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Release and Activity of Bound β-Amylase in a Germinating Barley Grain[1]

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ABSTRACT

In resting grains of Triumph barley (*Hordeum vulgare* L. cv Triumph) about 40% of the β-amylase could be extracted with a saline solution, the remaining 60% being in a bound form. During seedling growth (20°C), the bound form was released mainly between days 1 and 3. When a preparation containing bound β-amylase was incubated with an extract made of endosperms separated from germinating grains, release of bound β-amylase took place and could be studied *in vitro*. The release was almost completely prevented by leupeptin and antipain, specific inhibitors of a group of SH-proteinases, but it was not inhibited by pepstatin A or EDTA, which inhibit some other barley proteinases. It is thus very likely that in a whole grain, at least the bulk of the bound β-amylase is released by the proteolytic action of one or several SH-proteinases. When the bound β-amylase was released by papain, its molecular weight was about 5000 daltons smaller than that of β-amylase released by dithiothreitol. This indicates that the release is due to removal of a sequence of β-amylase itself. A similar decrease in size took place during seedling growth. Bound β-amylase showed some activity against native starch and it hydrolyzed maltotetraose at a rate that was about 70% of the rate the same amount of bound β-amylase gave after release. Bound β-amylase is thus not inactive and it is likely that the slower rate of hydrolysis is due to steric hindrances which prevent substrates from reaching the active site.

In germinating barley grain, starch present in the starchy endosperm is hydrolyzed into glucose by a concerted action of α- and β-amylases, debranching enzyme and α-glucosidase (maltase) (2, 6). In contrast to the three other groups of enzymes, β-amylase is not synthesized during germination but accumulates during development of the grain (9). At the end of development, when the grain is drying, a portion of the β-amylase is attached, apparently via S-S-bridges, to insoluble constituents of the starchy endosperm (16, 18). In a mature grain a great portion of bound β-amylase is associated with the periphery of starch granules (8, 15).

The bound β-amylase can be released *in vitro* either with reducing agents or with papain (27). On this basis it could be supposed that the bound β-amylase is released *in vivo* during germination either by a disulfide reductase or by a proteolytic enzyme(s). Isoelectric focusing separates β-amylase into several components, and the patterns of the free forms from ungerminated and germinated grains are different (3). Extraction of β-amylase with β-mercaptoethanol from ungerminated grains does not change this pattern, but after extraction with papain the pattern is different, resembling that obtained with germinated grains. Therefore, it has been suggested that bound β-amylase is released by proteolysis (3).

Our aim in the present study was to determine whether bound β-amylase is released *in vivo* by the action of proteolysis and, if so, to identify the proteinase in question.

Bound β-amylase has often been cited to be latent (4, 14, 23). Here we show that it can hydrolyze different substrates with a rate depending on the size of the substrate.

MATERIALS AND METHODS

Plant Material

Grains of barley (*Hordeum vulgare* L. cv Triumph) were obtained from SECOBRA (78580 MAULE, France). They were dehusked with 50% H_2SO_4, surface-sterilized with 1% NaOCl, and allowed to germinate aseptically on agar gel at 20°C in the dark (20). In these conditions the coleoptile was about 2 cm long after 3 d.

Extraction of the Free and Bound β-Amylase

Twenty whole resting grains or endosperms from 20 germinating grains were homogenized at 20°C in a mortar with a small amount of quartz sand in the presence of 0.5 to 2 mL of 0.1 M NaCl. After homogenization, more NaCl solution was added, the total volume used being 8 mL. After centrifugation for 10 min at 30,000*g*, the pellet was extracted a second and then a third time in the same way. The sum of β-amylase in these three extracts was taken as the free β-amylase. To release the bound β-amylase, the residual pellet was homogenized in 4 mL of 50 mM sodium acetate buffer (pH 5). Thereafter, 4 mL of 0.5% papain in the same buffer were added, the suspension was incubated at 30°C for 30 min and centrifuged as above, and the β-amylase in the supernatant was assayed.

