REFERENCES

Albright, F., Bauer, W., Ropes, M. & Aub, J. C. (1929). *J. clin. Invest.* **7**, 139.
Bauer, W., Aub, J. C. & Albright, F. (1929). *J. exp. Med.* **49**, 145.
Bodansky, A., Blair, J. E. & Jaffe, H. L. (1929–30). *Proc. Soc. exp. Biol., N.Y.*, **27**, 708.
Bülbring, E. (1931). *Arch. exp. Path. Pharmak.* **162**, 209.
Burns, C. M. & Henderson, N. (1935). *Biochem. J.* **29**, 2385.
Burns, C. M. & Henderson, N. (1936). *Biochem. J.* **30**, 1207.
Burrows, R. B. (1938). *Amer. J. Anat.* **62**, 237.
Cox, W. M. & Imboden, M. (1936). *J. Nutrit.* **11**, 147.
Dickens, F. (1941). *Biochem. J.* **35**, 1011.
Greenberg, D. M. & Miller, W. D. (1941). *J. Nutrit.* **22**, 1.
Greenwald, I. & Gross, J. (1926). *J. biol. Chem.* **68**, 325.
Hunter, D. & Turnbull, H. M. (1931–2). *Brit. J. Surg.* **19**, 203.
Jaffe, H. L. & Bodansky, A. (1930). *J. exp. Med.* **52**, 669.
Johnson, J. L. (1932). *Amer. J. med. Sci.* **183**, 761.
Selye, H. (1932). *Endocrinology*, **16**, 547.
Silberberg, M. & Silberberg, R. (1943). *Amer. J. Path.* **19**, 839.
Taylor, H. (1935). *Brit. J. Surg.* **22**, 561.

The β-Amylase of Barley

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The amylase of the resting barley grain is known to consist almost entirely of β-amylase, the form which promotes hydrolytic fission of starch into β-maltose and a limit dextrin (α-amylodextrin) (Baker, 1902). During ripening, and again during germination, the α-amylase also is active but its activity is minimal at the resting stage and, in exceptional cases, has been reported to be nil (Hills & Bailey, 1938).

The hypothesis that β-amylases attack the molecules of starch at the non-reducing ends of the glucosidic chains, removing maltose molecules successively, one at a time from each chain or branch, seems to be well established (Ohlsson, 1926; Hanes, 1937). Most starches are now considered to consist of 'amylose', the molecules of which are unbranched, and 'amylopectin' consisting of branched or laminated molecular chains. The action of β-amylase on the former should be complete, 100 % of the theoretical yield of maltose being obtained, whereas its action on the latter is brought to a standstill at the near approach of a branching point (Haworth, Kitchen & Peat, 1944). On these premises we can deduce the proportions of the amylopectin molecule contained respectively in the branchings and other parts provided we know (*a*) the yield of maltose from the pure amylopectin fraction or from the starch by the action of β-amylase and (in the latter case) (*b*) the percentage of amylose in that starch. For this reason alone, the purification of β-amylase and the determination of the exact limit of its action are of importance. Furthermore, with pure β-amylase it would be possible both to define more accurately and to prepare the limit dextrin whose investigation by end-group assay and other methods would be of great value.

The limit of maltose formation from native and soluble starches by β-amylase (usually barley amylases of varying purity) recorded in the literature varies from 59 to 67 % (Hanes, 1937; Blom, Bak & Braae, 1936). By pretreatment of an aqueous extract of barley with acetic acid at pH 3·6 and 0°, Blom *et al.* claimed to have freed the preparation from α-amylase. When such barley extract was allowed to act upon soluble starch at pH 3·3–3·5 at 25° the limit of action was sharply defined at 53·1 ± 0·5 %, and was independent of enzyme concentration and the presence of added maltose. This important observation has encouraged some workers, but by no means all, to adopt similar precautions in preparing β-enzyme from cereal grains. In particular, Kneen, Sandstedt & Hollenbeck (1943) freed β-amylase from α at pH 3·3 at 30° after dialysis. However, the barley extract used by Blom *et al.* contains much reducing matter which shows a marked increase with time at pH 3·4 and 25°—thus introducing an avoidable source of error. In repeating and extending their work in these laboratories, we have encountered several important points with respect to the preparation of the enzyme and the interpretation of the limit of its action. The important question is whether the hydrolysis of starch by pure β-amylase is complete at the 53 or the 60 % stage. If the former, then enzyme preparations which yield 60 % may contain an α-amylase impurity. Alternatively, the 53 %

limit, which was obtained by Blom *et al.* only in acid solutions at pH 3·3–3·5, may be due to some change in the substrate such as a form of retrogradation. The latter phenomenon is known to be encouraged by acid reactions.

