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Amylases of Developing Wheat, Barley, and Oat Grains[1]

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ABSTRACT

Four kinds of amylase activity are distinguished in the developing wheat grain, including β-amylase. A very high level of amylase activity, determined by the amylograph, is associated with the earliest stages of development and with the outer layers of the grain. Two other activities, determined by saccharogenic breakdown of soluble starch, are distinguished by their relative thermal stabilities. During wheat grain development in three successive years, the newly described activities have presented in patterns characteristic of their cultivars. No thermostable activity remains at ripeness, but some thermolabile activity persists beyond ripeness. Similar activities are observed in barley, and in lesser degree in oats.

Developing cereal grain possesses amylolytic activities (1-10) that are generally assumed to be α- or β-amylases. α-Amylase is usually defined as an *endo*, α-1,4-glucan 4-glucanohydrolase (E.C. 3.2.1.1) and β-amylase as an *exo*, α-1,4-glucan maltohydrolase (E.C. 3.2.1.2). The definition of amylases can, in our view, usefully be enlarged to include all enzymes splitting starch linkages, i.e., α-glucan-ases, since the α-1,6-glucan hydrolases (debranching enzymes, E.C. 3.2.1.9 and 10) (11) and disproportionating enzymes (E.C. 2.4.1.3 and 25) (12) also may have considerable effects on the starch properties used for assay of amylases. We assume that the amylase synthesized during the germination process is an α-amylase.

Here we are reporting three amylase activities of developing grain that clearly are neither α-amylase of the germination process nor β-amylase as defined above, but nevertheless are amylase activities in the wider sense and therefore of technological importance. We will show that these three activities rise and fall in characteristic manner during grain development.

For want of more definitive terminology we have followed Sandstedt and Beckord (9) in calling one activity "pericarp amylase". The other two activities, measured by their saccharogenic effects and differentiated by their thermal stabilities, we have called "stable" and "labile" enzymes.

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The pattern of grain development has been followed by sequential sampling, from ear emergence to beyond ripeness, for four cultivars of wheat, one of barley and one of oats, the wheats being replicated during several crop years. Amylase activities have been determined by the amylograph, and by a saccharogenic method, using an organo-mercurial as a selective inhibitor of *exo*-(β) amylase activity.

MATERIALS AND METHODS

Crops were grown in open plots and harvested as described previously (13). We emphasize that the grains must be deep-frozen (-20°C.) for several days before freeze-drying to avoid decay of enzyme activities. Grains frozen, freeze-dried, and stored at -20°C. in closed jars have retained substantially full activities of the various amylases for at least 18 months. The entire dry grains have been ground in a laboratory mill immediately before sampling for analyses.

Four bread wheat cultivars have been studied: Hilgendorf 61 and Aotea are New Zealand bred, Cappelle Desprez from France, and Raven from Australia. All four were studied in 1971, only the first three in 1970, and only Hilgendorf 61 in 1969. A crop of barley (*Hordeum distichum* L.), cv. Carlsberg, was also studied in 1971, and a crop of oats (*Avena sativa* L.), cv. Mapua, in 1972.

The amylograph technique was as previously described (14) but adding either 120 or 600 mg. ground grains to a flour slurry in which the native enzymes had been inactivated by acidification and neutralizing (15). The ratio of peak viscosities for the flour in the absence and presence of the added wheat, minus one, leads to arbitrary units, discussed later, that have sufficed for this investigation.

Saccharogenic method: 500 mg. ground grain was extracted with 10 ml. sodium acetate buffer (0.1M, pH 4.7, containing 5 mg. calcium acetate and 0.25 mg. Thiomersal) for 20 min. at 27°C. and filtered. Filtrate (0.1 ml.) plus 0.9 ml. water were incubated 15 min. at 27°C. with 2.5 ml. soluble starch (BDH, 2% in 0.2M acetate buffer) and reaction terminated by addition of 10 ml. alkaline ferricyanide for determination of reducing power iodimetrically according to AOAC method 20.62 (6th ed.). Units were derived from the titration volumes, duly corrected for titration values of reducing power in the wheat extract. Thiomersal (sodium ethylmercurithiosalicylate) was Sigma brand.

A thermostable fraction of the saccharogenic activity was determined by incubating the buffered grain filtrates in a water bath at 52.5°C. for 20 min. before cooling to 27°C. and adding the starch. The difference between this value and an unheated one has been interpreted as a thermolabile activity.

