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A MODEL FOR STARCH BREAKDOWN IN HIGHER PLANTS

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Abstract—An *in vitro* system for the breakdown of starch granules by mixtures of α- and β-amylase is developed and discussed with reference to information concerning the degradation of starch *in vivo* β-Amylase has no action on starch granules and has very little effect on the rate of starch granule digestion by α-amylase It does, however, affect the product distribution in an α-amylase digest and is considered to attack dextrin intermediates produced by the action of α-amylase on the starch granules

INTRODUCTION

STARCH is composed of two components, amylose and amylopectin, which form a paracrystalline network within the granule. Amylose is essentially a linear polymer containing α-1,4-linked glucose residues, whereas amylopectin is a multiply branched glucan containing both α-1,4- and α-1,6-linkages. A model for the degradation of this polysaccharide *in vivo* must be able to explain its digestion in a storage tissue, such as a cereal endosperm or potato tuber, as well as in leaves where it is laid down as a temporary reserve prior to translocation in the form of sucrose Enzymes must not only be able to break the α-1,4- and α-1,6-linkages in the soluble starch derivatives, but at least one of them must be capable of attacking granular starch itself.

As early as 1890 Brown and Morris[1] recognized that germinating barley grains contain two amylases, only one of which is capable of attacking native starch granules. It is now known that this component is α-amylase (E.C. 3.2.1.1.) and that β-amylase (E.C. 3.2.1.2) has no action on this substrate [2] Walker and Hope[3] have shown that crystalline α-amylases can degrade raw starch as well as the crude extracts from which they are purified and therefore the special raw starch degrading enzyme, postulated by Blish *et al.*,[4] is unnecessary.

The adsorption of α-amylase onto starch granules obeys the Freundlich adsorption isotherm[3] and this information has been used by McLaren[5] to develop an equation to describe the α-amylolytic attack on raw starch. He assumed that if amylase action were dependent on adsorption the rate of digestion would be proportional to the amount of enzyme bound to the granules. This is presented as follows:

$$\text{amount of adsorbed amylase} = c.A.E^n$$

[1] BROWN, H T and MORRIS, G H (1890) *J Chem Soc* **57**, 458
[2] SANDSTEDT, R M (1955) *Cereal Chem (Suppl)* **32**, 17
[3] WALKER, G J and HOPE, P M (1963) *Biochem J* **86**, 452
[4] BLISH, M J, SANDSTEDT, R M and MECHAM, D K (1937) *Cereal Chem* **14**, 605
[5] MCLAREN, A D (1963) *Enzymologia* **26**, 237

where A is the total surface area of the granules, E the total concentration of α-amylase and c and n are constants Therefore:

$$\text{velocity of reaction } (v) = k\, A\, E^n$$

where k is also a constant. This can be expressed in the form:

$$\log_{10} v = \log_{10} k\ .A + n.\log_{10} E$$

so that a plot of $\log_{10} v$ against $\log_{10} E$ should be a straight line of slope n and intercept $\log_{10} k\,.A$ on the $\log_{10} v$ axis. The results of Walker and Hope[3] fit this equation as do the more recent data of Sandstedt and Ueda.[6]

It is assumed below that the above equation is valid and a few cases of *in vitro* starch granule digestion are considered to obtain further information on the action of amylases on this substrate. This information, together with that obtained from the literature, is then used to build a simple model for the pathway of starch breakdown in plant tissues