A reaction mixture hydrolyzed by β-amylase to its limit possesses reducing power (R.P.) due to the maltose present, the residual dextrin and the enzyme preparation. The latter must obviously be deducted, that of the dextrin is negligible, but there remains a consideration of the R.P. of the original starch. Blom *et al.* employed soluble starch but it is not clear whether its R.P. value was deducted. Many Lintner and other soluble starches possess R.P. equivalent to 2 or 3 % maltose, an amount highly significant when considering the difference between a 53 and 60 % stage of hydrolysis. In this work deduction has been carried out since much of the R.P. of soluble starches may be due to maltose or simple dextrins the R.P. of which latter, measured by the hypoiodite method, carries the same value as that of the maltose split from them. It is considered that in the circumstances the *increase* in R.P. during the reaction (after allowing for increase in the enzyme preparation alone) is the truest measure of the number of α-glucosidic bonds split.

METHODS

Substrates. Lintner soluble starches (selected commercial preparations) of R.P. varying from 1·7 to 3·3 % of theoretical maltose (by hypoiodite) were boiled with water for 5 min. Starch pastes were prepared by stirring at 90–92° for 1 hr., 0·1 % NaCl being included in the liquid to assist dispersion.

Reaction mixtures. These contained 2 % (w/v) dry weight of starch, buffered with 0·02 N-phosphate-citrate (pH 3·4 or 4·6) with toluene plus nitrobenzene as antiseptic; incubation temp. was 25°. Reducing powers were determined by hypoiodite titration (Willstätter & Schüdel, 1918; modified by Hinton & Macara, 1924), those of initial substrate and of enzyme preparation being deducted. In certain experiments where it was desirable to have approximately equal initial velocities of reaction (Tables 1 and 4) three different quantities of each enzyme preparation were included in separate reaction mixtures and the one exhibiting the most suitable reaction velocity selected for continuation, the others being discarded.

Important precautions. In withdrawing duplicate samples from the reaction mixture the pipette must be free from all traces of iodine which may have contaminated the jet when running the previous withdrawals into the standard iodine. The top of the pipette should contain a cotton-wool plug to prevent possible contamination of the reaction mixture with saliva, a potent source of α-amylase.

Buffers should be freshly prepared. A stock solution of phosphate citrate buffer at pH 3·4 which had been stored for some months developed a small growth of white mould from which enzymes such as α-amylase or maltase might conceivably gain access to reaction mixtures.

Toluene was found to be imperfect as a bacterial antiseptic; nitrobenzene prevented growth of bacteria but not of moulds and yeasts. Toluene and nitrobenzene employed simultaneously were effective. Care must be taken in the hypoiodite titration to withdraw only the quantity of antiseptic dissolved in the water.

Controls for the action of the enzyme on the constituents of its preparation and on maltose were performed under parallel conditions with each experiment and the necessary corrections made. If maltase activity was found, the results of the experiment were rejected. None was detected in the barley mostly used (English of the 1938 season, kiln dried). Occasionally in more recent work with other barley samples a small maltase activity at both pH 3·4 and 4·6 has been detected in prolonged reactions.

RESULTS

The β-amylase preparation of Blom et al. (1936). Ground barley was extracted with water at 50°, the extract purified by freezing and after discarding large quantities of precipitated matter, brought to pH 3·6 with acetic acid and stored at 0°. This preparation hydrolyzed Lintner starch at pH 3·4 to 53 % of the theoretically obtainable maltose (R.M. value), whereas the non-acidified extract acting at pH 4·4 yielded 63·0 % R.M. The enzyme dose was varied and more than one specimen of barley and of Lintner starch were employed, but the limit at pH 3·4 was always 53·0 ± 0·8 %. The enzyme was still active when the limit was reached but would not carry the hydrolysis further even when the pH was changed to 5·0. Thus the experimental work of Blom *et al.* with β-amylase had been repeated and their results fully confirmed.

