CCXLIX. THE PROTEOLYTIC ENZYMES OF SPROUTED WHEAT. II.

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In a previous communication the author [1936] has described some of the principal characteristics of a proteinase and a dipeptidase which appear in the aqueous extracts of germinated wheat seeds. The aim of the present paper is to supply further data with regard to these two enzymes and to amplify certain of the findings already recorded, principally those concerned with the relative stabilities of the proteinase and dipeptidase and with the effect of cyanide upon enzymic activity.

EXPERIMENTAL.

The technique adopted was substantially the same as that already outlined, the Sørensen method of formaldehyde titration in two stages, (a) and (b), forming the basis of the investigation. In a few cases $N/10$ NaOH was employed in place of $N/20$ NaOH so as to furnish a sharper end-point. For the sake of uniformity, however, all results are recorded in terms of $N/20$ NaOH. Edestin, leucylglycine (LG.) and glycyglycine (GG.) were again used as substrates, the former for the investigation of proteinase activity and the two latter for the investigation of dipeptidase activity.

The use of 40% formaldehyde adjusted colorimetrically to pH 8.5 calls for comment, more especially in those instances in which the quantity of amino-acid present in the titrated liquid is low, as, for example, in the reaction mixtures prepared during the first 2 days of the tests on the effect of germination time on proteinase activity (see below). The addition of the adjusted formaldehyde to an equal volume of reaction mixture after its titration to stage (a) sometimes resulted, not in the expected discharge of the pink colour, but in its intensification. Addition of the formaldehyde to an equal volume of water produced a similar result. After the discharge of the intensified pink colour by a known amount of $M/20$ potassium hydrogen phthalate (usually 1 ml.) back-titration with NaOH revealed the extent of the apparent development of alkalinity on dilution of the formaldehyde. An example may perhaps clarify this statement: 10 ml. of 40% formaldehyde (containing 10 drops phenolphthalein) adjusted colorimetrically to pH 8.5, after the addition of 10 ml. water, 1 ml. $M/20$ potassium hydrogen phthalate and 11 more drops of indicator (to maintain its concentration at 1 drop per ml.) required 0.24 ml. $N/20$ NaOH to restore the pink colour to that of the pH 8.5 standard. Deducting this latter value from that required by the $M/20$ potassium hydrogen phthalate (namely, 1 ml.) gives 0.76 ml. $N/20$ NaOH as the apparent development of alkalinity on mixing 10 ml. formaldehyde at pH 8.5 with 10 ml. water. This value will vary according to the accuracy of the preliminary colorimetric adjustment of the formaldehyde to pH 8.5 and with the amount of formaldehyde required in the titration. Thus all quantitative determinations of amino-acids by the above method require individual correction by the addition of the NaOH equivalent of the apparent development of alkalinity. Results obtained by difference between titres at the

beginning and end of a given period are, of course, not subject to this correction providing the same procedure is observed throughout. Correction is necessary, however, in those instances in which the initial titre (a) is less than, and the final titre (b) is greater than, the apparent development of alkalinity figure (cf. initial stages of germination experiments).

The apparent development of alkalinity is, of course, due to the increase in the pK' value of phenolphthalein in the 40 % formaldehyde. This alteration in pK' commences at some point between 20 and 40 % concentration since below 20 % the pK is the same as in aqueous solution [Richardson, 1934].

RESULTS.

Effect of germination time on proteinase activity.

The germination of wheat seeds results in increase in proteinase activity: the rate of increase is shown in Fig. 1. 50 g. portions of the same wheat were allowed to germinate for varying lengths of time at 18°. The grains were first immersed

Fig. 1. Effect of germination time. Concentration of edestin in reaction mixture: 2.0 %. $M/7$ acetate buffer at pH 4.1. Enzyme dilution 2 : 7. Formaldehyde titration of 4-hour samples. Temperature 40°. Titres as ml. $N/20$ NaOH per 5 ml. sample in excess of a control solution without edestin.

in tap water at 18° for 24 hours, being afterwards transferred to perforated porcelain germinating trays and kept covered with moist absorbent cotton-wool. After the appropriate period each portion was minced finely and its gain in weight made up to 100 % with water. The mass was then extracted with 67 ml. water containing a few drops of toluene for 2 hours at 30° and filtered. The 24-hour immersion period was considered as part of the germination period. The initial value was obtained by the use of an extract prepared from 50 g. of finely ground wheat seeds. Germination was not continued beyond the seventh day as mould growth appeared after that time.