Free β-amylase was determined in a similar extract of ground grain in acetate buffer (without calcium or Thiomersal). Filtrate (0.5 ml.) plus 0.5 ml. water were incubated in a water bath at 52.5°C. for 20 min. to inactivate the labile enzyme. After cooling it was further incubated at 27°C. for 20 min. with 3 ml. M/3 ammonium oxalate (to mainly inactivate α-amylase) and either 1.5 ml. water (test) or 1.5 ml. Thiomersal (20 mg. per liter) solution (blank). Then 2.5 ml. soluble starch was added and incubated for exactly 15 min. at 27°C. The resulting reducing power was titrated iodimetrically as in the saccharogenic method already described. The difference between titration values for test and blank led to values for enzyme activity in arbitrary units comparable to those already described.

The enzyme characteristic plots of Fig. 4 were obtained by saccharogenic

digestion of soluble starch using methods similar to those described above, and by determining blue values in similar digests but at ten times the enzyme concentrations.

Many of the results are plotted not only on an enzyme activity concentration basis, i.e., per unit weight of ground grain (Figs. 1 and 2), but also on an amount of activity per kernel basis (Fig. 5), which is frequently more significant. The amylograph-determined amylase activity, for example, is calculated on 600 mg. dry matter basis (one-hundredth the amount of flour used in the amylograph), and the activity per kernel is arrived at by dividing by 600 and multiplying by the average dry kernel weight in mg. The activities of β-amylase and of thermostable and thermolabile enzymes are in comparable units, one concentration unit corresponding to liberation of 1.82 mg. maltose (or its equivalent in reducing power) from 50 mg. starch by 50 mg. wheat under the specified conditions in 15 min. The activity per kernel is then derived by dividing by 50 and multiplying by the kernel weight.

Experience has led us to abandon anthesis as a datum for stage of development of wheat; greater consistency has been obtained using ear emergence from the leaf sheath as an initial datum. From this point anthesis is a variable number of days but our arbitrarily defined point of ripeness is relatively constant in our climate at about 65 days after emergence, independent of cultivar and season. Our barley samples could not be fitted into this scheme since fertilization took place before emergence. The curve of dry weight per kernel for barley has been fitted to those for wheat and the barley development expressed on the equivalent wheat time scale. On this scale, our barley was ripe at 55 days. The oats was ripe 52 days after emergence.

Ripeness varies considerably according to what variable is being used as parameter. We have arbitrarily chosen that day on which the moisture content of entire heads is first reduced to 20% dry basis, or would have reached that level if rain had not interfered. This point is deduced from a sequential plot of head moisture contents.

RESULTS AND DISCUSSION

Pericarp Amylase

Using the amylograph technique we have observed amylolytic activity (Fig. 1) associated with the very early stages of development of the grains and which we assume is similar to that recorded by Sandstedt and Beckord (9). We have not found this activity in parts of the wheat plant other than the flowers and grains; there was no activity in the anthers.

We dissected wheat grains approximately into white pericarp and green inner part and separately determined the enzyme activities in each. A sample of Hilgendorf 61 wheat, 21 days after emergence, showed 6.5 units activity in the "pericarp" and 4.7 units in the central portion. A sample of Aotea wheat, 23 days after emergence, showed 8.7 and 4.7 units, respectively, for the two portions. The higher values for the pericarp portions thus support the conclusion of Sandstedt and Beckord that this is an enzyme of the pericarp, but the distribution is not clear cut. The pericarp of wheat at this stage contains massive amounts of small granular starch (our observations), and we may presume that this amylase activity is associated with metabolism of the pericarp starch.

Our calculation of units is alternative to the existing AACC "malt index" (Method 22-10) to allow for the different inherent pasting strengths of the starches of different flours (15) and the higher activities we are dealing with. In the present work, the extreme dilution of the enzyme-containing material by the base flour used in the amylograph ensures that the starch and other materials of the wheat under test have little effect on the result. Calculating in these units the response to varying doses of amylase-containing wheat is almost linear, and almost independent of the peak height achieved by the inactivated base flour.