Further investigation showed that barley extract if stored at pH 3·4 for 24 hr. or more but allowed to act on starch at pH 4·6 would promote hydrolysis beyond 53 % and gave variable and indefinite limits between 53 and 60 % R.M. It was noted that the R.P. of the barley extract in the reaction mixtures in all the above experiments was equivalent to about 12 % of the theoretical maltose equivalent of the starch present. This involved a high 'control'. It was decided to use a purer enzyme preparation and the next investigations were devoted to methods of securing this.

Purification of β-amylase

Freezing aqueous extracts. Alternate freezing and thawing were unsuccessful. After each freezing the ice portion was separated, thawed, refrozen and the ice fraction again separated, this being repeated several times. Similarly the first liquid portion was frozen and the new liquid fraction recovered, refrozen, etc. The final liquid portion was found to be only 1·17 times as active as the final ice fraction, though much precipitated matter had been rejected. There were indications, however, that the final liquid preparation was freer from α-amylase than the original extract.

Extraction of β-amylase from resting barley. The conditions of extraction were investigated and the activities of the extracts (measured by standardized procedure) expressed

as % of theoretically obtainable maltose (R.M.) formed. It was found that both at 25 and 50° the enzyme was rapidly extracted by water in the first few minutes and at decreasing rates with the progress of time. At 25°, extraction was about two-thirds as fast as at 50°; and in both cases the extract promoted hydrolysis to 60% R.M.

100 g. of ground barley were extracted at room temperature with 200 ml. of 20% (v/v) ethanol for 1 hr. (extract A). The solid residue was re-extracted similarly twice (the combined extracts becoming extract B) and again for two further successive 2 hr. periods yielding extract C. Extracts A, B and C were precipitated in 80% (v/v) ethanol and the washed and dried enzyme quantitatively recovered from each. The residue of extracted barley was dried, divided, and the halves extracted respectively with water (D) and 1% papain (E) and similar precipitates from these extracts were prepared. The activities of the five preparations were found to be (relatively): A, 100; B, 26; C, 15; D, 1·3; E, 6; D and E being half portions. D and especially E carried starch hydrolysis further, to 65% R.M. with corresponding loss of iodine coloration. For a yield of enzyme relatively free from α-amylase a short extraction with 20% (v/v) ethanol is indicated.

Precipitation of β-amylase by ethanol. Many precipitants have been used but ethanol possesses certain advantages, notably the power of inactivating the α-form. Aqueous extracts were precipitated fractionally: A, with 50% (v/v) ethanol; B, the filtrate from A raised to 65%; C, the filtrate from B raised to 83% (v/v) ethanol. The activities of the precipitated fractions were as follows (aqueous extract 100): A, 10; B, 50; C, 12; and whilst the hydrolysis limits for the aqueous extract and A were 59–60%, those for B and C were 56% under parallel conditions. It seems that preliminary precipitation by 50% (v/v) ethanol removes relatively little β-amylase, some α-amylase and, incidentally, a quantity of inactive matter. The subsequent raising to 83% gives the purest and most useful fraction. Similar conclusions for barley malt were made by van Klinkenberg (1931) and Caldwell & Doebbeling (1935) and for ungerminated wheat by Meyer, Bernfeld & Press (1940).

Precautions as to sterility. Gelatin plate cultures of washings of barley gave colonies of *B. mesentericus*, and *B. subtilis*, spore-forming thermophilic rods known to secrete α-amylase. The first ethanolic precipitates from barley extracts contain such rods, which even if dead may have excreted amylase into the medium during the aqueous extraction. Ordinary barley carries a microflora beneath the paleae, so that washing before grinding may not remove all bacteria. That such bacteria secrete α-amylase has been experimentally demonstrated in these laboratories. Fortunately, acidification of the extracts or preparations to pH 3·4 inactivates α-bacterial amylases.