Extracts containing both free and bound β-amylase were obtained by homogenizing 20 whole resting grains or endosperms from 20 germinating grains in 4 mL of acetate buffer, after which 4 mL of 0.5% papain were added and the suspension was incubated at 30°C for 2 h before centrifugation.

Assay of Release of β-Amylase *in Vitro*

In order to obtain a preparation containing bound β-amylase but free of soluble β-amylase, ungerminated grains were

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finely ground in a ball mill, and 4 g of the flour were extracted 5 times with 40 mL of 0.1 M NaCl for 30 min with stirring. The final extracted pellet was homogenized in a mortar, suspended in 40 mL of 0.1 M NaCl, and divided into aliquots which were stored at −20°C.

Extracts containing the releasing factor were obtained by separating 30 endosperms from 3 d germinated grains and homogenizing them at 0°C in 3 mL of distilled water in a mortar with quartz sand and then separating the supernatant by centrifugation.

The release of bound β-amylase was studied by incubating 0.4 mL of the suspension containing the bound β-amylase with 0.2 mL of 50 mM sodium acetate buffer (pH 5), 0.1 mL of the extract of 3 d endosperms, and 1.3 mL of water or of the test solutions. The incubation was done at 20°C for 18 h with gentle shaking. After incubation 2 mL of 50 mM sodium phosphate buffer (pH 6) were added and the suspension was centrifuged for 10 min at 3000*g*. The pellet was rinsed twice by suspending it in 4 mL of the phosphate buffer and centrifuging. The bound β-amylase remaining in the pellet was released by adding 1 mL of 0.5% papain in 50 mM sodium acetate buffer (pH 5) and incubating the suspension at 30°C for 1 h. After the incubation, 3 mL of the buffer were added, the suspension was centrifuged as above, and β-amylase activity in the supernatant was assayed. The total amount of bound β-amylase in the sample was assayed in the same way except the suspension was incubated with water, for 18 h, instead of extract.

Assay of β-Amylase Activity

β-Amylase was assayed using the Testomar amylase test (Behring Diagnostics, La Jolla, CA) which contains *p*-nitrophenyl-α-maltopentaoside, *p*-nitrophenyl-α-maltohexaoside, and a microbial α-glucosidase. This reagent, originally made to assay α-amylase for clinical purposes, has been suggested by Mathewson and Seabourn (19) to be specific for cereal β-amylases. For barley β-amylase, the reagent is reconstituted with 47 mM NaH_2PO_4 to give pH 6. We have modified the assay as follows: 0.2 mL of the substrate solution were incubated with 0.2 mL of suitably diluted enzyme (dilution in 50 mM Na-phosphate buffer, pH 6, very gentle mixing) for 20 min at 30°C. Then, 0.8 mL of 150 mM Na_2CO_3 were added and the absorbance was read at 405 nm in microcuvettes. The absorbances were changed into enzyme units ($\mu mol \cdot min^{-1}$) using the molar absorption coefficient of 18800 cm^{-1} M^{-1} (19).

Hydrolysis of Maltotetraose

Samples of β-amylase (35 μL) were incubated with 70 μL of 60 mM maltotetraose in 50 mM sodium phosphate buffer (pH 6) for 20 min at 30°C with slow agitation. The reaction was stopped by treatment at 100°C for 2 min. After centrifugation, samples of the supernatant were analyzed for maltose by HPLC.

Hydrolysis of Starch

Samples of 0.25 mL of β-amylase were incubated with 0.5 mL of 1% soluble starch (Merck No. 1252) in 50 mM sodium phosphate buffer (pH 6) for 20 min at 30°C with slow agitation. The reaction was heat killed in a boiling water bath for 5 min and the supernatant was separated by centrifugation. The amount of reducing sugars in the supernatant was assayed with 3,5-dinitrosalicylic acid (13) using maltose as standard.

Assay of α-Amylase and Proteinase Activity

α-Amylase was assayed by measuring the release of soluble products from an insoluble, colored starch derivate (Phadebas-test, Pharmacia, Sweden) (24).