The activities found are extremely high, such that they could not readily be put

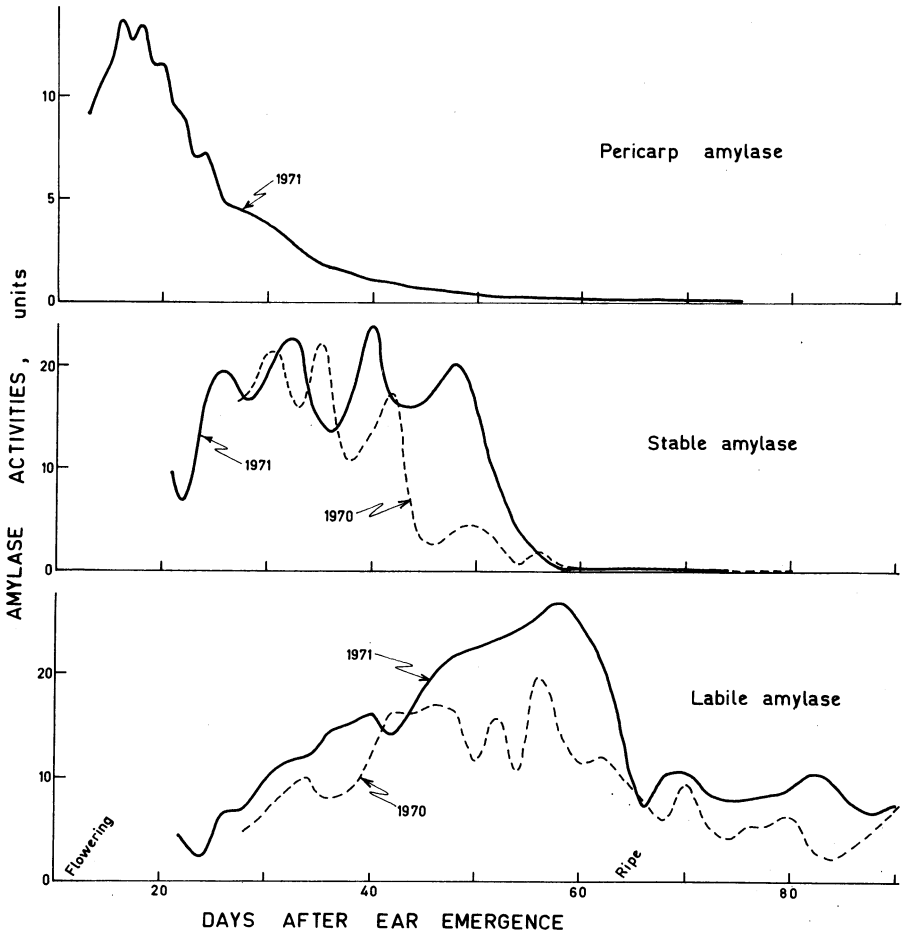

Fig. 1. Amylase activity concentrations in developing wheat grains as functions of stage of development. (upper curve) Activity per 600 mg. grain by amylograph method, 1971 harvest only. (middle curve) Thermostable activity and (lower curve) thermolabile activity per 50 mg. grain, by saccharogenic method, 1970 harvest (dashed line) and 1971 harvest (solid line). Each curve is the mean of similar curves for three cultivars. Each cultivar was sampled about 30 times during the period; hence each curve is the result of about 90 experimental points.

