LXXX. THE PROTEOLYTIC ENZYMES OF SPROUTED WHEAT.

By JAMES DENIS MOUNFIELD.

From the College of Technology, University of Manchester.

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ALTHOUGH previous work has shown the presence of proteolytic activity in wheat and wheat flour, attention has been focused on the fate of the wheat proteins rather than on the nature of the enzymic processes [Sharp and Elmer, 1924; Johnson *et al.*, 1929; Brownlee and Bailey, 1930]. Although the proteolytic activity of wheat is very slight [Cairns and Bailey, 1928; Herd, 1931; Landis, 1935] it may be enhanced considerably by germination of the seeds [Bach and Oparin, 1922]. In an endeavour to compare the proteolytic enzymes of wheat with those demonstrated in barley and malted barley [Hopkins *et al.*, 1929; 1930; 1931; Linderstrøm-Lang *et al.*, 1929; Sato, 1931] the principal characteristics of a protease and a dipeptidase have been determined in aqueous extracts of germinated wheat seeds. The results serve to confirm and amplify the similar conclusions of Pett [1935] which have appeared very recently.

EXPERIMENTAL.

Various methods are available for the measurement of proteolytic activity [Cairns and Bailey, 1928] and of these the determination of liberated amino- or carboxyl groups by the Sørensen formaldehyde titration was chosen, as the procedure is simple and gives accurately a set of data which has a recognised meaning. The comparator technique of Grünhut [1919] and Lüers [1920], slightly modified, was found satisfactory. Formaldehyde concentration at the final end-point was 16–20 % [Harris, 1929] and indicator concentration was carefully maintained at one drop of 0.2 % phenolphthalein per ml. solution in the comparator tube. Titration of aliquots at stated time intervals was carried out with $N/20$ NaOH in two stages: (a) directly in aqueous solution to an end-point corresponding to p_H 8.5 (compared with a buffer solution of that p_H), and (b) from this end-point to the same colour after formaldehyde addition. Titre (b) gives a first approximation to amino-N liberated by proteolysis, while titre (a) + (b) represents liberated carboxyl groups [Grünhut, 1919; Richardson, 1934; 1935]. Since titre (b) will be deficient with respect to some of the polypeptide amino-N (which is already partly titrated in the (a) titration), titre (a) + (b) is probably a more reliable estimate of the total scission of peptide linkages and has been used in the present work except where otherwise stated.

Preparation of enzyme extract. The naturally feeble proteolytic activity of wheat seeds was reinforced by germination for not longer than 5 days; with longer periods mould growth was occasionally observed on the damp wheat. Uniformity of germination was secured by the following procedure: 150 g. of English wheat (1934 crop) were soaked in an excess of tap water for 24 hours at 18°, after which the grains were transferred to perforated porcelain germinating trays and kept covered with moist absorbent cotton-wool for 4 more days.

After weighing, the germinating seeds were minced finely and the gain in weight (usually about 90%) was made up to 100% with distilled water. The mass was finally extracted for 2 hours at 30° with an additional 200 ml. distilled water containing a few drops of toluene and filtered. The clear golden-brown filtrate was stored, with additional toluene, in the dark at 18°. It invariably darkened in colour and deposited a fine dark brown precipitate, the change usually being noticeable within 24 hours. Loss of activity (Fig. 6) and changes in p_H with age necessitated the use of freshly prepared extracts only.

p_H measurement. All p_H values were determined with a glass electrode of the Hughes [1922] type coupled to a Harrison [1930] triode valve amplifier unit.

Reaction conditions. Edestin and leucylglycine were chosen as substrates for the investigation of protease and peptidase activities respectively. Reaction mixtures, except where specified, had a total volume of 35 ml. and consisted of varying quantities of edestin dissolved in $N/5$ acetic acid together with enzyme and appropriate acetic acid-sodium acetate buffers. The enzyme extract, brought to 40°, was added from a specially calibrated pipette to the mixed substrate and buffer solutions, also at 40°. Flasks were closed with cork stoppers since rubber ones were shown by Sato [1931] to retard the reaction in the case of malted barley protease. 5 ml. samples were withdrawn within 2 min. and again after 4 hours at 40° and titrated with $N/20$ NaOH as previously indicated.

