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Mechanism of action and the substrate-dependent pH maximum shift of the α-amylase of *Bacillus coagulans*

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Abstract

The α-amylase of *Bacillus coagulans* is a saccharifying α-amylase which hydrolyses the disaccharide maltose [L. Keating, C. Kelly, and W. Fogarty, *Biochem. Soc. Trans.*, 24 (1996) 44S]. The pH maximum for maltose hydrolysis is pH 5.0, differing from the pH maximum for starch hydrolysis which is pH 6.0. Studies using reducing end ^{14}C-labeled maltooligosaccharides revealed a substrate-dependent pH maximum shift; hydrolysis of radiolabeled maltotriose (G_3*) was maximal at pH 5.0 while the pH maximum for hydrolysis of radiolabeled maltopentaose (G_5*) and maltohexaose (G_6*) was pH 6.0. With maltotetraose (G_4*) however, the pH maximum was pH 5.0–6.0. In addition, the bond cleavage pattern of G_4* was dependent on pH. At pH 5.0, the pH maximum for maltose hydrolysis, the frequency of hydrolysis of the reducing end terminal bond of G_4* was maximal. Determination of the pH maximum of the productive binding modes of the cleavage patterns of G_3* to G_6* illustrated the possible role of the occupation of subsite $r+2$ in the pH control mechanism of *B. coagulans* α-amylase. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: *Bacillus coagulans*; α-Amylase; pH maximum shift; Maltose-hydrolysing

1. Introduction

α-Amylases (α-(1→4)-D-glucan glucanohydrolase, E.C.3.2.1.1, endo amylase) hydrolyse α-(1→4)-glucosidic linkages in starch and glycogen by an endo-acting hydrolytic mechanism, by-passing α-(1→6)-glucosidic linkages. They have little, if any, affinity for small maltooligosaccharides, and maltose is rarely hydrolysed. In general bacterial α-amylases hydrolyse maltotriose slowly in contrast to fungal α-amylases, some of which have a high affinity for this trisaccharide [1].

α-Amylases are usually stable in the pH range pH 5.5–8.0, with maximal activity normally occurring between pH 4.8 and pH 6.5. α-Amylases may differ in the values of their pH maxima and in the shape of their pH activity profiles. However, the pH maximum of any one α-amylase does not vary with substrate, except in the case of a small number of mammalian α-amylases [2].

The active site of an α-amylase is assumed to be composed of a definite number of subsites, each of which specifically interacts with a certain glucose

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unit in the substrate. A number of methods have been described to determine the affinities of these subsites [3–6]. In all of these theories two assumptions are made; firstly, k_{int}, the intrinsic rate constant, is independent of the substrate chain length, and secondly, the subsites are independent of each other, so that the affinities are simply additive. However, for porcine pancreatic α-amylase (PPA) Ishikawa et al. [7] state that this latter assumption does not hold true.

In this paper the unusual substrate specificity of *Bacillus coagulans* α-amylase is examined in conjunction with the substrate-dependent pH maximum shift and action pattern of the enzyme. The data presented indicate that the theories currently employed to determine subsite affinities are not applicable to the α-amylase of *B. coagulans*.

2. Results

Hydrolysis of small maltooligosaccharides (maltosidase activity).—The saccharifying α-amylase of *B. coagulans* hydrolyses maltose and starch [8], with the pH maximum for maltose hydrolysis at pH 5.0, differing from that for starch at pH 6.0 (Fig. 1). In addition to atypical hydrolysis of maltose, the α-amylase of *B. coagulans* hydrolysed maltotriose at a relatively high rate. The pH profile