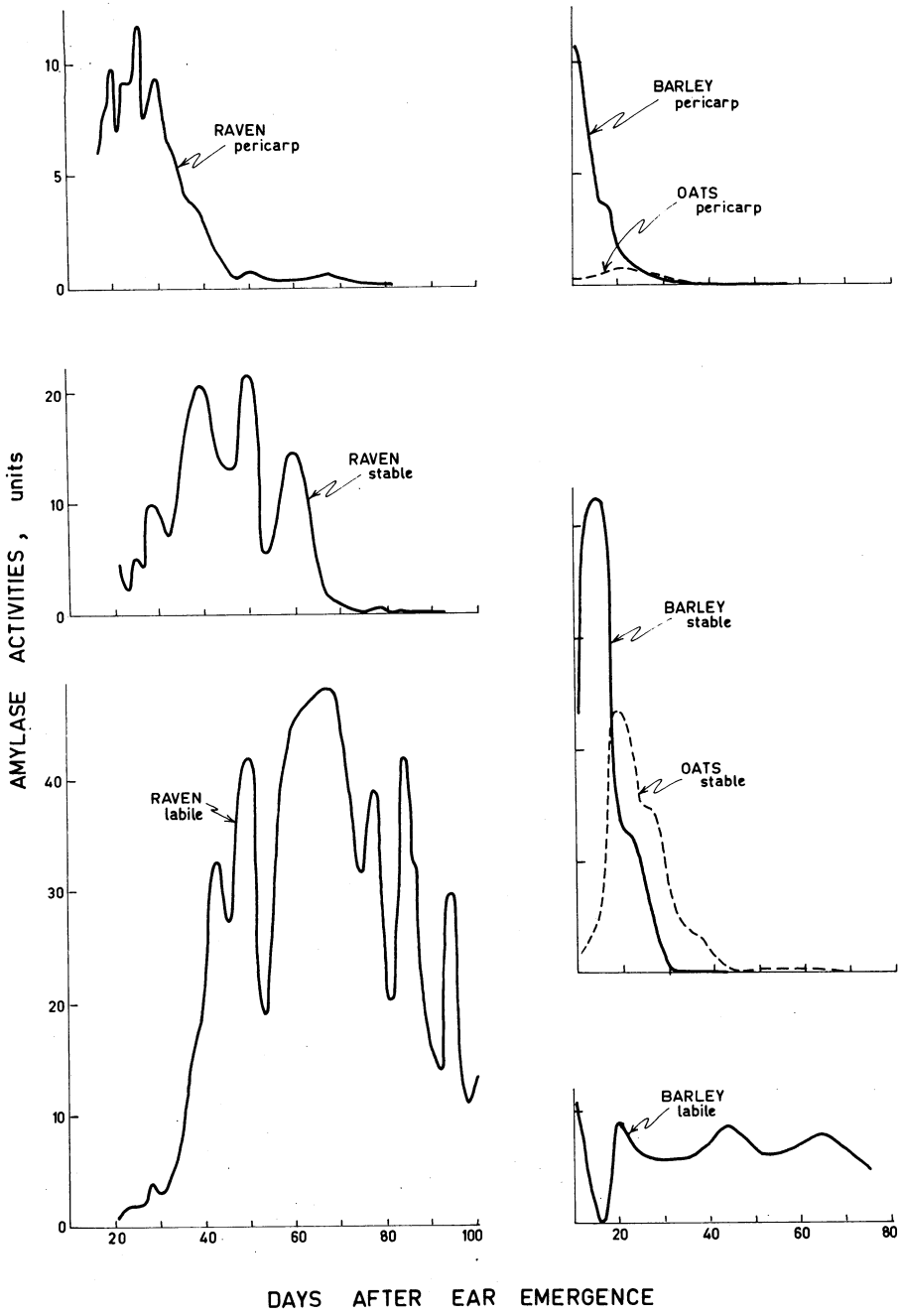

Fig. 2. Amylase activity concentrations in developing grains as functions of stage of development. Ordinate scales comparable with Fig. 1, but abscissae (time) scales compressed. Oats shown by dashed lines.

into conventional "malt index" values. An example will make clear the need for the 1:100 or 1:500 dilution and for the units. A sample of Aotea had activity 15.9 units; i.e., 120 mg. of this wheat added to 60 g. base flour lowered the amylogram peak viscosity from 815 to 195 B.U. In other units, this activity approximates 600 sprout units (16), 2,200 Farrand units (17), or 20 S.K.B. units (18).

These activities are vastly greater than those normally encountered in wheat damaged by premature sprouting at harvest. Sprout damage in New Zealand wheat, when present, is in the range 0.1 to 1.0 amylograph units, while sound wheat activity is up to 0.03 units.

This amylograph-determined activity declines rapidly during maturation of wheat and barley, as shown in Figs. 1 and 2, until a negligible amount of it is left by ripeness. It declines much more rapidly than does the green color of the grain, for example. The pattern of disappearance suggests again the association with the pericarp. We tentatively equate this activity with band A of Stoddart's (10) separation of barley amylases, and with band A of Frydenberg and Nielsen's (3) separation. Only low levels of this activity were found in oats (Fig. 2).

The Saccharogenic Enzymes

The patterns of stable and labile saccharogenic activities during grain development are presented in Figs. 1 and 2 as activities (unit weight basis) and in Fig. 5 as per kernel basis. Comparison with activities of β-amylase is in Table I.