Ageing of wheat seeds.

The effect of ordinary storage on dry wheat seeds is illustrated in Fig. 2. The curve is constructed from figures obtained over a period of approximately 2 years. A quantity of English wheat (1934 crop) was kept in a glass-stoppered bottle at 18° and in the dark. Samples were withdrawn at intervals, allowed to germinate for 5 days, and the proteolytic activity of an aqueous extract determined in the usual way with edestin as substrate.

Influence of glycerol on dipeptidase stability.

In the previous communication it was shown that the proteinase activity of aqueous extracts of sprouted wheat seeds diminishes to half its original value in about 18 days on storage in the dark at 18° and in the presence of toluene whereas

Fig. 2. Ageing of wheat seeds. Details as in Fig. 1.

the activity of the dipeptidase is reduced to zero in 5 days under similar conditions. The inclusion of glycerol in the wheat extract was found to increase considerably the stability of the dipeptidase (see Table I), activity being maintained almost at the original level even after 21 days at 18°. In Table I are included for convenience the results already recorded for the stability of the dipeptidase in

Table I.

Days of storage	...	0	1	2	4	7	14	21
Increase in	Aqueous	1.34	1.16	0.62	0.06	—	—	—
titre (b):	Glycerol	1.12	1.08	1.14	1.10	1.13	1.10	1.08

Concentration of LG. in reaction mixture: *M*/25. Enzyme dilution 2 : 7.

Concentration of glycerol in reaction mixture: 14%.

Titration of 3-hour samples. Reaction at pH 7.3.

aqueous extract. Extracts containing glycerol were prepared by replacing 200 ml. of the extraction water with an equal quantity of 98% glycerol per 150 g. wheat used. The concentration of the glycerol in the extraction mixture was approximately 45%. Other conditions remained unaltered. The colour of the glycerol extracts invariably remained a deep golden yellow in contrast to the aqueous ones which usually deepened to a dirty brown shade.

The pH value of the aqueous extracts in the majority of cases fell rapidly on storage from about 6 to a value between 4 and 5. The glycerol extracts, however, did not vary in this manner: their acidity was either maintained at the original value of pH 6 or in some few cases tended even to rise slightly. Representative figures for aqueous and glycerol extracts are given in Table II. The pH values of the glycerol extracts were calculated from the millivolt readings in the usual manner, no allowance being made for any change in dielectric constant etc. by the inclusion of 14% glycerol in the reaction mixture.

Table II.

Days of storage	...	0	1	2	4	7	14	21
pH value:	Aqueous	6.02	5.10	4.83	4.64	4.50	4.41	4.33
	Glycerol	6.05	—	6.12	6.23	6.40	6.52	6.64

Effect of pH on stabilities of proteinase and dipeptidase.

From the data presented in the previous section it appears probable that the increase in acidity on storage of aqueous extracts is responsible for the destruction of the dipeptidase. The stabilities of both proteinase and dipeptidase were therefore measured during the storage of aqueous extracts at various pH values.

Three separate portions of the same fresh aqueous extract were adjusted by means of acetate buffer or NaOH solution to pH 4, 6 and 8, respectively. The innate tendency of the extracts to develop acidity on standing necessitated the frequent readjustment of the alkaline extract to pH 8 by the cautious addition of dilute NaOH. At no time did the reaction of this extract vary by more than 0.2 unit from the desired figure. The two acid extracts maintained constant pH values. The extract adjusted to pH 4 remained a pale brown, slightly turbid liquid, whereas that at pH 6 rapidly darkened to a dirty brown shade. The extract at pH 8 remained throughout a fairly clear golden yellow. The activity of each extract was determined at intervals both on edestin at pH 4.1 and on leucylglycine at pH 7.3. The results are shown graphically in Figs. 3 and 4.