Final preparation of β-amylase

As a result of the above experiments, β-amylase was prepared by extracting ground barley with 20% (v/v) ethanol with mechanical stirring for not more than 30 min. at room temperature, centrifuging, filtering and raising the ethanol content to 50%. After settling overnight, the clear portion was treated with ethanol to 80%, the precipitate collected, washed and dried *in vacuo*. An aqueous extract of it was cooled to 0–1°, and simultaneously, sufficient acid to bring it to pH 3·4 and some phosphate citrate buffer at pH 3·4. Then, at 1°, the buffer followed by the acid were added to the enzyme and the mixture kept (with antiseptics) for 24 hr. or more until required for use, when the calculated amount of alkali was added if necessary before allowing it to assume ordinary temperature.

Effect of time of exposure to acid. The effect of exposure to an acid pH of 3·4 (Table 1) at 25° is clearly to destroy α-amylase, but storage for short periods at 0° as employed by Ohlsson (1926) in the preparation of 'saccharogenamylase' from malt is not quite safe. Barley samples and enzyme preparations from them vary in this respect. At pH 3·4 the α-amylase is inactivated rapidly and in this case evidently completely within 24 hr., whereas the β-enzyme partly survived and was further weakened after that time. In other experiments a precipitated enzyme lost about 80% of its saccharifying activity in 2 hr. but little thereafter.

Criterion of purity. The criterion of freedom from α-amylase finally adopted was the hydrolysis limit of a fast reaction at pH 4·6 and is based on evidence furnished in Tables 3 and 4 and discussed later. When this limit is 56% R.M. or less, and further pretreatment of the enzyme at pH 3·4 and 0° does not reduce the limit, the β-amylase is regarded as pure.

Table 1. *Pretreatment of precipitated enzyme at* pH 3·4 *and* 0°

Time of pretreatment of enzyme at pH 3·4 ...	1 min.	1 min.	2 hr.	3 hr.	24 hr.	50 hr.	125 hr.
Vol. of enzyme/100 ml. reaction mixture (ml.)	1	1·6	1·8	2·1	5	10	10
pH of reaction mixture	4·6	3·4	3·4	3·4	4·6	4·6	4·6
Time of reaction (hr.)	Amylase action as percentage of theoretically obtainable maltose						
1	42·6	42·0	41·9	43·9	45·8	45·5	—
24	55·9	51·6	51·8	51·8	52·4	—	52·3
46	58·1	52·5	52·1	51·8	—	—	52·7
94	60·6	53·4	53·0	53·0	—	54·1	—
121	—	—	—	—	54·0	54·2	53·6
145	—	—	—	—	54·3	—	—
192	—	—	—	—	54·5	54·4	—

The limit of reactions promoted at pH 3·4 was established by employing a very wide range of enzyme concentration, the smaller doses of active enzyme being made up to the volume of the highest dose with well-boiled and inactivated enzyme. The results are given in Table 2. After 504 hr. action, the surviving enzyme activity was measured by the addition of fresh starch to the concentration of 2 %. The results of 3 hr. renewed action are expressed on the same basis as the others in the table. In the absence of inactivation these latter results should agree with those in the main table for 3 hr., except that allowance should be made for dilution (7:10) of enzyme on adding fresh starch. However, in col. 1 of Table 2 it will be seen that the surviving enzyme could after dilution to 7/10 perform in 3 hr. what the original enzyme performed in 0·5 hr., i.e. about 24 % of the original had survived. These and the corresponding values for the other columns are included in Table 2. Thus an effective enzyme activity had survived in the two mixtures which had received the most initial enzyme and these had reached 53·5–54 % R.M. Although hydrolysis had virtually ceased in the presence of active enzyme, inspection of the results, especially the bottom six lines of Table 2, leads to the conclusion that 54 % is not necessarily the extreme limit of action of β-amylase on this preparation of Lintner soluble starch at pH 3·4. In the three most dilute enzyme systems, before 54 % hydrolysis could be attained, the enzyme became sufficiently inactivated to bring progress to a standstill without attaining the limit. It seems probable that even the highest enzyme doses were acting so slowly at the end that the limit was not actually reached. The figures in Table 2 also confirm proportionality in the early stages between hydrolysis and the product of enzyme concentration and time. The R.M. of the enzyme control, which was deducted in arriving at the figures in the table, constitutes a serious correction under these conditions, increasing markedly with time.