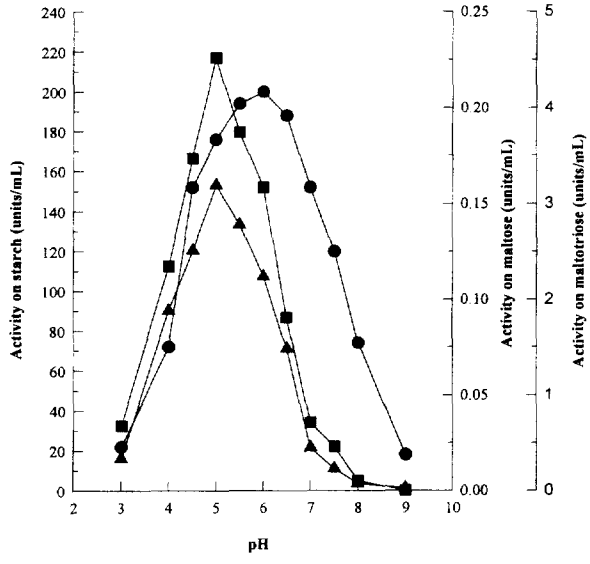

Fig. 1. Determination of the pH profiles for various substrates of *Bacillus coagulans* α-amylase. —●— Activity on starch; —■— Activity on maltose; —▲— Activity on maltotriose. Activity was assayed using 1% (w/v) substrate on 0.1 M Universal buffer, at the pH values indicated, at 40 °C for 30 min.

for hydrolysis of maltotriose was similar to that for maltose hydrolysis, with the pH maximum also occurring at pH 5.0 (Fig. 1). The affinity of the enzyme for the trisaccharide was determined at pH 5.0 and the K_m value was 1.9 mM (data not shown).

Bond cleavage pattern.—To further analyse the substrate-dependent shift of the pH maximum of *B. coagulans* α-amylase, studies using reducing end ^{14}C-labeled maltooligosaccharides were carried out. The bond cleavage pattern of the α-amylase was determined at pH 5.0 and at pH 6.0 (Fig. 2). The findings were independent of the buffer systems used and the substrate concentrations employed (20–500 μM). For the hydrolysis of maltotriose (G_3*), maltopentaose (G_5*) and maltohexaose (G_6*) no significant differences between the cleavage patterns at pH 5.0 and 6.0 were observed. However, for maltotetraose (G_4*) the bond cleavage pattern was pH dependent. A decrease in pH from pH 6.0 to 5.0 resulted in increased hydrolysis of the reducing end terminal bond. In effect, at pH 5.0 more G_1* was produced than at pH 6.0. To further examine the pH dependence of the bond cleavage pattern for maltotetraose, the effect of a range of pH values (pH 4.5–6.5) was determined (Fig. 3). As the pH was increased from pH 5.0 to 6.5 the frequency of hydrolysis of the reducing end terminal bond decreased, reducing the production of G_1* from G_4*. A decrease in pH from pH 5.0 to 4.5 also decreased G_1* production. That is to say that at pH 5.0, the pH maximum for maltose hydrolysis,

	G-G-G* G3*			
pH 5.0	0.05	0.95		A
pH 6.0	0.05	0.95		A
	G-G-G-G* G4*			
pH 5.0	0.54	0.46		A
pH 6.0	0.57	0.43		B
	G-G-G-G-G* G5*			
pH 5.0	0.16	0.74	0.10	A
pH 6.0	0.16	0.74	0.10	A
	G-G-G-G-G-G* G6*			
pH 5.0	0.05	0.54	0.38	0.03 A
pH 6.0	0.05	0.54	0.38	0.03 A

Fig. 2. Determination of the bond cleavage pattern of α-amylase of *Bacillus coagulans* for the maltooligosaccharides G_3* to G_6*. G^* = ^{14}C-labeled reducing end glucose; maltotriose = G_3; maltotetraose = G_4; maltopentaose = G_5; and maltohexaose = G_6. The bond cleavage pattern was independent of the buffers employed (acetate buffer, pH 5.0, phosphate buffer, pH 6.0, Universal buffer, pH 5.0 and pH 6.0) and of the substrate concentrations examined (20–500 μM). F-test (x).

	G - G - G - G*	G4*	
pH 4.5	0.570	0.430	(B/C)
pH 5.0	0.540	0.460	(A)
pH 5.5	0.555	0.445	(A/B)
pH 6.0	0.570	0.430	(C)
pH 6.5	0.610	0.390	(D)

Fig. 3. Determination of the pH dependence of the bond cleavage pattern of ^{14}C-labeled maltotetraose displayed by α-amylase of *Bacillus coagulans*. $G^* = {}^{14}$C-labeled reducing end glucose; maltotetraose = G_4. The bond cleavage pattern was independent of the buffers employed (acetate buffer, pH 5.0, phosphate buffer, pH 6.0, Universal buffer, pH 4.5 to 6.5) and of the substrate concentrations examined (20–500 μM). F-test (xx).

there was the highest frequency of hydrolysis of the reducing end terminal bond of G_4*. From this it may be inferred that there are two productive binding modes for the hydrolysis of G_4* which have different pH dependencies.