Proteinase activity was assayed using casein at pH 5.4 as substrate (26). The samples were dialyzed against 20 mM Na-succinate buffer, containing 0.1 mM DTT, at 4°C for 20 h.

SDS-PAGE and Immunoblotting

SDS-PAGE was done according to King and Laemmli (12) in 10% acrylamide gels and immunoblotting was carried out according to Towbin *et al.* (29). The mol wt markers were obtained from Pharmacia.

All the enzyme assays have been made in duplicate and all the experiments have been carried out at least twice.

RESULTS

Validity of the β-Amylase Assay

The modified assay of β-amylase (see "Materials and Methods"), which is cheaper and more suitable for a large number of samples than the original one (19), gave values which were directly proportional to the time of incubation and to the amount of extract. However, when the values of absorbance were above 0.6, the reaction slowed down (data not shown).

Because the test was originally developed for human α-amylase, interference by barley α-amylase was tested. A sample (0.5 μg, 2 units, see Ref. 24) of purified α-amylase from barley gave an absorbance of 0.4 in the Testomar-assay. This indicates that the test is not fully specific for β-amylase.

To test the effect of α-amylase in the actual assay conditions, an endosperm extract was made using grains germinated for 4 d, at which time the activities of α- and β-amylases are virtually maximal. When β-amylase in the extract was inactivated by treatment at 70°C, the activity in the Testomar-assay decreased to 0.5% of the activity before the treatment (Table I). Because α-amylase had remained fully active, the interference caused by it can be at the highest 0.5%. On the other hand, α-amylase was completely inactivated by a treatment with EDTA, but this treatment had virtually no effect on the β-amylase assay. These results show that high amounts of barley α-amylase give some activity in the test, but in the conditions used to assay β-amylase in extracts of germinating barley, the interference by α-amylase is less than 0.5% of the activity of β-amylase. The assay can therefore be used to measure specifically β-amylase activity even in extracts of germinating barley grains, as already suggested by Mathewson and Seabourn (19). This conclusion has been further supported by immunoprecipitating the β-amylase with a specific antiserum (15). Immunoprecipitation removed 98% of the activity (data not shown).

Table I. *Effect of Various Treatments on the Testomar-Assay of β-Amylase*

Ten endosperms separated from grains germinated for 4 d were extracted with 1 mL of 0.2 M sodium acetate buffer (pH 5.5) containing 1 mM $CaCl_2$. To inactivate β-amylase a part of the extract was diluted 5-fold with 50 mM Tris-HCl buffer (pH 7.5) containing 3 mM $CaCl_2$, and a portion of the mixture was incubated at 70°C for 15 min (22) while the rest was kept at 0°C. To inactivate α-amylase, the extract was diluted 10-fold with 25 mM EDTA in 0.1 M sodium acetate buffer (pH 5.5) while another portion was diluted with 1 mM $CaCl_2$ in the acetate buffer. Both samples were kept at 30°C for 20 h.

Treatment	Activity in	
	Testomar assay (β-amylase)	Phadebas assay (α-amylase)
	units·mL⁻¹	
0°C	60.3 (100%)	356 (100%)
70°C	0.3 (0.5%)	347 (97%)
−EDTA	59.4 (100%)	353 (100%)
+EDTA	58.0 (98%)	3 (0.8%)

Release of Bound β-Amylase with Papain and DTT

In our assay conditions (30°C, 50 mM sodium acetate buffer, [pH 5] 30 min), maximal amount of β-amylase was released by 0.1% papain, a 10-fold increase of papain having the same effect (data not shown). DTT at 50 mM (50 mM sodium phosphate buffer [pH 6], 30°C, 30 min) apparently released all β-amylase, with a 3-fold increase in DTT having no further effect. Treatment with papain always gave values which were slightly higher (10–20%) than treatment with DTT. When bound β-amylase was first released with DTT and then the released β-amylase was treated with 0.5% papain, the activity increased by 16%. This indicates that papain can have an activating effect on β-amylase.