Table 2. *Limit of action of β-amylase at* pH 3·4

Vol. of enzyme preparation/100 ml. of reaction mixture (ml.) ...	10	5	2·5	0·5	0·1	10 (no starch)
Time of reaction (hr.)	Results as percentage of theoretically obtainable maltose					
0	—	—	—	—	—	1·3
0·25	33·3	—	—	—	—	—
0·5	40·1	32·8	24·4	—	—	—
1·0	44·6	40·4	34·4	12·1	—	—
2·5	—	45·9	41·7	24·1	—	—
3	48·2	46·8	43·7	27·0	8·5	—
4	—	—	44·7	31·2	10·9	—
24	49·3	48·4	47·5	43·0	30·6	2·0
96	51·3	49·9	49·0	46·4	39·4	2·6
144	52·1	51·1	50·1	47·4	40·7	3·7
168	53·9	53·0	—	—	—	—
240	53·9	53·1	50·6	48·5	41·3	6·0
312	53·9	53·2	50·5	48·6	41·5	—
504	54·1	53·4	50·6	48·7	41·8	6·0
Fresh starch added 3 hr. later ...	40·0	31·2	14·6	0·4	0·0	2·0
	Amount of enzyme still active after 504 hr.					
(*a*) as percentage of the amount originally added	24	22	10	1·5	0	
(*b*) as ml. of original preparation (compare top line of table)	2·4	1·1	0·25	0·007	0	

Table 3. *Limit of action of β-amylase at* pH 4·6

Vol. of enzyme preparation/100 ml. of reaction mixture (ml.) ...	1·0	0·5	0·4	0·25
Time of reaction	Results as percentage theoretically obtainable maltose			
1 hr.	38·0	27·6	23·1	16·2
24 hr.	53·3	54·2	53·0	51·9
6 days	54·7	54·8	54·2	53·2
13 days	56·4	—	—	—
20 days	—	56·0	54·2	—
27 days	56·5	56·9	55·8	54·3

The limit of action of the enzyme, pre-restricted at pH 3·4 but promoting hydrolysis at pH 4·6 was now determined using a wide range of enzyme additions (see Table 3). The enzyme had been stored at pH 3·4 at 0° for several hours before use. There was considerable residual enzymic activity in the final reaction mixtures in all cases. The limit is not so clear cut when the reaction is carried out at pH 4·6, a slight 'drift' continuing beyond 54 % R.M. as far as 56–57 % in this case. In other experiments, such as the following one, the final limit was about 56–58 %.

Action of β-amylase on different starches. Many different samples of barley were employed at one time or other in these investigations but little difference was found. It was also considered desirable to extend these experiments to other preparations of soluble starch, and to potato starch paste. One example of such is given.

Potato starch paste and a new Lintner soluble starch (commercial preparation) were used as substrates under conditions identical with those of the previous two experiments except that a fresh barley sample was used as source of amylase. The reaction mixtures at pH 3·4 were split after 46 hr., one part receiving a fresh dose of enzyme equal to

Table 4. *Limits of action of β-amylase on potato and soluble starches*

	Potato starch		Soluble starch	
Substrate concentration in reaction mixture (%) ...	1·25		1·55	
pH	3·4	4·6	3·4	4·6
Vol. of enzyme/100 ml. of mixture (ml.)	20	5	20	5
Time of reaction (hr.)	Results as percentage of theoretically obtainable maltose			
0·5	39·1	39·4	41·3	42·5
1·0	—	43·9	46·4	51·2
17·5	51·2	55·0	52·6	53·4
42	51·4	56·8	53·3	54·2
	pH 3·4 pH 4·6		pH 3·4 pH 4·6	
66	53·5 52·0	57·6	53·3 54·4	54·0
90	53·5 54·1	57·8	53·3 55·5	54·4
144	53·7 56·2	58·2	54·6 57·5	54·7

that previously added, the other being brought to pH 4·6 with a small volume of NaOH. The reactions were now continued. The results are shown in Table 4. Whilst the reaction at pH 3·4 reached the same fairly sharp limit as before, the 'drift' at pH 4·6 is again in evidence. The enzyme clearly survived 42 hr. at pH 3·4. This and other experiments also indicated that a well-dispersed potato starch paste will give the same small range of limits of hydrolysis as Lintner starches at both values of pH and that Lintner starches vary a little among themselves in this respect.