Substrate-dependent pH maximum shift.—The hydrolysis rates of the enzyme for a range of radiolabeled substrates (G_3*–G_6*) were measured at low substrate concentrations (20–100 μM). The plots of $\ln(\Sigma G_i^*/G_n^*)$ versus reaction time were linear, in accordance with first-order kinetics, where ΣG_i* represents the sum of the radioactivities of all radioactive species (G_1*–G_n*). From the slope (k) of each plot, the catalytic efficiency (k_{cat}/K_m) of the enzyme was determined for each substrate (G_3*–G_6*) over the pH range pH 4.5–6.5. The pH profiles of the hydrolytic activities for G_3*–G_6* (Fig. 4) were, thus, obtained. In addition to this, the pH profiles for the various cleavage patterns of these substrates were determined, as demonstrated for G_1* and G_2* production from G_4* at pH 5.0 and 6.0 (Fig. 5(a) and (b)). However, it was not possible to determine the pH profiles for production of G_2* from G_3*; G_1* and G_3* from G_5*; and G_1* and G_4* from G_6* because of the extremely low level of production of these products. Again, the findings were independent of the buffer systems used and the substrate concentrations employed. In all cases, no maltooligosaccharide with a degree of polymerisation greater than that of the substrate were observed, indicating the absence of transglycosylation reactions. As concentrations of buffer greater than 50 mM are reported to effect the determination of the kinetic parameters [7], experiments were carried out at 25 mM concentrations.

The pH maximum for hydrolytic activity was dependent on the chain length of the substrate. The

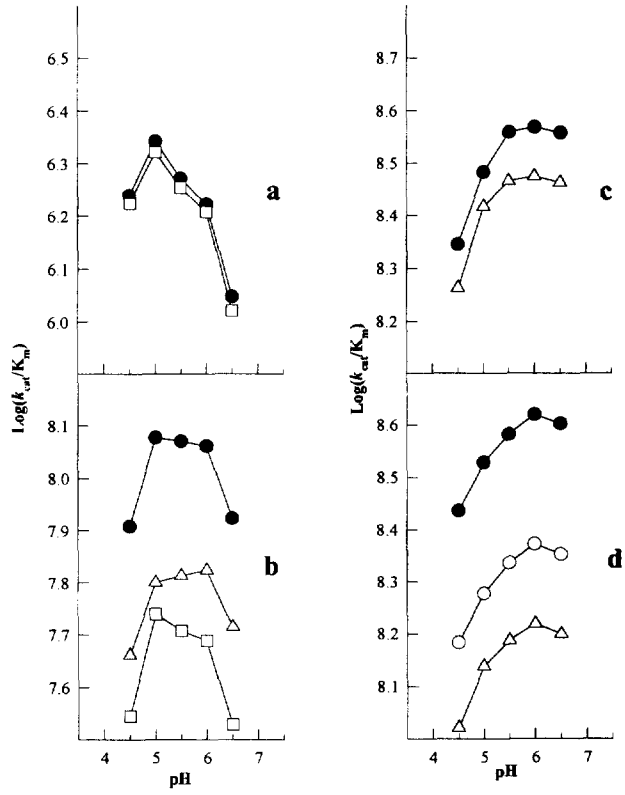

Fig. 4. Determination of the pH profiles of the determinable cleavage patterns and of the total hydrolytic activity of *Bacillus coagulans* α-amylase for G_3* (a), G_4* (b), G_5* (c) and G_6* (d). —●— Total hydrolysis; —□— G_1* production; —△— G_2* production; —○— G_3* production.

pH maximum for G_3* hydrolysis was pH 5.0 (Fig. 4(a)), as had been determined previously by the glucose oxidase method, with the pH profile for total hydrolytic activity identical to that for G_1* production from G_3*. The pH maximum for hydrolysis of G_5* and G_6* was pH 6.0 (Fig. 4(c) and (d)), similar to starch. Furthermore, the pH profiles for the one determinable cleavage pattern of G_5* and the two determinable cleavage patterns of G_6* were identical to those for total hydrolysis of these sugars. For G_4*, however, the pH profiles of the two cleavage patterns were different from one another and the pH maximum of the total hydrolytic activity of the two productive binding modes was pH 5.0–6.0 (Fig. 4(b)). The pH maximum for production of G_1* from G_4* was pH 5.0, whereas the pH maximum for G_2* production was pH 6.0.