We distinguish β-amylase activity by its sensitivity to inactivation by Thiomersal (19). This use of a mercurial, or temperature, inactivation is the only method presently used to distinguish between the saccharogenic activities of α- and β-amylases. Even then, the presence of large activities of α-amylase makes difficult the determination of β-amylase activity because of the high blanks encountered. It is perhaps for this reason that there is no accepted official method for the determination of β-amylase. In the present work we have attempted to minimize the effect of α-amylase in the β-amylase determination by treatment with oxalate. We have further added a thermal treatment to minimize interference from the thermolabile saccharogenic enzyme discussed below.

We are not completely satisfied that the Thiomersal technique applied here gives results distinguishing the labile enzyme from β-amylase in an absolute manner, but we are satisfied for reasons given below that there are two distinct activities that we call labile enzyme and β-amylase. If there is confusion between the two, it is that much activity ordinarily determined as β-amylase is actually due to thermolabile amylase.

Given the foregoing definitions and distinctions, the pattern of activities during wheat grain development shown in Table I clearly demonstrates the non-identity of β-amylase activity and the other two saccharogenic amylase activities. In the early stages, β-amylase activity is low while the other two are elevated. Later, the values of the labile activity are falling while the β-amylase activity remains static. The stable activity, high initially, rapidly diminishes to negligible proportions while the β-amylase is elevated, but does not follow the same decay pattern as the amylograph-determined activity (Fig. 1). The maximal values of β-amylase activity never approach the values attained by the other saccharogenic activities.

The pattern of iodine-determined "α-amylase" shown by MacGregor et al. (5) for developing barley compares with our limited data (Fig. 2) for barley "pericarp

TABLE I. ACTIVITIES OF SACCHAROGENIC ENZYMES (ARBITRARY UNITS PER UNIT GRAIN WEIGHT) IN DEVELOPING GRAIN, HILGENDORF 61, 1970 SEASON

Days after Emergence	Thermolabile Enzyme	Thermostable Enzyme	β-Amylase
28	6.1	21.1	0.3
33	15.8	21.4	0.6
47	24.0	29.1	1.0
54	17.1	16.3	3.3
59	24.1	0.6	4.1
68	11.9	0.1	3.6
80	11.6	0.1	3.6
87	7.3	0	3.4
102	10.3	0	3.2

amylase" or stable enzyme or both. We suggest from our observations that the labile enzyme would not have survived the pH used by Stoddart (10) for electrophoresis but would be present in major amount in his gel-filtration experiment sample 2, and that the stable enzyme is represented by Stoddart's band B. We agree with Stoddart's interpretation that bands C are β-amylases.

Band B of Frydenberg and Nielsen (3) is probably analogous to our stable enzyme, while again the labile enzyme would not have survived their electrophoresis conditions. Olered and Jönsson (6) have applied to unripe wheat grains an electrophoretic technique similar to that of Frydenberg and Nielsen but with slightly milder conditions of pH that should allow some of the labile enzyme to survive. The activities that they call "green" amylases are clearly to be equated with our stable and labile enzymes.

Properties of the Enzymes

When we first observed saccharogenic activity in developing wheat that was neither α- nor β-amylase activity, we systematically investigated the effects of temperature and pH on its stability. The thermal inactivation curve was not smooth but proceeded in a series of steps (Fig. 3). The characteristic temperatures of these steps were not widely separated for wheat, but were much more clearly separated for barley. Shift of pH in an alkaline direction at 27°C. gave substantially the same discrimination of activities.

The activity from wheat that at pH 4.7 is stable to 47°C. treatment but is inactivated by 52.5°C. treatment is, in the overall grain development picture, a relatively minor component of somewhat irregular occurrence. It is conceivably a fungal contaminant. We have chosen for the moment not to consider it separately but to make the stability distinction at 52.5°C. and so include the minor activity with the major labile one.