It was invariably noticed that the reaction mixtures whose hydrolysis terminated at 53–57 % remained fairly clear to the end and the iodine coloration blue, whereas those which reached 60 % and presumably contained a trace of α-amylase flocculated and gave a purple iodine colour. Baker (1902) and Meyer *et al.* (1940) have reported similarly, but Hanes (1935) has observed the same flocculation phenomenon with α-malt amylase, acting alone, and it has been repeatedly observed by us. Presumably it is associated with a certain early reaction product of the α-enzyme.

Since the work already described was performed (in 1938–40) a possible explanation of the failure of β-amylase to promote hydrolysis to 60 % at pH 3·4 has presented itself in the phenomena of retrogradation of starch components, particularly amylose. To test this point 2 % Lintner starch was brought to pH 3·4 and divided. One portion was allowed to stand at room temperature at pH 3·4 for 2 days with antiseptics, another after titration back to pH 4·0, others similarly at pH 5 and 7. Finally, all were adjusted to pH 5 and digested under the same conditions as previously with pre-restricted β-amylase. There was no significant difference between the rates or limits of the enzyme actions. Apparently no retrogradation occurred at pH 3·4 to render the starch more resistant to the amylase. It is even less likely to have occurred in our reaction mixtures at 25° which attained the 50 % stage of hydrolysis in a few hours. It seems, therefore, that the true limit of action of β-amylase from barley is about 56 % ± 1 R.M., and varying somewhat with the starch preparation. Higher figures than this obtained from Lintner soluble and potato starch which have received no abnormal pretreatment must be due to α-amylase which, apart from the contribution of the natural barley-grain microflora, is almost invariably present.

Effects of α-amylase impurity. As an example of the effect of a most minute trace of α-amylase the following experiment is illustrative:

A bacterial amylase made from *B. subtilis* and purified by repeated ammonium sulphate precipitation, kindly supplied by Dr Leo Wallerstein, New York, which has been described by Hopkins & Kulka (1942), was dissolved and a solution (0·1 %) prepared such that 1 ml. possessed α-amylolytic activity equal to that of about 1 g. of average barley malt. A hydrolysis of 2 % soluble starch by β-amylase at pH 4·6 of the type reported earlier in the paper—attaining about 40 % hydrolysis in the first hour—proceeded normally. The addition of 0·001 ml. of the α-amylase solution to another 100 ml. of the reaction mixture modified the hydrolysis only in the final stages after 55 % R.P. had been attained, the final limit being 63 %. The mixed enzymes behaved, in fact, like a typical barley amylase, which had not been pretreated at pH 3·4.

Furthermore, by heating unpurified barley amylase solution to 70° for 15 min. as in Ohlsson's method of preparing α-amylase from malt extract, a preparation possessing α-amylase activity was obtained which hydrolyzed starch in the manner characteristic of such enzymes, as indicated both by kinetics of reducing power formation and of iodine coloration.

Also, ethanol-precipitated barley amylase was inactivated by iodine treatment, 10 ml. (equivalent to 30 g. barley) was treated with 0·4 ml. of 0·1 N-I_2 in KI for 2 hr. at room temperature. The saccharifying power was thereby reduced to 1/4000 of the original. There remained, however, a substantial liquefying power, roughly one-fortieth of that of the original enzyme, as measured by the method of Blom & Bak (1938). Thus the liquefying power, another criterion of α-amylase activity, was relatively concentrated by destruction of the β-amylase, and its presence more easily demonstrated.

Finally, the hypothesis was tested that the α-amylase of ungerminated barley is actually and solely due to the presence of bacteria. Ground barley was extracted for 30 min. only, centrifuged, the supernatant liquid filtered, the clear extract passed through a Chamberland filter and preserved sterile. This was used directly as an enzyme instead of precipitating with ethanol. Action on 2 % soluble starch at pH 4·8 and 25°, commenced and continued as far as possible under sterile conditions, gave the following results (Table 5).