Action of Papain and DTT on Free β-Amylase

Because papain seemed to have an activating effect on released β-amylase, its effect on the free β-amylase was tested. When free β-amylase extracted from ungerminated grains was incubated with small amounts of papain, β-amylase activity in the extract increased by about 20% (Fig. 1). DTT had a similar activating effect (Fig. 1). When papain and DTT were added together, the activation was the same as with papain alone. When the β-amylase was extracted from grains germinated for 5 d, no effect of papain on the activity was observed.

Changes in Free and Bound β-Amylase during Seedling Growth

In ungerminated grains of Triumph barley, the amount of free β-amylase was somewhat lower than that of bound β-amylase. During the seedling growth, the free β-amylase activity increased, especially between d 1 and d 3, and at the same time the amount of bound β-amylase decreased (Fig. 2). At d 4, virtually all bound β-amylase had been released.

The sum of free and bound activity increased somewhat during seedling growth. However, when total β-amylase was extracted with papain, the activity was the same at 0 and 5 d. Probably at least a part of the increase in the sum of free and bound β-amylase activity can be explained by activation of the free β-amylase by proteolytic enzymes in the grain.

Identification of the Releasing Factor

In order to assay the release of bound β-amylase by factors present in the germinating grain, endosperms of germinating grains were extracted and samples of this extract were incubated with the preparation containing bound β-amylase at pH 5, the pH of the starchy endosperm (see "Materials and Methods"). Because the extract used contained high amounts of β-amylase, the increase of β-amylase activity in the supernatant due to release was rather small in relation to the initial activity and could therefore not be reliably assayed.

More reliable and reproducible results were obtained when the release of β-amylase was assayed indirectly: after incubation, the pellet containing bound β-amylase was separated from the supernatant and rinsed, and then the bound β-amylase remaining in the pellet was released by papain and assayed. In optimal conditions it could be shown that after incubation with an extract the increase of β-amylase activity in the supernatant was similar to the β-amylase activity lost from the insoluble preparation. This shows that the decrease in the amount of bound β-amylase caused by the extract was really due to release and not, *e.g.* to inactivation.

The amount of β-amylase released in the assay conditions used increased directly proportionally to the amount of extract until about half of the total amount was released, whereafter the proportionality was lost (Fig. 3). With small amounts of extract, the rate of release was linear for at least 20 h (data not shown). In further experiments the amount of extract was chosen to be in the linear range.

To find out whether the release of β-amylase was due to

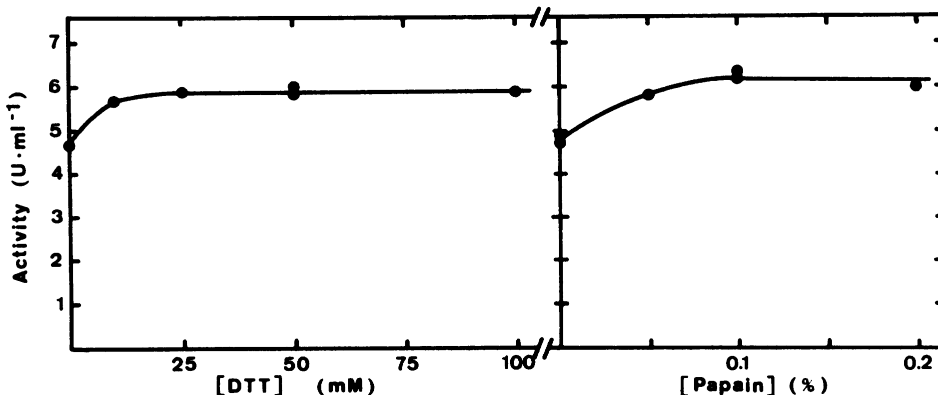

Figure 1. Action of papain and DTT on free β-amylase. One g of barley flour was extracted with 10 mL of 100 mM NaCl for 30 min at room temperature and centrifuged. Samples of 0.2 mL of the extract were preincubated for 30 min at 30°C with either 0.2 mL of papain solutions in 50 mM sodium acetate buffer (pH 5) or DTT solutions in 50 mM sodium phosphate buffer (pH 6). After dilution with the phosphate buffer the β-amylase activity was assayed.

