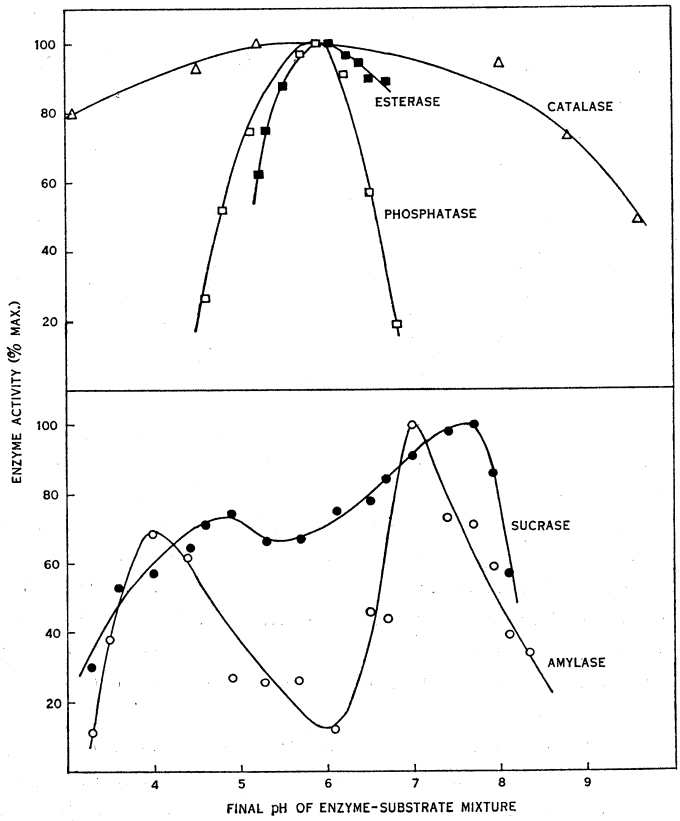

Fig. 11.—pH-activity curves for some non-proteolytic mould enzymes.

The thermal stability of the enzymes was measured by heating aliquots of unbuffered aqueous solution at pH 6.8 to various temperatures between 40 and 75°C. The resistance to heat was found to increase in the following order: catalase, phosphatase, esterase, amylase, viscometric protease, gravimetric protease, sucrase. The enzymes were found to have similar relative stabilities when heated as a suspension in 67 per cent. ethanol.

(e) Effect of pH on the Digestion of Enzymes during Incubation

During incubation of mixed enzyme concentrate for several days at 40°C without prior adjustment of the pH, catalase and phosphatase were almost

completely inactivated in 1 day, but the other enzymes, particularly esterase, were more resistant.

The protein N precipitable with 2 volumes ethanol followed the protease and amylase activities closely (Fig. 12).

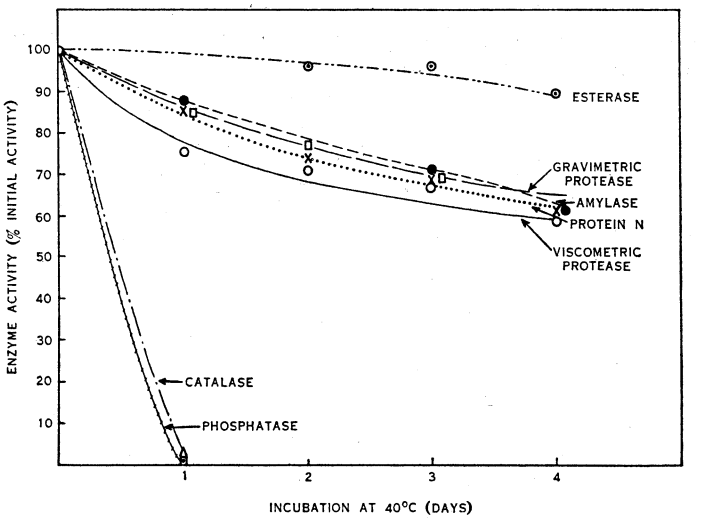

Fig. 12.—Fall in activity during incubation of mixed enzyme concentrate at 40°C.

The effect of pH on the disappearance of enzymic activity was investigated using a standard suspension of the enzymes prepared by vacuum concentrating the culture filtrate, precipitating with 67 per cent. ethanol, and storing at 2°C to prevent proteolysis. The precipitate was dissolved in 0.1M acetate buffer and held at the required pH for 17 hr at 40°C. After dialysis in the rotating dialyser (Lennox 1949) and adjustment to a constant volume, residual activities were determined (Fig. 13).