These thermal stabilities are, of course, highly pH dependent; hence when we determine similar stability curves at pH 4.7 for the other amylase activities (Fig. 3), we find that the α-amylase of germination is very unstable, whereas we know it to be stable to 70° to 80°C. at higher pH in the amylograph. The pericarp enzyme, on the other hand, has higher stability than any of the other enzymes we are considering. β-Amylase of wheat is stable at pH 4.7 to the inactivation temperature 52.5°C. that we have used. Therefore, it cannot be confused with the labile enzyme that also occurs in highest concentration late in development. Similar arguments for

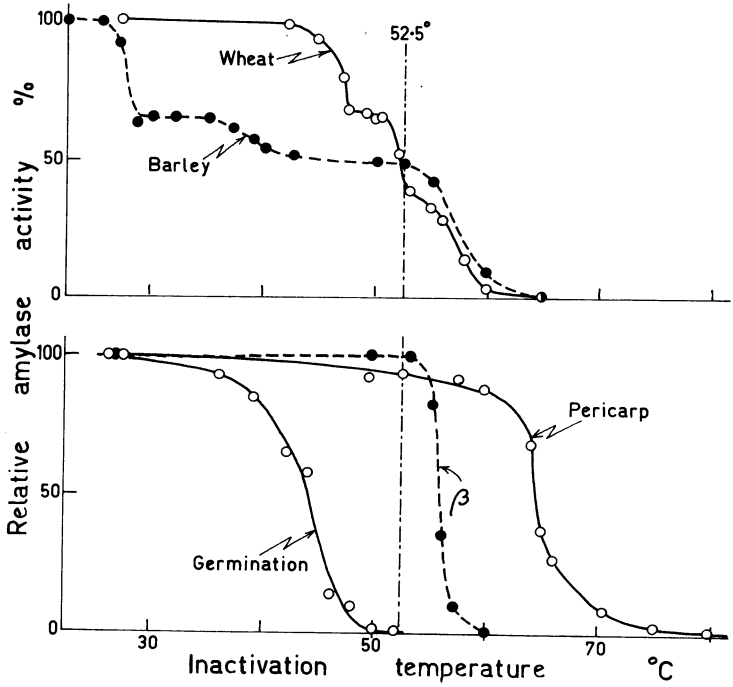

Fig. 3. Thermal inactivation curves of amylase activities in acetate buffer, 0.17M, pH 4.7. Freshly prepared extracts were inactivated for 20 min. at stated temperature and assayed for 15 min. at 27°. (top—solid line) Curve for composite of wheat samples, Hilgendorf 61, 1970 harvest, 28 days and 56 days. (dashed line) Curve for composite of barley samples, Carlsberg, 1971 harvest, 18 and 47 days. (bottom) Germination-amylase curve for a partly purified preparation from a commercial sprout-damaged wheat; β-amylase curve for a wheat sample, Hilgendorf 61, 1970 harvest, 83 days after emergence; pericarp-amylase curve for a wheat sample, Hilgendorf 61, 1971 harvest, 24 days after emergence, assayed in amylograph after inactivation of extract.

the stability of germination α-amylase at this pH show us that the major amylase activity of the developing wheat grain is not identical with the germination amylase activity. Further, the pattern of activity during development measured by the saccharogenic method is completely different from the pattern shown using the amylograph.

The metal dependence of these enzymes has been tested by assaying them in the presence and absence of versene (ethylene-diamine tetracetic acid, disodium), with suitable controls. A sample having pericarp enzyme activity 16.0 units assayed only 1.1 units in the presence of versene. We conclude that this enzyme is metal-dependent. Most α-amylases are calcium dependent, so this result is not unexpected. A sample having thermostable activity originally 28.0 units assayed 27.2 units in the presence of versene. A sample having its major thermolabile activity originally 21.9 units assayed 25.2 units in the presence of versene, whereas corresponding figures for the minor thermolabile activity in the same sample were 4.2 and 12.9 units, respectively. We thus conclude that neither the thermostable

activity nor the thermolabile activity is metal-dependent and that, indeed, one or both of the labile activities are sensitive to the presence of metals as inhibitors.

We have made preliminary attempts to characterize the actions of these enzymes. Testing their activities against soluble starch at pH 4.7, the thermolabile enzyme does not completely digest to the achromic point when assayed by iodine color, but stops at about 30% blue value. By contrast, the thermostable enzyme and α-amylase of germination both readily digest to the point of no color with iodine.

More elaborately, the characteristic patterns of digestion have been determined by the dextrinogenic and saccharogenic methods, as originally suggested by Bourne et al. (20), but acting on soluble starch. The pericarp enzyme gave a pattern (Fig. 4) almost identical with that of salivary amylase, suggesting that it is a true α-amylase. The labile enzyme gave a pattern similar to β-amylase, while the stable enzyme gave a curve between these two extremes (as did the α-amylase of sprout-damaged wheat, not shown). The plots are open to considerable latitude in their interpretation because of the large effects that traces of other amylases can have on the position of a curve and because of the crude substrate; the enzyme samples were not purified except as indicated in the caption. Different enzyme concentrations were used for the two determinations.