Fig. 3.

Fig. 4.

Fig. 3. Proteinase stability. Concentration of edestin in reaction mixture: 1.6%. $M/7$ acetate buffer at pH 4.1. Enzyme dilution 2:7. Titration of 3-hour samples. $\odot$ pH 4. $\times$ pH 6. $\square$ pH 8.

Fig. 4. Dipeptidase stability. Concentration of LG.: $M/25$. Enzyme dilution 2:7. Reaction at pH 7.3. Titration of 3-hour samples. $\odot$ pH 4. $\times$ pH 6. $\square$ pH 8.

Activation of proteinase by cyanide.

The rate of hydrolysis of edestin by wheat proteinase is markedly accelerated in the presence of cyanide, the pH optimum at the same time being shifted towards the alkaline side from pH 4.1 to 4.8. In Fig. 5 are reproduced the pH

optimum curves for the action of the proteinase on edestin in the presence and in the absence of cyanide. The latter substance was added to the reaction mixtures in the form of sodium cyanide.

Fig. 5. Activation of proteinase by cyanide. Concentration edestin: 1.6%. Concentration NaCN: 0.01 *M*. Enzyme dilution 2:7. *M*/7 acetate buffers. ○ Normal. × With CN.

Relation between cyanide concentration and proteinase activation. The effect of variation in cyanide concentration on the rate of hydrolysis by wheat proteinase is shown by the graph in Fig. 6. Reaction mixtures containing cyanide were maintained at the appropriate optimum, pH 4.8: the initial mixture containing no cyanide was adjusted to pH 4.1, the optimum in the absence of cyanide.

Fig. 6. Activation with varying cyanide concentration. Concentration edestin: 1.6%. Enzyme dilution 2:7. *M*/7 acetate buffers.

Activation of dipeptidase by cyanide.

Cyanide also accelerates the action of wheat dipeptidase on both glycylglycine and leucylglycine. In both cases the optimum is shifted towards the alkaline side, to a greater extent with LG. as substrate than with GG. (Figs. 7 and 8.)

DISCUSSION.

Germination studies on wheat in which particular reference has been made to the proteolytic enzyme content of the seeds have been reported by Bach & Oparin [1922], Bach *et al.* [1927] and Pett [1935]. Bach *et al.* found the proteinase activity of wheat to be increased about 40 times on germinating up to the

Fig. 7.

Fig. 8.

Fig. 7. Activation of dipeptidase by cyanide. Concentration LG. = $M/25$. Concentration NaCN: 0.01 M . Enzyme dilution 2:7. $\odot$ Normal. $\times$ With NaCN.

Fig. 8. Activation of dipeptidase by cyanide. Concentration GG. = $M/25$. Concentration NaCN: 0.01 M . Enzyme dilution 2:7. $\odot$ Normal. $\times$ With NaCN.

eighth day. Enzymic activity was determined by precipitation of the amino-acids as their copper salts with subsequent estimation of the N content of the precipitate by Kjeldahl's method. Much of the work was unfortunately carried out without due regard to such factors as optimum pH, effect of drying etc. Pett's contribution was a study of the distributions of both proteinase and dipeptidase in five easily dissected parts of the seed, namely, hull, endosperm, scutellum, plumule and radicles. His observations showed clearly that although hull and endosperm possess little or no proteolytic activity either initially or on germination, scutellum and embryo are more liberally endowed with proteinase and dipeptidase even in the dormant state. On germination these latter two portions of the seed show a rapid increase in activity. In the scutellum this increase continues up to 36 hours and in the embryo up to 12 hours, activity afterwards diminishing. The present author's observations (see Fig. 1) show little significant increase in proteinase activity until the third day of germination, increases thereafter being rapid until about the sixth day. On the seventh day the activity is approximately ten times that in the dormant seed. The apparent differences between Pett's results and the present ones may possibly find an explanation in the methods by which the two sets of results were obtained. Pett's determinations were carried out on dissected portions of seeds whereas the present ones involved the extraction of whole germinated seeds and hence slight variations in the activities of the various parts would tend to be levelled out. The lag phase extending over the first 2 days may be occasioned by an incomplete diffusion of enzyme into solution during extraction owing to the imperfect opening up of the scutellum and embryo cells. By the third day of germination the condition of these latter portions may allow a better release of enzyme content on mincing and extracting. Support is gained for this view by the observa-