3. Discussion

B. coagulans α-amylase was similar to a large number of α-amylases in displaying maximal

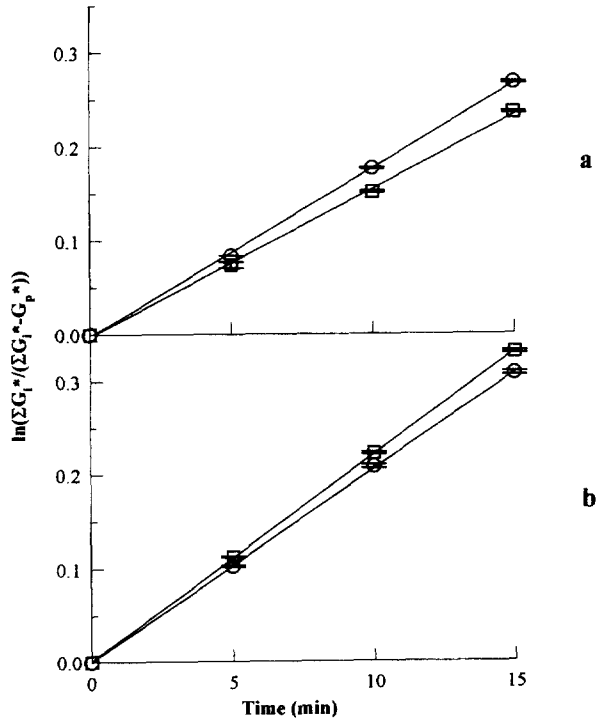

Fig. 5. First order plot of the production of labeled G_1 (a) and G_2 (b) from ^{14}C-labeled maltotetraose at pH 5.0 and pH 6.0 by α-amylase of *Bacillus coagulans*. —○— pH 5.0; —□— pH 6.0. The reaction mixture contained 20–100 μM reducing end radiolabeled maltotetraose (G_4*) and 7×10^{-9} M enzyme, in 25 mM Universal buffer at the appropriate pH. Aliquots of the reaction mixture were removed at the time intervals indicated and boiled to stop the reaction. The digests were analysed by paper chromatography. ΣG_1* = the sum of the radioactivities of all radioactive species (G_1*–G_4*) at time t. G_p* = the radioactivity of the product (G_1* or G_2*) at time t. Slope, $k = (k_{cat}/K_m)e_o$.

activity on starch at pH 6.0 [9,10]. However, the enzyme displayed maximal activity on maltose at pH 5.0. It is not the value of the pH maximum that is extraordinary but rather the occurrence of a substrate-dependent pH maximum shift. Such a shift has only ever been observed in a small number of mammalian α-amylases [2].

The pH maximum for maltotriose hydrolysis is also pH 5.0. The enzyme displays a relatively high affinity for this substrate compared to other bacterial α-amylases which have little, if any, affinity for this sugar [11,12].

Examination of the bond cleavage pattern of the enzyme at pH 5.0 and 6.0 using a series of reducing end ^{14}C-labeled maltooligosaccharides, G_3* to G_6*, revealed that, for the hydrolysis of G_4*, the cleavage pattern was pH dependent while pH did not appear to effect the cleavage patterns of G_3*, G_5* and G_6*. Further examination over a range of pH values demonstrated that hydrolysis of the reducing end bond of G_4*, resulting in the production of G_1*, was maximal at pH 5.0, the pH maximum for maltose hydrolysis.

In examining the difference in the action patterns on G_4* of maltose-hydrolysing α-amylases and non-maltose-hydrolysing α-amylases with *B. coagulans* α-amylase, an interesting observation is made (Fig. 6). The preferential bond cleaved in G_4* by the majority of the enzymes examined, regardless of whether the enzyme hydrolyses maltose or not, is bond 2, with G_2* as the major product. However, the maltose-hydrolysing α-amylases produce substantial levels (26–56%) of G_1* from G_4*. The α-amylases which are unable to hydrolyse maltose produce very low, if any, G_1* from G_4*, with the exception of the α-amylase of *Bacillus amyloliquefaciens*. This enzyme, however, is distinct from the maltose-hydrolysing α-amylases in producing G_3* (10%) from G_4*. It, thus, appears that the active site of maltose-hydrolysing α-amylases is structured such that G_1* production from G_4* is increased, if not favoured. This is best demonstrated by the work of Matsui et al. [17] on the maltose-hydrolysing α-amylase of *Saccharomycopsis fibuligera*. The authors produced a mutant (K210R) with enhanced maltase activity and found that the bond cleavage pattern for G_4* of the enzyme from the mutant was altered from that of the wild-type, resulting in increased G_1* production from G_4*. The enzyme of a second mutant (K210N) produced similar levels of G_1* from G_4* but also produced G_3* from the tetrasaccharide and displayed reduced maltase activity.