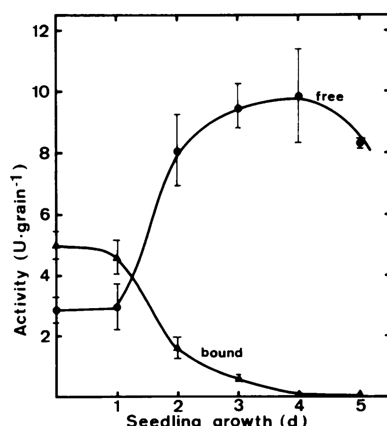

Figure 2. Changes in bound and free β-amylase activity during seedling growth. The grains were allowed to germinate for 0 to 5 d, and the endosperms were separated, extracted, and the activities of free and bound β-amylase were assayed (see "Materials and Methods").

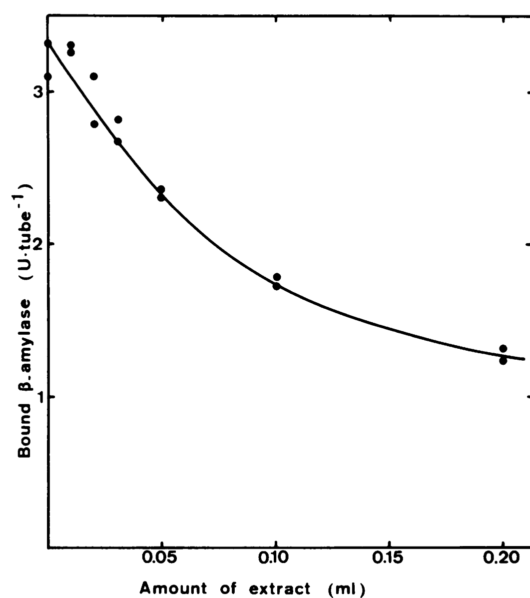

Figure 3. Release of β-amylase *in vitro*. A sample of 0.4 mL of the preparation containing bound β-amylase was incubated with different amounts of extract from endosperms of grains germinated for 3 d, as described in "Materials and Methods". After 18 h, the remaining bound β-amylase was released and assayed.

proteolytic enzymes known to be present in germinating barley, the effect of some inhibitors of proteolytic enzymes on the release was tested (Table II). Pepstatin A, a specific inhibitor of aspartyl proteinases already present in ungerminated barley grains (Takkinen, T. Sopanen, unpublished results), had no effect on the release. EDTA, which inhibits a group of metalloproteinases in barley (28) was also without effect. Leupeptin which inhibits specifically a group of SH-proteinases (7), prevented the release of β-amylase virtually completely. It is thus likely that the bulk or all of β-amylase is released by one or several enzymes belonging to the leupeptin-sensitive group of plant proteinases. Antipain, another specific inhibitor of the same group of proteinases, also prevented the release of β-amylase.

Table II. *Effect of Various Inhibitors of Proteinases on the Release of Bound β-Amylase*

Samples of 0.1 mL of an endosperm extract from grains germinated for 3 d were incubated for 18 h with bound β-amylase with or without proteinase inhibitor and the remaining amount of bound β-amylase was assayed. The β-amylase activity released by the extract was obtained by substracting the remaining bound β-amylase activity after incubation with extract from that after incubation in buffer only (3.45 units/tube).

Inhibitor	Amount of Bound β-Amylase Released	Inhibition of Release
	units/tube	%
None	1.75	0
Pepstatin A (20 μM)	1.72	2
Leupeptin (40 μM)	0.16	91
Antipain (20 μM)	0.16	91
EDTA (5 mM)	1.69	3

Figure 4. Comparison of the sizes of bound and free β-amylase by SDS-PAGE and immunoblotting with a specific antiserum (15). Bound β-amylase was released either with DTT (lane A) or papain (lane B). Free β-amylase was extracted from ungerminated grains (lane C) and grains germinated for 3 d (lane D).