Control tests. Control solutions for each reaction mixture were made up as follows: (1) substrate + buffers + boiled enzyme, (2) substrate + buffers only, (3) buffers + fresh enzyme only.

The increases in (1) and (2) were always negligible. (3) however almost invariably provided an appreciable correction titre (0.07–0.10 ml. NaOH in the protease experiments and up to 0.32 ml. in the corresponding dipeptidase controls, Fig. 8). These values are no doubt attributable to the action of the proteolytic enzymes of the wheat extract on those wheat proteins rendered soluble under the conditions of extraction (*e.g.* leucosin and globulin). Toluene was used throughout as antiseptic.

RESULTS.

A. Protease.

Determination of p_H optimum for edestin. The effect of variation in p_H on protease activity was determined by the formaldehyde titration method as outlined above and checked by Harris's [1923] method of titration in 85% alcohol using thymolphthalein as indicator (Fig. 1). Both curves indicate an optimum at about p_H 4.1.

Rate of reaction. The rates of activity of protease at 30° and 40° respectively are compared in Fig. 2.

Relation between initial reaction velocity and substrate concentration. The saturation point of wheat protease for edestin as substrate is shown in Fig. 3. The curve is constructed from values for increase in titre (*b*) only. Details as in Fig. 2 except for substrate concentration.

Effect of variation in enzyme concentration with constant substrate concentration. Fig. 4 indicates influence of gradually increasing enzyme concentration when substrate concentration is maintained constant, and in Table I are presented values for Schutz's constant (K) calculated from $x = K\sqrt{et}$ (where x = amount of protein digested, e = enzyme concentration, and t = time).

Fig. 1.

Fig. 2.

Fig. 1. Variation of protease activity with pH. Conc. of edestin in reaction mixture: 1.4%. *M*/7 acetate buffer. Enzyme dilution 2 : 7. Titres as ml. *N*/20 NaOH per 5 ml. sample in excess of a control solution without edestin. × Formaldehyde titration of 4-hour samples. ○ Alcohol titration of 10-hour samples.

Fig. 2. Effect of temperature. Edestin 1.4%. Enzyme dilution 2 : 7. *M*/7 acetate buffer at pH 4.1. ○—○ 30°; ×—× 40°.

Fig. 3.

Fig. 4.

Fig. 3. Effect of edestin concentration. Formaldehyde titration of 4-hour samples.

Fig. 4. Effect of enzyme concentration. Edestin 0.7%. *M*/7 acetate buffer at pH 4.1. *a* ml. enzyme, (10 - *a*) ml. water. Formaldehyde titration of 4-hour samples.

Table I.

Enzyme concentration in ml. (= <i>e</i>)	1.25	2.5	5.0	10.0
Titre (<i>b</i>) (increase ml. <i>N</i> /20 NaOH = <i>x</i>)	0.16	0.24	0.34	0.44
Schutz's constant (<i>K</i>)	0.072	0.076	0.076	0.070

Heat-inactivation of wheat protease. The critical inactivation temperature, *i.e.* the temperature at which an enzyme is half destroyed in 1 hour, was determined by heating quantities of enzyme extract to 40, 50, 55 and 60° respectively before adding to the substrate-buffer mixture. For this purpose, 12 ml. fresh extract at its natural p_H value of 6.0 were heated as rapidly as possible to the desired temperature, maintained there for 1 hour and cooled rapidly to 18°. Evaporation losses were made good with distilled water and 10 ml. of well-mixed extract transferred to reaction flask.

Fig. 5. *Critical inactivation temperature of protease.* Edestin 2.1%. Enzyme dilution 2:7. M/7 acetate buffer at p_H 4.1. Normal value, 0.70 ml. N/20 NaOH.