In the case of the bond cleavage pattern of G_4* by *B. coagulans* α-amylase, the frequency of hydrolysis of the reducing end terminal bond of

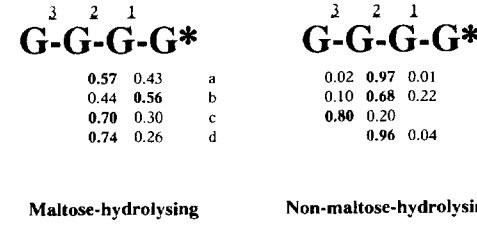

Fig. 6. Comparison of the bond cleavage pattern of G_4* of maltose-hydrolysing α-amylases and non-maltose-hydrolysing α-amylases. 1 = bond 1; 2 = bond 2; 3 = bond 3; a = *Bacillus coagulans* α-amylase; b = *Bacillus subtilis* α-amylase [13]; c = procine pancreatic α-amylase [7]; d = *Saccharomycopsis fibuligera* α-amylase [14]; e = *Aspergillus oryzae* α-amylase [5]; f = *Bacillus amyloliquefaciens* α-amylase [11]; g = *Saccharomonospora viridis* α-amylase [15]; h = *Thermoactinomyces vulgaris* α-amylase [16].

this sugar is maximal at pH 5.0, the pH maximum for maltose hydrolysis. PPA also displays a pH dependent bond cleavage pattern for G_4* hydrolysis [7], with a shift in the pH from pH 6.9 (the pH maximum for starch hydrolysis) to pH 5.2 (the pH maximum for maltose hydrolysis) resulting in an increase in the frequency of cleavage of the reducing end terminal bond. Kobayashi et al. [18] chemically modified the α-amylase of *Aspergillus oryzae*. The modified enzymes showed enhanced maltosidase activity despite reduced α-amylase activity and, unlike the native enzyme, produced glucose on hydrolysis of G_4 (as measured by the glucose oxidase method). So again, a structural change, this time brought about by chemical modification, may have caused an increase in hydrolysis of the reducing end terminal bond of G_4 and resulted in increased hydrolysis of small maltooligosaccharides. Radiolabeling experiments would be necessary, however, to determine if it is the hydrolysis of the reducing end terminal bond or the non-reducing end terminal bond, or both, that gives rise to the production of glucose from G_4.

It, thus, appears that an active site which accommodates hydrolysis of the reducing end terminal bond of G_4 also facilitates maltosidase activity in α-amylases. However, if the subsite structure also permits hydrolysis of the non-reducing end terminal bond of G_4, as with the *S. fibuligera* mutant, K210N, and also demonstrated in *B. amyloliquefaciens* α-amylase, then hydrolysis of maltose does not take place.

The pH maximum for hydrolytic activity in *B. coagulans* α-amylase was dependent on the chain length of the substrate. A simple two-catalytic residue model, in which the shape of the pH profile for k_{cat}/K_m is independent of the substrates [19], therefore can not be applied to the α-amylase of *B. coagulans*. Ishikawa et al. [20] proposed a three-catalytic residue model to explain the substrate-dependent pH maximum shift of PPA. This model may be applicable to the α-amylase of *B. coagulans*.

In studying the interaction between the substrate and the subsites of an enzyme the subsite theory is commonly used [13,14,16,21]. However, as the data for G_4* hydrolysis indicates that the function of the catalytic residues are not independent of the binding modes in *B. coagulans* α-amylase, the subsite theory does not appear to be applicable to this enzyme. For this reason, the number of the subsites and the location of the catalytic site is not deduced. Rather, a schematic model is drawn in which it is assumed that the active site of *B. coagulans* α-amylase is composed of at least four subsites (Fig. 7). The arbitrary notations assigned to the subsites by Suganuma et al. [5], prior to the application of their theory, are used. As such, the catalytic site is located between the r-th and the $(r+1)$-th subsites.