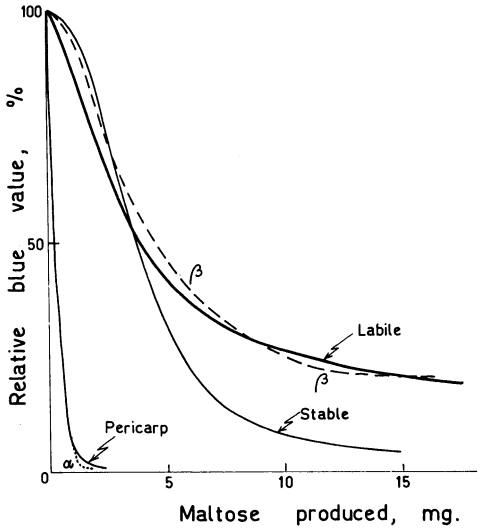

Fig. 4. Characteristic curves for actions of crude amylases on soluble starch, comparing change of blue value with production of reducing power, at different enzyme concentrations, during the course of enzymatic digestion, in 0.17M acetate buffer, pH 4.7. Pericarp amylase from Hilgendorf 61 wheat, 1971 harvest, 18 days, heated to 62°C. to further reduce the low levels of thermostable and thermolabile activities, with Thiomersal. Salivary amylase (labeled α) is filtered human saliva. Thermostable amylase from Hilgendorf 61 wheat, 1971 harvest, 31 days, incubated 20 min. at 52.5°C. to reduce its low thermolabile activity, with Thiomersal. Thermolabile amylase from Hilgendorf 61 wheat, 1971 harvest, 60 days, almost free of thermostable and pericarp amylases, with Thiomersal. β-Amylase (labeled β), prepared commercially from barley, by Wallerstein Company, New York, α-amylase-free.

Varietal Differences

The levels of activities attained and the patterns of rise and fall of activity have been relatively consistent during 3 years. Analyses for 2 years and three cultivars are presented in Fig. 5, the data for the first year being omitted because sampling was insufficiently frequent to give more than a general indication. For both