To find out which types of proteinase activities were present in the starchy endosperm, where the release takes place *in situ*, starchy endosperms were separated after 4 d of seedling growth and extracted. The effect of the inhibitors mentioned above on the proteolytic activity in the extract was assayed. The bulk (95%) of the proteolytic activity against casein was due to one or several leupeptin-sensitive proteinases, the other inhibitors had a negligible effect (data not shown). The leupeptin-sensitive proteinase appeared in the endosperm between d 1 and d 2 of germination.

SDS-PAGE and subsequent immunoblotting showed that the bound β-amylase released with DTT had a mol wt of about 64000 D (Fig. 4, lane A). However, when the release was done with papain, the mol wt was 5000 D smaller (Fig., 4, lane B). This was true even when the time of treatment with papain was decreased to 10 min, at which time only 40% of the β-amylase was released. A component having the intact size was not detected. This suggests that the reduction of the size does not take place after the release but is an essential part of release. SDS-PAGE and immunoblotting also showed

that the free β-amylase extracted from ungerminating grains had the same size as the bound β-amylase released with DTT (Fig. 4, lane C). On the other hand, after germination for 3 d the size of soluble β-amylase was smaller and similar to that of bound β-amylase released by papain (Fig. 4, lane D).

Activity of Bound β-Amylase

The activity of samples of bound β-amylase was tested using starch, the Testomar reagent or maltotetraose as substrates. For comparison, the bound β-amylase in similar samples was first released with papain and then assayed using the three substrates. The activity of bound β-amylase on starch was small, about 5% of the activity obtained after release with papain (Table III). This activity indicated, however, that even starch can be slowly hydrolyzed by bound β-amylase. The activity of bound β-amylase on the Testomar reagent, a mixture of *p*-nitrophenyl maltohexaoside and maltopentaoside was 12% of the activity after release and the activity on maltotetraose was about 70% of the activity after release.

DISCUSSION

The results presented above show that release of β-amylase takes place when a sample of bound β-amylase is incubated with an extract made from endosperms separated from germinating grains at a moment when the bound β-amylase is decreasing. The rate of release *in vitro* caused by an amount of extract corresponding to 1 grain was 3.6 units·d^{-1} and the release in the intact germinating grain proceeds roughly at a rate of 4 units·d^{-1} (between d 1 and 3). Thus, the assay conditions seem to reflect the events taking place in the intact germinating grain and, *e.g.* a substantial inactivation of the releasing factor is not likely.

The fact that the release is virtually completely prevented by leupeptin suggests that the main factor causing the release is a SH-proteinase (or a group of similar proteinases). Leupeptin is a small peptide-like compound resembling the substrates of proteinases and therefore it is unlikely that it would act on other types of enzymes. Inhibition of release by antipain, another specific inhibitor of SH-proteinases, gives further support to this conclusion. Furthermore, the fact that leupeptin-sensitive proteinase activity is present in the starchy endosperm, where the bound β-amylase is located (15) and the fact that this activity appears between d 1 and d 2 of germination, when the release of β-amylase begins, are consistent with the role of this proteinase in the release. This proteinase is induced by GA_3 (7, 11), thus a gibberellin is indirectly necessary for the release of β-amylase.

At least in the assay conditions with casein as substrate, the leupeptin-sensitive proteinase accounts for 95% of proteinase activity in the starchy endosperm. Therefore it is not surprising that it seems to play the main role in the release of β-amylase. At the present, it is not clear whether this action is specific for only SH-proteinases or whether the other proteinases known to be present in the germinating barley grain (28) (but not necessarily in the starchy endosperm) could also release bound β-amylase if present in sufficiently high amounts.