Having determined the pH profiles of a number of the cleavage patterns of the substrates G_3* to G_6*, it was possible to determine the pH maximum of the productive binding modes of those cleavage patterns. For simplicity, only the productive binding modes of *B. coagulans* α-amylase for G_3* to G_5* are illustrated. It appears that there may be two different hydrolytic modes depending on pH; mode A, with subsite $r+2$ unoccupied, results in G_1* production, the pH maximum of which is pH 5.0, while mode B, with subsite $r+2$ occupied, results in the production of G_2*, the pH maximum of which is pH 6.0 (Fig. 7(a)). Although not shown in Fig. 7(a), production of G_3* from G_6* is also maximal at pH 6.0. This productive mode also belongs to mode B, with subsite $r+2$ occupied.

This suggests that the composition of the active site in *B. coagulans* α-amylase may switch from one pair of catalytic residues to another pair exhibiting different pH dependencies as a result of the occupation of subsite $r+2$ by a glucosyl residue of the substrate, assuming there are three catalytic residues. This may be brought about by a con-

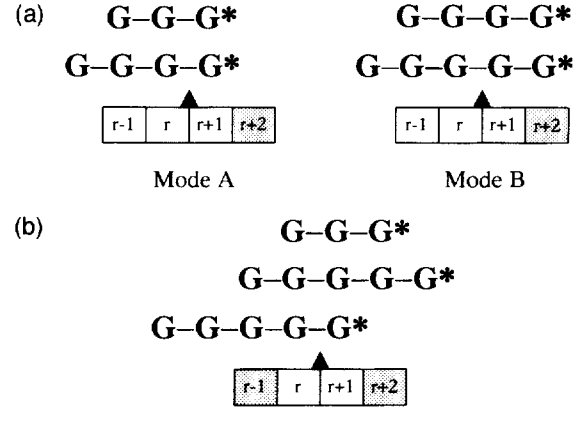

Fig. 7. (a) Schematic binding modes of *Bacillus coagulans* α-amylase and maltooligosaccharides (G_3*, G_4* and G_5*) for which the pH maximum for cleavage is determinable. The up arrowhead shows the catalytic site and the boxes represent the subsites. G^* = ^{14}C-labeled reducing end glucose. (b) Schematic binding modes of *Bacillus coagulans* α-amylase and maltooligosaccharides (G_3* and G_5*) for which the pH maximum for cleavage is not determinable. The up arrowhead shows that catalytic site and the boxes represent the subsites. G^* = ^{14}C-labeled reducing end glucose.

formational change of the active site induced by the interaction between the bound substrates and the subsites. It, thus, appears that the binding of subsite $r+2$ with a glucosyl residue of the substrate controls the catalytic activity, including the maximum pH, of *B. coagulans* α-amylase. Ishikawa et al. [7,20] similarly found that subsite 5 (numbered from the non-reducing end) in PPA was important in controlling the pH maximum for catalytic activity of the mammalian enzyme. For ease of comparison, the subsites of PPA will be numbered as previously described for *B. coagulans* α-amylase. As such, subsites 3 and 4 of PPA, adjacent to the active site, are the r-th and $(r+1)$-th subsites, respectively, and subsite 5 is the $(r+2)$-th subsite. The correlation between the $(r+2)$-th subsite of PPA and that of *B. coagulans* α-amylase is noted. The productive binding modes covering subsite $r+2$ appear to modulate the kinetic parameters in both of these enzymes, depending on pH. It would, thus, appear from the data presented that the subsite affinities of *B. coagulans* α-amylase cannot be determined by the subsite theory, as previously found for the mammalian α-amylase PPA [7], because in this theory each subsite is defined independently and the function of the catalytic residues are assumed to be independent from the binding modes.

Ishikawa et al. [22] further examined the substrate dependence of pH activity in PPA using a series of *p*-nitrophenyl maltooligosaccharides. Analysis of the multiple productive binding modes of these substrates revealed that another binding site (subsites r-1 and/or r-2), in addition to subsite $r+2$, was involved in the mechanism controlling the pH maximum of PPA. Although the α-amylase of *B. coagulans* hydrolyses both G_3* and G_5* at more than one bond, the low frequency of hydrolysis of particular bonds (e.g. bond 2 in G_3*) did not allow the accurate determination of the pH maximum of those cleavage patterns (Fig. 7(b)). If the pH maximum for production of G_2* from G_3* could be estimated then the role played by subsite r-1 in controlling the pH maximum of the α-amylase of *B. coagulans* could be determined.