tion that the quantity of protein extracted during the process corresponded closely with the quantity of enzyme, being almost negligible during the first 2 days of germination, afterwards increasing rapidly up to the sixth or seventh day. The increase in enzymic activity on germination ran parallel with the physical changes in the seeds: the embryo developed slowly for the first 2 days and then showed a considerable increase in size on the third and fourth days. By the seventh day the acrospires had attained an average length of 2 in. and were tipped with green whilst the radicles were about $2\frac{1}{2}$ in. long. Barley, on germination, underwent changes similar to those described above [Lüers, 1936].

The percentage germination of wheat seeds is usually taken as a measure of the vitality of the seeds. Under ordinary conditions of storage vitality persists only for about 10 years [Bradley, 1935] although under special conditions vitality has been demonstrated in seeds 20 years' old [Whymper, 1935]. It is reasonable to assume that loss of vitality is accompanied by loss of enzymic activity and the results depicted in Fig. 2 bear out this assumption. It is somewhat surprising however to find that in the comparatively short period of 2 years the power to develop proteinase activity on germination dwindled to about one-third of its initial value.

The stability of the dipeptidase of sprouted wheat extracts is enhanced very considerably by the presence of glycerol, no loss of activity being observed after 21 days in the presence of 45 % glycerol whereas in its absence all activity disappeared after 5 days at 18° and in the presence of toluene. These observations are in close accord with those of Linderstrøm-Lang & Sato [1929]. The observation that glycerol extracts, unlike aqueous ones, did not develop acidity on storage (see Table II) led to the investigation of the effect on enzyme stability of storage of the extract at various *pH* values. From the results presented in Figs. 3 and 4 it is evident that the influence of *pH* during storage is considerable. The proteinase is comparatively stable at *pH* 4 and 6 but is rapidly destroyed at *pH* 8, whilst the dipeptidase remains quite active at *pH* 6 and 8, losing all activity however by a short exposure of less than 2 days to a medium of *pH* 4. Lüers & Malsch [1929] found the peptidase of green malt to be destroyed rapidly below *pH* 5. At *pH* 6 both wheat enzymes are fairly stable although the loss of activity by the dipeptidase occurs at an appreciably greater rate than that sustained by the proteinase. At *pH* 4 the wheat extract is deprived of four-fifths of its dipeptidase content within a few minutes. The proteinase is not so readily destroyed at *pH* 8: it still retains slight activity after 2 days. Storage of sprouted wheat extracts at *pH* 4 and 8, respectively, furnishes therefore an additional means of separation of proteinase from dipeptidase. After 2 days at 18° at *pH* 4 the extract is dipeptidase-free, whilst after 3 days at *pH* 8 the extract is proteinase-free. The results also provide further proof of the separate identities of the two enzymes.

From Fig. 4 it is clear that the increased stability of dipeptidase in presence of glycerol is due largely to the action of the latter in maintaining the acidity of the wheat extract at or about *pH* 6. The effect of the glycerol however is not merely to retard acid production since at *pH* 6 and even at *pH* 8 the dipeptidase still suffers an appreciable loss of activity in the comparatively short time of 7 days, whereas in the presence of glycerol the enzyme retains its original vigour for at least 21 days. The precise action of glycerol is not certain but it is probable that its main effect depends upon a lowering of the rates of the various reactions which undoubtedly proceed in an aqueous extract of sprouted wheat and which are probably responsible for the destructive effect. Hydrolytic processes in

particular would almost certainly be retarded by concentrations of glycerol as high as 45 %. The absence of pigment formation and acid production in glycerol extracts supports this view.