Fractionation of the concentrate with ethanol at pH 4.0 yielded four preparations, the first containing a high proportion of the catalase and little protease, the second containing mainly amylase and sucrase with little protease, and the third and fourth fractions consisting largely of protease and esterase (cf. Crewther and Lennox 1953). When portions of fraction 1 were incubated at 40°C with native and with heat-denatured fraction 3 inactivation of catalase was found to proceed much more rapidly in the presence of the native protease. Similarly the amylase and sucrase of fraction 2 were rapidly inactivated by the proteases of fraction 4.

The pH of optimum stability of the proteases has been found to depend on the conditions of the experiment. For example, addition of heat-denatured fractions 3 and 4 to fractions 1 and 2 respectively changed the pH of optimum stability from 5.5 to 5.0 in each case. On the other hand the proteases in a solution of crystalline material, containing 0.025 mg/ml, were most stable at pH 7.0.

V. Discussion

The results shown in Figures 2 and 3 indicate that the production of enzymes by *A. oryzae* in the culture medium is not directly associated with mycelial growth since there is no correlation between weight of mycelium and enzyme production. It may be significant that rapid release of enzymes into the medium occurred when the sucrose of the medium had been almost completely consumed and the invert sugar concentration was decreasing. Table 1 indicates that the mycelium can store a considerable amount of protease either as such or as a precursor which is converted to the protease on disruption of the cell structure. The decrease in mycelial weight from the eighth to the 13th day is probably due to release of stored enzymes into the medium.

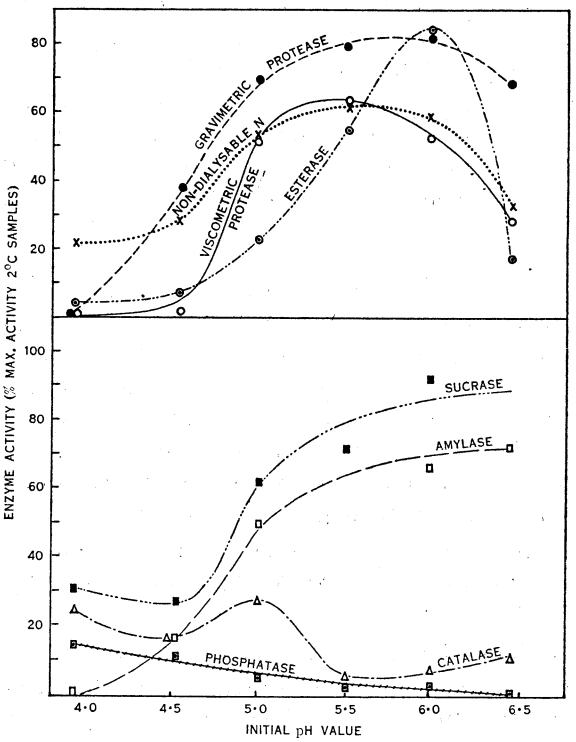

Fig. 13.—Effect of pH value on loss in activity after incubation for 17 hr at 40°C.

The evidence that the proteases estimated by the reduction in viscosity of gelatin and by the digestion of lower-molecular-weight components of gelatin in the gravimetric technique are different must be considered conclusive. Thus the ratio of the protease activity as measured by the two methods varies greatly according to the medium of growth, and also with the presence of sulphydryl-reacting substances, oxidizing or reducing agents, or metal chelating agents during activity measurement (Tables 2, 3, and 4). The pH-activity curves obtained with the two methods are dissimilar, and it has been possible to

separate one of the enzymes almost completely from the other by the physical methods of ethanol fractionation, paper electrophoresis, or chromatography on silica gel. Figure 8 reveals the presence of two components attacking low-molecular-weight gelatin and also suggests that catalase and esterase should be separable from the other enzyme constituents.

The pH-activity curves of sucrase and amylase similarly indicate the presence of more than one component in each enzyme (Fig. 11).

The inactivation of the enzymes during storage in concentrated solution is apparently dependent on at least two factors, one of which is the protease activity of the solution. It is apparent that over the pH range 5.5-10.5 there is considerable protease activity, yet the proteases, amylase, and sucrase are most stable in the pH range 5.0-7.0. It has been shown that inactivation in the presence of active protease is much more rapid than in the presence of heat-inactivated protease, and furthermore the curve for non-dialysable nitrogen is similar to that for the stability of the proteases (Fig. 13). It is probable therefore that the enzymes are more susceptible to proteolytic attack when in a certain state of ionization. This state may correspond with the reversibly denatured form of enzymes postulated by Kunitz and Northrop (1934), or irreversible denaturation may take place readily at 40°C in certain pH ranges.

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