Fig. 6. *Effect of keeping.*

Inactivation of wheat protease with age. The storage of an aqueous extract of sprouted wheat in the dark at 18° and in the presence of toluene results in progressive loss of activity: the rate of loss is depicted in Fig. 6 (details as for Fig. 5). Increases are those of titre (b) only. In Table II are calculated the values for k (referred to later) from the equation $k = \frac{1}{t} \log \frac{a}{a-x}$.

Table II.

Days of storage ...	0	2	4	7	14	21	28	42
Increase in titre (ml. N/20 NaOH)	0.70	0.65	0.60	0.54	0.42	0.32	0.25	0.16
Velocity constant k	—	0.0161	0.0167	0.0161	0.0159	0.0162	0.0160	0.0153

On ageing, the extract deepened in colour and a brown precipitate appeared. Moreover, the p_H value of the extract examined fell from 6.04 to 5.17 in the 42 days occupied by the tests.

Inhibition of protease action. Attempts to use buffers such as sodium citrate-HCl, potassium hydrogen phthalate-HCl, and glycine-NaCl-HCl, in place of acetic acid-sodium acetate mixtures in the preceding experiments met with little success, increases in titre being either nil or but a small fraction of the corresponding value in the presence of acetate buffers. It will be evident that all except the latter mixtures contain HCl as common ingredient and it therefore seemed reasonable to suppose that chloride ions were responsible for the inhibitory effect. Fig. 7 shows the influence of varying concentrations of NaCl on protease activity. The graph is drawn from data for increase in titre (b) only. A thick precipitate of edestin was observed in those mixtures containing 0.2 and 0.3 M NaCl, a slight precipitate in that containing 0.1 M NaCl, whilst the remaining ones were clear.

Fig. 7. *Effect of NaCl on protease activity.* Edestin 2.0%. Enzyme dilution 2 : 7. $M/7$ acetate buffer at p_H 4.1. x ml. 10% NaCl. $(5-x)$ ml. water.

It should be pointed out that the use of sodium citrate-citric acid buffers also yielded no increase in titre at p_H 4.1. Data have also been obtained which show that phosphate ions slightly reduce the activity of protease.

Investigations now in progress have established that wheat protease is markedly activated by cyanide in concentrations as low as 0.005 M , the p_H optimum at the same time being shifted towards the alkaline side. It is hoped to publish full confirmation later.

B. Dipeptidase.

The existence of a dipeptidase in the aqueous extract of sprouted wheat is demonstrated in the following experiments by the use of glycylglycine and leucylglycine as substrates. The formaldehyde comparator technique was again adopted for all mixtures having a p_H value below 8.5. For more alkaline values back-titration was practised: to the 5 ml. sample + indicator in the comparator tube was added a known quantity (usually 1 or 2 ml.) of $M/20$ potassium hydrogen phthalate sufficient to reduce the alkalinity to below p_H 8.5. Titration then proceeded with $N/20$ NaOH to the usual end-point.

The buffers used were acetic acid-sodium acetate, potassium dihydrogen phosphate-NaOH, dipotassium hydrogen phosphate-NaOH. In addition, simple mixtures of NaOH and water were tried, the natural buffering effect of the

enzyme extract being found sufficient to maintain remarkably constant p_H values throughout the reaction period, which in these experiments was 3 hours instead of 4.

p_H optima for leucylglycine and glycylglycine. The corrected curves in Figs. 8 and 9 indicate the respective p_H optima for leucylglycine and glycylglycine, namely 7.3 and 7.9. In each figure are included the experimental test and control curves from which the corrected curves were calculated by difference. The control curve was determined on mixtures containing no dipeptide.

Fig. 8.

Fig. 9.

Fig. 8. p_H optimum for leucylglycine. Leucylglycine $M/25$. Enzyme dilution 2 : 7. $M/10$ phosphate-NaOH buffers. Titration of 3-hour samples. $\square$ Test curve. $\times$ Control curve. $\circ$ Corrected curve.