The releasing action of the proteinase could be either on the protein to which β-amylase is attached or on β-amylase itself. When the main soluble component of β-amylase was purified and then treated either with papain or with an extract containing the proteolytic enzymes of germinating barley, the isoelectric point of this component increased and the size decreased (17). This gave rise to two transitory and one more long-lived components, which are also present in the germinating grain. The enzymic properties of β-amylase did not change during this process. It is, thus, clear that proteolytic action on β-amylase itself is possible. Most interestingly, in the sequence removed from the C-terminal end of the polypeptide chain there is a cysteine residue (14), and already Lundgard and Svensson (17) speculated that β-amylase is bound via this cysteine; the release would take place when the proteinase splits a peptide bond between this cysteine and the bulk of the molecule. Our results, which show that the size of β-amylase decreases by about 5000 D when it is released with papain, give experimental support for this hypothesis. There are two facts which suggest that the release in the germinating grain is similar to that caused by papain: first, it has been shown that papain belongs to the same family of proteolytic enzymes than an SH-proteinase of barley (25); second, in germinating barley, the size of the free β-amylase is similar to that liberated by papain.

The activation of free β-amylase by papain or DTT is somewhat surprising since it has been shown that the action of papain on one purified component of β-amylase does not at all change the enzymic characteristics of β-amylase (17). However, in crude extracts of wheat grains, there are two components of β-amylase, the minor one apparently being a complex of β-amylase and another protein (4). When this component is treated with β-mercaptoethanol, its mobility in immunoelectrophoresis changes to that of the β-amylase monomer and the activity increases by 17%. In barley extracts, a rather high portion of β-amylase is bound to protein-Z (10), and it is possible that release from this complex by DTT or papain increases the enzymic activity as in wheat. However,

Table III. *Activity of Bound β-Amylase on Different Substrates*

Three samples (0.5 mL) of the preparation containing bound β-amylase were first preincubated for 1 h at 30°C. Two samples were preincubated with 0.5 mL of 50 mM sodium acetate buffer (pH 5); one was centrifuged and the activity in the supernatant was assayed (residual free β-amylase), the other was used without centrifugation (bound + residual free β-amylase). The third sample was preincubated with 0.5 mL of 1% papain in the same acetate buffer and the activity of the supernatant was assayed (released β-amylase + residual free β-amylase). The activity of the residual free β-amylase (0.5–2% of the activity after the release) was substracted from the two other values to get the activity of bound β-amylase or that of bound β-amylase after release.

Substrate	Activity of		Bound as Percentage of Released
	Bound β-amylase	Released β-amylase	
	μmol·min⁻¹·mL of preparation⁻¹		%
Starch	0.30	6.22	5
Testomar-assay	0.86	7.22	12
Maltotetraose	3.60	5.08	71

this does not explain the activation by papain of β-amylase released with DTT.

The bound β-amylase has often been said to be inactive or latent (4, 14, 23). The present results show, however, that the bound β-amylase has a small but clear activity even on starch, but especially it rapidly hydrolyzes the small substrate, maltotetraose. Soluble polymers of β-amylase are active (21) as well as the soluble complex of β-amylase and protein-Z (10). Thus, the attachment of β-amylase by S-S-bridges does not abolish its activity. It is probable that the rather small activity of bound β-amylase on starch is only due to steric hindrances which prevent the substrate from reaching the active site. This notion is supported by our results which suggest that the smaller the substrate the higher is the activity of bound β-amylase.

The physiological significance of the release of bound β-amylase is not evident. If the free β-amylase in the grain has the same activity in the assay conditions and if its activity at 20°C is half of its activity at 30°C, it can be calculated that the free β-amylase in one grain could hydrolyze all the starch in a grain (roughly 40 mg) if in the form of amylose, in about 18 min. Because the germination takes place in about 6 d (144 h), the amount of β-amylase seems to be in very large excess (500-fold). Even the bound β-amylase could hydrolyze all the starch in one grain in about 6 h, if the starch would be in a suitable form. The very large excess of β-amylase as well as the great variation in the amount of bound β-amylase among different varieties of barley (16) indicate that the binding of β-amylase does not have a crucial physiological function. The fact that some barley (1) and rye mutants (5) having very low amount of β-amylase germinate as well as the original strain also indicates, that at least in usual conditions the grain could do with a much smaller amount of β-amylase than that actually present. The excess of β-amylase may, however, have some role in exceptional conditions, or it may have some other function in addition to hydrolysis of starch and smaller substrates.

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