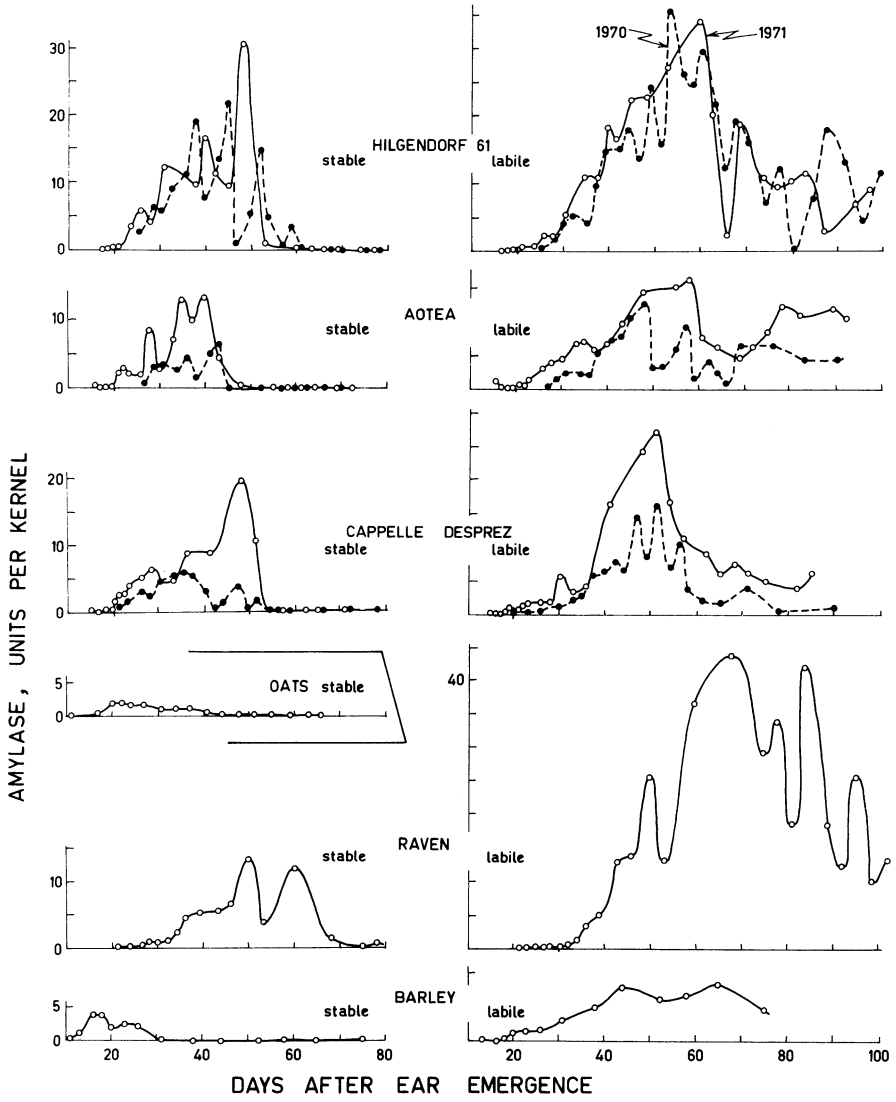

Fig. 5. Thermostable (left) and thermolabile (right) amylases expressed on activity per kernel basis as function of grain development, including experimental points for each cultivar. Where there are two curves to a plot, solid line for 1971 harvest and dashed line for 1970 harvest. Raven wheat and barley are 1971 harvest, and oats is 1972 harvest.

enzymes, Hilgendorf 61 attained the highest levels and Aotea the lowest. Although Hilgendorf 61 showed the rapid decline of labile enzyme at ripeness, residual activity is much higher than for the other varieties.

The fourth wheat cultivar, Raven, has only been examined in one season (Figs. 2 and 5), but it has shown a level of labile enzyme far above those for the other three. Like other Australian-bred wheat cultivars grown in New Zealand, it has a superior starch-pasting strength.

Barley has also been included in 1 year for comparison (Figs. 2 and 5). The concentration attained by the stable enzyme was extraordinarily high (Fig. 2) but since this peak of activity also occurred while the grains were very small, the amount of stable enzyme activity per kernel (Fig. 5) was very small, as also was the amount of labile enzyme.

Oats (Fig. 2) possessed negligible activity of labile enzyme (as judged by 52.5°C. treatment), but stable enzyme in pattern comparable to that of barley and in peak concentration similar to the wheats. Again, because this peak of stable enzyme activity occurred while the grains were very small, the enzyme per kernel (Fig. 5) was correspondingly small.

The Shapes of the Curves

Figures 1 and 2 have been set out to show the time relations between the pericarp and stable and labile enzymes. The stable enzyme increases as filling of the endosperm begins, reaches a peak when starch deposition is maximal, and declines to negligible proportion before ripeness. The labile enzyme starts to appear at about the same stage but takes longer to reach peak activity. There is a very sharp decline in this activity at ripeness but some activity remains after the grain is ripe.

We do not think the lines drawn to the experimental points necessarily portray exactly the fluctuations of enzyme activities that occur, but have been drawn to give minimal fluctuation while giving the points their full weight. The large fluctuations observed are not experimental error of determination, nor of sampling, and are not a function of weather conditions, but they portray in an imperfect manner what may well be a rather sluggish feedback control. Such fluctuations are suggested in Fig. 7 of Olered and Jönsson (6). We have shown them more definitely and reproducibly in the rate of starch deposition, which will be the subject of a separate communication.

The observation by the same authors (6) that "the alpha-amylases in this group become more temperature-sensitive as the kernels approach the stage of full ripeness" is immediately understandable in terms of our patterns of increase and decline of the stable and labile enzymes during development. Their work contains the important concept that the "green" enzyme becomes reactivated during germination, a possibility we have not investigated.

We are not prepared at this stage to suggest what is the action or function of the saccharogenic enzymes except to observe that there is a circumstantial association between their levels and the rate of starch deposition in the developing wheat grain. Further characterization will await separation and purification of the enzymes.

The fluctuations observed in the levels of pericarp enzyme, of lesser magnitude and shorter period, are distinct from the foregoing discussion. They probably represent anatomic transformations as the several layers of pericarp decay and other tissues proliferate.

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