Activation of plant proteinases by cyanide is a well-known phenomenon, papain [Willstätter & Grassmann, 1924], pineapple proteinase [Willstätter *et al.* 1926], yeast proteinase [Grassmann & Dyckerhoff, 1928, 2] and the proteinase of green malt [Lüers & Malsch, 1929] being among the principal enzymes affected in this way. The proteinase of sprouted wheat extract is also activated by cyanide, the increase in rate of reaction as shown in Fig. 5 being about 64 %. Actually the extent of activation varied between 50 and 64 % for different preparations of wheat extract. Lüers & Malsch reported a 50 % increase in the activity of green malt infusions in the presence of cyanide. Papain, on the other hand, showed a 200–300 % increase in activity. The *pH* optimum of the latter enzyme was unaffected by the addition of the cyanide although its specificity was widened. Cyanide however caused a shift in the respective *pH* optima of the proteinases of both green malt and sprouted wheat. Using gelatin as substrate Lüers & Malsch found an optimum of *pH* 4.9–5.0 for green malt proteinase; with cyanide the optimum was lowered slightly to *pH* 4.6 and 4.7. The effect of cyanide on sprouted wheat proteinase was to cause a shift in the optimum for its action on edestin from *pH* 4.1 to 4.8.

Fig. 6 shows also that the activation of the proteinase reaches a maximum at a cyanide concentration of about 0.001 *M*, the extent of activation thereafter remaining the same even up to 0.05 *M*, the highest concentration investigated.

The effect of cyanide upon the majority of peptidases so far tested in this way is one of inhibition, not of activation. Among those inhibited are erepsin [Euler & Josephson, 1926], the yeast peptidases [Grassmann & Dyckerhoff, 1928, 1] and yeast polypeptidase [Grassmann & Dyckerhoff, 1928, 2]. The dipeptidase of wheat, on the contrary, is activated when both LG. and GG. are used as substrates (Figs. 7 and 8). The extent of activation in both instances is less than that exhibited by the proteinase. For LG. the increase varied from 5 to 20 % with an accompanying shift in the optimum from *pH* 7.3 to about 7.8. For GG. an increase of about 10 to 30 % was recorded with a slight shifting of the optimum from *pH* 7.9 to 8.1.

SUMMARY.

1. The proteinase activity of germinating wheat seeds, after a lag phase of 2 days, increases tenfold in 7 days at 18°.
2. Wheat seeds, stored in the dark at 18°, display a steady loss of power to develop proteinase activity on germination, the loss amounting to about 67 % in 2 years.
3. The stability of the dipeptidase of sprouted wheat extract is raised considerably in the presence of 45 % glycerol; the activity remaining at the original level for at least 21 days whereas in aqueous solution all activity is lost in 5 days at 18°.
4. Wheat proteinase is relatively stable in buffer solutions maintained at *pH* 4 and 6 respectively but is destroyed at *pH* 8 at 18° in less than 3 days.
5. Wheat dipeptidase loses its activity comparatively slowly at *pH* 6 and 8 but is almost immediately destroyed by exposure to a medium of *pH* 4.
6. Glycerol doubtless owes much of its stabilizing power to its ability to maintain the acidity of sprouted wheat extracts at or about their initial value of *pH* 6: aqueous extracts almost invariably develop acidity, falling generally from *pH* 6 to between *pH* 4 and 5 in 1 or 2 days.

7. Both the proteinase and dipeptidase of sprouted wheat are activated by cyanide. The former enzyme is increased in activity by about 60 %, suffers a shift in the optimum for its action on edestin from pH 4.1 to 4.8 and shows a maximum requirement of 0.001 M cyanide. The dipeptidase is stimulated to a less extent than the proteinase but shows similar shifts in pH optima towards the alkaline side. For leucylglycine the shift is from pH 7.3 to 7.8 and for glycylglycine from pH 7.9 to 8.1.

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