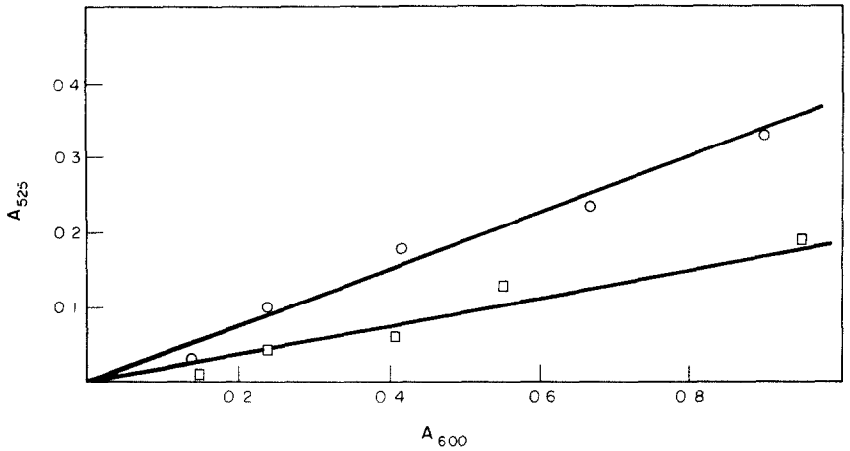

FIG 1 GLUCOSE PRODUCTION DURING THE AMYLOLYTIC DIGESTION OF STARCH GRANULES
Digests contained 50 mg of α-amylase alone (○), or 50 mg of α-amylase and 50 mg of β-amylase (□) 0 2 ml samples were removed at intervals and the amount of glucose produced (A_{525}) plotted against the increase in reducing power (A_{600}) Digest conditions were as described in the text

RESULTS

The production of glucose during the digestion of starch granules by α-amylase was directly proportional to the total reducing power generated This was also the case when mixtures of α- and β-amylase were used, but in these digests the glucose produced for a given level of digestion was depressed (Fig 1) This was confirmed using TLC which showed that the addition of β-amylase to an α-amylase digest stimulated the formation of maltose but decreased the rate of production of glucose and maltotriose Native starch granules were not susceptible to attack by β-amylase and this enzyme stimulated the production of reducing power by α-amylase by only very small amounts For example, the addition of 50 mg of β-amylase to a digest containing 50 mg of bacterial α-amylase stimulated the rate of granule digestion, as measured from reducing power increases, by less than 10%

[6] SANDSTEDT R M and UEDA S (1969) *Japan J Starch Sci* **17**, 215

Partially digested starch granules were prepared for further studies on the actions of α- and β-amylase. An initial digest was prepared containing 50 mg of starch and 50 mg of bacterial α-amylase in 5 ml of buffer. This was incubated for 60 min at 40° to give approximately 10% digestion of the granules. Then 100 mg of maltose was added to inhibit α-amylase binding to the starch granules,[7] and the mixture centrifuged (2 min in a bench centrifuge). The starch pellet was resuspended in 5 ml of 2% maltose solution in buffer. The mixture was again centrifuged and the pellet resuspended in 5 ml of buffer containing no maltose. Further centrifugation and resuspension of the pellet in fresh buffer yielded a preparation of partially digested starch granules free of α-amylase and reducing sugar. These granules were susceptible to α-amylase attack but were still completely resistant to β-amylase.

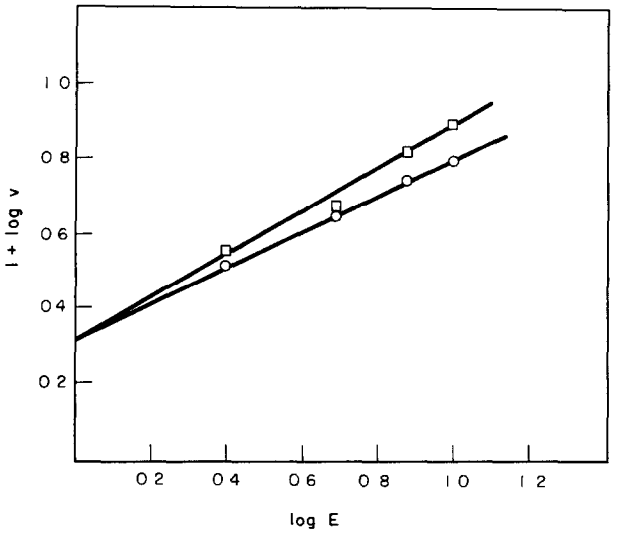

FIG 2 KINETICS OF STARCH GRANULE DIGESTION
Digests contained α-amylase alone (○), or equal amounts (weight) of α- and β-amylase (□) For each α-amylase concentration (E) the reaction rate (v) was measured and the data plotted in the form of $1 + \log_{10} v$ against $\log_{10} E$ Digest conditions were as described in the text