Table 5. *Limit of action of sterile barley extract (Chamberland-filtered)*

Time	Percentage of theoretically obtainable maltose: 5 ml. extract/200 ml. reaction mixture	Percentage of theoretically obtainable maltose: 20 ml. extract/200 ml. reaction mixture
0·5 hr.	39·8	53·9
2 hr.	53·3	—
4 days	56·9	58·2
7 days	58·6	59·1
14 days	60·8	61·6
21 days	60·9	61·8
28 days	60·9	61·8

Apparently the enzyme which is responsible for extending the hydrolysis limit from 56 to 60 % is not removed by these precautions, and it is not solely due to living bacteria. There remained the possibility that amylase-secreting organisms present in the barley may have excreted α-amylase into the medium during the extraction. However, similar results were obtained using commercial pearl barley (i.e. possessing no 'husks' beneath which bacteria could lurk) which was first repeatedly washed with water containing chloroform, rapidly dried in a draught, ground and extracted with water containing antiseptic. This process removed bacteria before the initial ½ hr. extraction. These precautions, whilst advisable, are probably rendered unnecessary by subsequent treatment of the enzyme at pH 3·4.

The conclusion is drawn that all the resting barleys we have examined contain a little active α-amylase. Only barley amylase preparations which have been treated to inactivate the α-enzyme can be employed as pure β-amylase.

SUMMARY

1. The amylase of resting barley grain is mostly β-amylase but in all cases examined contained a trace of α-amylase. Unless adequate precautions are taken, bacteria present on the grain contribute to the α-amylolytic activity.

2. The β-amylase may be prepared free from α by the method of acidification to pH 3·6 applied by Blom *et al.* (1936) to aqueous extracts of barley. This, with precautions that are described, may also be applied to an ethanol-precipitated preparation of the enzyme.

3. Such β-amylase hydrolyzes potato starch paste and Lintner soluble starch alike to 53–54 % of theoretically obtainable maltose at pH 3·4 and to 56–57 % at pH 4·6. The latter is the truer result as considerable enzyme inactivation takes place in the reaction mixtures at pH 3·4. This result is of importance in calculations concerning the amylopectin molecule.

4. These limits vary a little with the starch preparation employed, but retrogradation phenomena are not responsible for them.

5. The many and confusing data in the literature concerning the limit of barley amylase action are largely accounted for by the presence of α-amylase which is responsible for the hydrolysis beyond 56 %. Maltase also may have contributed in some cases.

6. Suitable conditions for the extraction of the β-amylase from the grain and subsequent removal of α-amylase are described.

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REFERENCES

Baker, J. L. (1902). *J. chem. Soc.* **81**, 1177.
Blom, J., Bak, A. & Braae, B. (1936). *Hoppe-Seyl. Z.* **241**, 273.
Blom, J. & Bak, A. (1938). *Hoppe-Seyl. Z.* **256**, 197.
Caldwell, M. L. & Doebbeling, S. E. (1935). *J. biol. Chem.* **110**, 739.
Hanes, C. S. (1935). *Canad. J. Res.* B. **13**, 185.
Hanes, C. S. (1937). *New Phytol.* **36**, 101, 189.
Haworth, W. N., Kitchen, H. & Peat, S. (1944). *J. chem. Soc.* p. 619.
Hills, C. H. & Bailey, C. H. (1938). *Cer. Chem.* **15**, 351.
Hinton, C. L. & Macara, T. (1924). *Analyst*, **49**, 2.
Hopkins, R. H. & Kulka, D. (1942). *J. Inst. Brew.* **48**, 170.
van Klinkenberg, G. A. (1931). *Proc. Acad. Sci. Amst.* **34**, 893.
Kneen, E., Sandstedt, R. M. & Hollenbeck, C. M. (1943). *Cereal Chem.* **20**, 399.
Meyer, K. H., Bernfeld, P. & Press, J. (1940). *Helv. chim. Acta*, **23**, 1465.
Ohlsson, E. (1926). *C.R. Lab. Carlsberg*, **16**, 7.
Willstätter, R. & Schüdel, G. (1918). *Ber. dtsch. chem. Ges.* **51**, 780.