Fig. 9. p_H optimum for glycylglycine. Glycylglycine $M/25$. Other details as in Fig. 8.

Stability of dipeptidase. The inactivation of dipeptidase on storage of the aqueous extract of sprouted wheat at 18° in the dark and in the presence of toluene is shown in Table III, both leucylglycine and glycylglycine being used as substrate. Details as in Fig. 8.

Table III.

Days of storage	...	...	0	1	2	3	4
Increase in titre	{	Leucylglycine	1.34	1.16	0.62	—	0.06
		Glycylglycine	0.19	0.07	0.01	0.02	—

DISCUSSION.

It is clear from the experimental data that an aqueous extract of sprouted wheat contains at least two proteolytic enzymes, a protease and a dipeptidase, of which the former functions at acid, and the latter at alkaline, reactions. This accords with the results of Linderstrøm-Lang *et al.* [1929; 1932; 1934] on aqueous extracts of malted barley, in which the existence of similar enzymes was established. The corresponding p_H optima, namely 4.1 and 4.3 for the action of wheat protease and malt protease respectively on edestin at 40° , and p_H 7.3 and 8.6 for the action of wheat dipeptidase and malt dipeptidase respectively on leucylglycine at 40° , suggest substantial similarity between the proteolytic enzyme equipment of the two cereals. Furthermore, the behaviour of the two enzymes in the aqueous wheat extract during storage resembles that of the malt extract, in that the protease loses some activity but is much more stable than the dipeptidase. The stability of an enzyme is, of course, influenced

by several factors, such as temperature of storage, condition of the preparation, extent of exposure to light, p_H value during storage, presence of stabilisers such as glycerol *etc.* Tables II and III deal only with one set of conditions, namely storage of aqueous extracts at 18° with toluene and in the dark. Experiments now in progress point to a much higher stability of the dipeptidase in the presence of glycerol. The rate of destruction of the protease is slightly more rapid in the early stages of storage, the activity being halved in about 18 days. The graph in Fig. 6 bears a distinct resemblance to an inverted unimolecular reaction curve and, assuming a = the initial rate of proteolysis and x = diminution in activity in time t , the application of $k = \frac{1}{t} \log \frac{a}{a-x}$ yields values for k which are reasonably constant (see Table II) and indicate that spontaneous inactivation of wheat protease follows a unimolecular course. This suggests simple disintegration of the enzyme complex rather than its inactivation by combination with any other substance.

The course of proteolysis is generally too complex a process to follow any simple formula, yet Schutz [1885] found that the digestion of egg albumin by pepsin, as measured by peptone formation, varied as $\sqrt{\text{enzyme concentration}}$. Arrhenius [1907] extended this rule and derived the formula $x = K\sqrt{et}$ (where x = amount of protein digested, e = enzyme concentration, t = time and K is a constant). He found this to apply over a considerable range so long as t is neither too small nor too large. The application of this formula to the present work yields values for K which are fairly constant (Table I), so that during the initial stages, at least, the action of wheat protease on edestin appears to follow the Schutz-Arrhenius rule. The formula, unfortunately, does not offer much further insight into the nature of proteolysis. Nor is much help gained from the equation $K_m = x \left(\frac{V}{v} - 1 \right)$ (where x = concentration of substrate, v = velocity of reaction, V = limiting velocity, and K_m is a constant) developed by Michaelis and Menten [1913] and applied to the initial velocities of sucrose hydrolysis by yeast invertase. The constant, K_m , is a characteristic of the enzyme, but for the action of wheat protease on edestin (Fig. 3) no constant values were obtained. This is perhaps not surprising since the degradation of edestin is probably not accomplished by a single reaction but rather by a series of consecutive reactions, and as a consequence no single reaction constant can be expected to apply. Numerically, K_m should be equal to the substrate concentration at which half the limiting velocity of action is reached. For wheat protease this concentration is 0.5% edestin (Fig. 3), a value which may be compared with those reported by Northrop [1920; 1922] for the action of trypsin on caseinogen and gelatin (0.75 and 0.5%), and for the action of pepsin on ovalbumin (4.5%). No other data appear to have been published for proteolytic enzymes although numerous figures are available for other enzyme reactions. From Fig. 3 the maximum velocity of action of wheat protease on edestin appears to be reached at approximately 2% substrate concentration, whilst from 2 to 4.3% edestin the velocity of action is reasonably constant. No falling off is to be observed, although in the case of certain enzymes, notably that of yeast sucrase [Nelson and Schubert, 1928], the decrease in rate of reaction with increase in substrate concentration beyond the maximum point is quite pronounced and immediate. It was found impracticable to investigate higher substrate concentration than 4.3% edestin (Fig. 3).