The effect of enzyme concentration on the rate of granule digestion was studied and the results found to fit the equation derived by McLaren.[5] For α-amylase alone, up to 50 mg of enzyme was used in the 5 ml digests containing 50 mg of starch. For a mixture of α- and β-amylase, equal weights (up to 50 mg of each) of each enzyme were used in similar digests. β-Amylase had very little effect on the kinetics of granule digestion. $\text{Log}_{10}k.A$ was unaffected, but n changed from 0·5 to 0·6 (Fig. 2).

TLC using EtOAc–MeOH–H_2O (52:36:13) showed that the products were glucose, maltose and maltotriose. TLC using EtOAc–MeOH–H_2O (37·40.23) revealed oligosaccharides with a degree of polymerization of around 15–20 which were present in digests containing mixtures of α- and β-amylase as well as those containing α-amylase alone. These dextrin intermediates, which could be visualized using sulphuric acid or iodine vapour, were present in trace amounts and their levels and chromatographic mobilities did not appear to be influenced by the presence of β-amylase. Control experiments showed that these dextrins were not present in undigested starch samples.

[7] SCHWIMMER, S and BALLS, A K (1949) *J Biol Chem* **180**, 883

The glucose oxidase assay of starch granule digestion was developed so that amylolytic action could be measured in the presence of high concentrations of reducing sugars, such as maltose, which might have an important regulatory function in the plant cell The sample of maltose used contained less than 0·2% of glucose so that quite high concentrations could be used before there was any serious interference with the assay procedure. In these inhibition experiments it was found that maltose at 50 mg/ml had no effect on glucose production during barley α-amylase attack on maize starch granules.

DISCUSSION

The present results confirm earlier work in showing that α-amylase attacks native starch granules and that β-amylase does not. However, they go further in suggesting that β-amylase is incapable of attacking starch granules at any stage of their dissolution. β-Amylase had no action on partially digested granules and had very little effect on the rate of starch digestion by α-amylase. This small effect might have been caused by the different product distribution or perhaps a non-specific activation of α-amylase by added protein. If β-amylase has no action on the starch granules themselves, one must postulate the existence of dextrin intermediates to explain the increased production of maltose in these digests The β-amylase would then successfully compete with α-amylase for the degradation of these dextrins to simple sugars The existence of these intermediates was confirmed by TLC and so one can describe β-amylase as a "complement" to α-amylase, the latter being the fundamental enzyme concerned with the degradation of starch itself

Analytical studies on the *in vivo* breakdown of starch support the above conclusions. Fukui and Nikuni[8] isolated starch from ungerminated and germinated rice and found that the amylose/amylopectin ratio apparently increases during germination Greenwood and Thomson[9] carried out similar studies on germinating barley. They found that during germination there is an increase in the amylose content of the starch and limited decreases in the size of the amylose and average chain length and beta-amylolysis limit of the amylopectin Manners and Bathgate[10] obtained similar results when analysing the starch of ungerminated and malted oats. Kiribuchi and Nakamura[11] interpreted these results in terms of limited debranching of the amylopectin while Greenwood and Thomson[9] postulated that the changes originate from a limited α-amylolysis of the amylose and a limited β-amylolysis of the amylopectin, both components still being incorporated in the granule. However, Manners and Bathgate[10] when studying the *in vitro* digestion of starch granules, found that all the above changes can be explained by limited α-amylolysis alone

Although β-amylase, limit dextrinase (α-dextrin 6-glucanohydrolase) and α-glucosidase are present in the endosperm of ungerminated cereals there is no starch breakdown in this tissue without the prior secretion of α-amylase from the aleurone layer or scutellar epithelium [12] During cereal germination the initiation of starch breakdown is paralleled by the appearance of α-amylase activity,[13–15] and this picture is also obtained from germination