The destruction of wheat protease by heat appears to follow a normal course, the critical inactivation temperature at p_H 6.0 being about 54° (Fig. 5).

This critical temperature may vary, however, according to the p_H value adopted at the time of heating. Arrhenius [1907] gives a critical temperature of 65° for both trypsin and pepsin and 46° for rennin. Euler and Laurin [1919] found 59° for invertase at p_H 4.0 and Lüers and Wasmund [1922] 57° for malt amylase.

With regard to the inhibitory effect of chloride ions on wheat protease it seems clear from Fig. 7 that the phenomenon is caused by a decrease in the solubility of the edestin with increasing NaCl concentration. The precipitation of the substrate at concentrations of NaCl corresponding to 0.2 and 0.3 *M* doubtless removed the edestin from the sphere of activity of the enzyme. On the other hand, a similar precipitation of edestin was observed in several of the reaction mixtures made up with acetate buffer alone, particularly at about p_H 5, without an equivalent reduction of activity (Fig. 1).

The dipeptidase of wheat extract, although relatively unstable, is quite an active enzyme, especially with leucylglycine as substrate. Incidentally this instability renders it possible to obtain a dipeptidase-free preparation simply by keeping the extract for 4–5 days at 18° under toluene; this has no serious effect upon the activity of the protease. The dipeptidase induces hydrolysis of leucylglycine much more readily than hydrolysis of glycylglycine. In 3 hours at optimum p_H hydrolysis of glycylglycine is only 13 % that of leucylglycine (Figs. 8 and 9). It will be observed that the control curve (Fig. 8) has itself an optimum at about p_H 7.5, doubtless the optimum for dipeptidase activity on those dipeptides which have found their way into the enzyme extract.

The separate identities of protease and dipeptidase are clearly established, firstly, by the great difference in stability between the two and secondly, by the fact that at the p_H at which protease exerts its optimum activity on edestin the dipeptidase does not attack leucylglycine and *vice versa*.

SUMMARY.

1. The aqueous extract of sprouted wheat contains a protease which shows an optimum p_H of 4.1 for decomposition of edestin at 40° .
2. The protease requires a minimum concentration of about 2 % edestin for maximum rate of activity at 40° . The data reveal no constant value for the Michaelis constant, K_m . The substrate concentration at which half the maximum reaction velocity is reached corresponds to 0.5 % edestin. The course of reaction in the early stages appears to follow the Schutz-Arrhenius rule.
3. The critical inactivation temperature is 54° at p_H 6.0.
4. The protease is spontaneously inactivated on standing at 18° with toluene. The inactivation follows the course of a unimolecular reaction. The activity falls to half the original value in about 18 days.
5. The protease activity is considerably reduced by citrate and chloride ions, owing probably to precipitation of substrate.
6. The aqueous extract contains a dipeptidase which attacks leucylglycine optimally at p_H 7.3 and glycylglycine at p_H 7.9.
7. The dipeptidase is unstable in aqueous solution, all activity being lost in 5 days at 18° in the presence of toluene.

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