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[9] GREENWOOD, C T and THOMSON, J (1959) *J Inst Brew* **65**, 346
[10] MANNERS, D J and BATHGATE G N (1969) *J Inst Brew* **75**, 169
[11] KIRIBUCHI, S and NAKAMURA, M (1973) *J Agr Chem Soc Japan* **47**, 341
[12] BRIGGS, D E (1973) *Biosynthesis and its Control in Plants* (MILBARROW B V, ed), p 219 Academic Press, London
[13] KIRIBUCHI, S and NAKAMURA, M (1973) *J Agr Chem Soc Japan* **47**, 333
[14] ABBOTT, I R and MATHESON, N K (1972) *Phytochemistry* **11**, 1261
[15] MURATA T, AKAZAWA, T and FUKUCHI S (1968) *Plant Physiol* **43**, 1899

studies on peas.[16,17] α-Amylase also appears to be responsible for starch breakdown in senescing tobacco leaves,[14] photosynthesizing maize leaves[18] and sprouting potato tubers.[19] All the data point to the fundamental role which α-amylase plays in the dissolution of starch granules and this information can be used to formulate a simple model for the breakdown of starch granules which is expressed as follows: (a) α-amylase is the only degradative enzyme which has any action on the starch granule *in vivo*; (b) the function of the other "starch degrading" enzymes (β-amylase, limit dextrinase and α-glucosidase) is to degrade dextrins released into solution by the α-amylolytic attack on the granule itself.

The initial, rate limiting step in this pathway is the attack of α-amylase on the starch granule surface and therefore this is where control, if necessary, will be exerted It has been suggested[20] that maltose may have an important control function, although this could not be demonstrated in the present studies using purified barley α-amylase. It would not be surprising if there were no control in a cereal endosperm but one would expect control mechanisms to be found in photosynthetic tissue.[18]

The probable relationship between the breakdown of starch and other pathways of starch and sucrose metabolism has been discussed by de Fekete and Vieweg.[18] Although there is considerable controversy over the biosynthetic role of starch phosphorylase the present author does not consider that there is any reliable evidence for its involvement in the breakdown of starch as proposed by Lee *et al.*[21] However, one cannot rule out the possibility of the formation of some glucose-1-phosphate by the phosphorolytic breakdown of dextrins released from starch granules by α-amylase.

EXPERIMENTAL

Materials Maize starch was used in all digests incorporating native starch granules "Extra pure" maltose, used in inhibition studies, was obtained from B D H Ltd *Bacillus subtilis* α-amylase (39 units/mg as measured by the method of Manners and Wright[22]) and barley β-amylase (100 units/mg as measured by the method of Hobson *et al* [23]) were both commercial preparations A purified α-amylase preparation, free of α-glucosidase activity, was obtained from an extract of malted barley (*Hordeum distichum* cv Proctor) by a combination of starch granule adsorption,[24] ion exchange chromatography on DEAE-Sephadex[25] and gel filtration[26] on Sephadex G150

Digest conditions Typical digests were incubated at 40° and contained 50 mg of starch in 5 ml of 0 2 M acetate buffer, pH 5·0, (containing 10 mM $CaCl_2$) Where relevant, up to 50 mg of β-amylase was added and the mixture preincubated at 40° The digestion was started by the addition of up to 50 mg of bacterial α-amylase (or the equivalent amount of barley α-amylase) and duplicate 0 2 ml samples withdrawn, for reducing power[27] or glucose content[28] measurements, after periods up to 60 min The starch was kept in suspension by gentle shaking

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[24] SCHWIMMER, S and BALLS, A K (1949) *J Biol Chem* **179,** 1063
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[28] LLOYD, J L and WHELAN, W J (1969) *Anal Biochem* **30,** 467

TLC of products Samples from digests were analysed by TLC[29] (silica gel on aluminium) using EtOAc–MeOH–H_2O (52 36 13 or 37 40 23) After development (single ascent), the plates were stained using alkaline $AgNO_3$, iodine vapour or 18N H_2SO_4 plus heat

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