Apart from a brief reference on Taka-amylase A by Kobayashi et al. [18], the only other reports of the pH maximum being controlled by the substrate have been on two additional mammalian α-amylases, that from human pancreas [2] and human saliva [22]. It is interesting to note that Ishikawa et al. [22] attribute great significance to their elucidation of the substrate-dependent pH maximum shift of mammalian α-amylases stating that this mechanism, specific to mammals, is very interesting in terms of the biological evolution and protein engineering of α-amylases considering that the amino acid sequences relevant to the active site are highly conserved in both mammalian and microbial α-amylases. In light of this, a corresponding finding in a microbial α-amylase is of great importance. This is the first report of a microbial α-amylase with these attributes. The question then arises, is this characteristic peculiar to *B. coagulans* α-amylase among microbial α-amylases or is it a common attribute in α-amylases that has remained unrecognised to date. The latter is more likely the case, with the limited number of reports of this phenomenon more likely attributable to lack of discovery rather than lack of occurrence. If this exception turns out to be the rule then it may be possible to incorporate the new findings into the subsite theory.

4. Experimental

Organism and growth conditions.—Cultures of *B. coagulans* were grown at 30 °C on 1% (w/v) agar slopes containing 1% (w/v) starch, pH 7.0, and stored at 4 °C. The culture was inoculated into 250 mL Erlenmeyer flasks containing 50 mL medium with the following composition (g/L): starch, 10.0; bactopeptone, 6.0; yeast extract, 3.0; K_2HPO_4, 1.5; $MgCl_2.6H_2O$, 0.2; $MnSO_4.4H_2O$, 0.04; initial pH 7.0. After growth at 30 °C for 18 h, a 1% (v/v) inoculum was transferred into production flasks containing the optimised medium previously described [8]. The cultures were shaken at 200 rpm in a New Brunswick Orbital incubator (Model G25) at 30 °C. After 48 h, the cells were removed from the medium by centrifugation at 16,300 *g* for 20 min at 4 °C in a Sorvall RC2-B centrifuge and the cell-free supernatant (CFS) was used for subsequent enzyme purification.

Materials.—Bactopeptone and yeast extract were from Difco, Michigan, U.S.A. *Bacillus macerans* cyclodextrin glucanotransferase (CGTase) (600 Tilden and Hudson units/mL) was a generous gift from Mr. Y. Ohyabu, Amano Enzyme Europe Ltd., Milton Keynes, U.K., D-[U-^{14}C]glucose (specific activity 297 mCi/mmol) was from Amersham Life Science, U.K. and Ecoscint A from National Diagnostics, Georgia, U.S.A. Maltose (high grade)

was purchased from Calbiochem Novabiochem (U.K.) Ltd., England, soluble starch (for assay) from May and Baker Ltd., U.K. and aniline phthalate spray from E. Merck, Germany. All other chemicals and reagents used were of AnalaR grade and were purchased from BDH, Dorset, U.K.

Assay.—Reducing sugars produced on hydrolysis of starch were measured by the dinitrosalicylic acid method [23]. Glucose released from maltose and maltotriose was measured by the glucose oxidase peroxidase-*o*-dianisidine (GOPOD) method [24]. Assays were carried out by adding 0.5 mL of enzyme to 1% substrate in 0.1 M acetate buffer, pH 5.0 (maltosidase), 0.1 M phosphate buffer, pH 6.0 (α-amylase) or 0.1 M universal buffer, pH 3.0–9.0. An enzyme unit is defined as the amount of enzyme releasing 1 mg of glucose, or glucose equivalents, from the substrate per 30 min at 40 °C.

Enzyme purification.—Initial purification was achieved by acetone precipitation at room temperature. A $\frac{3}{4}$ volume of precooled solvent (−18 °C) was added dropwise to the CFS, with constant stirring, and allowed to stand for over 1 h before centrifugation at 16,300 *g* for 20 min at 4 °C. The precipitate obtained was discarded and the procedure was repeated with a second $\frac{3}{4}$ volume of acetone. This precipitate was dissolved in a minimum amount of 0.02 M phosphate buffer, pH 6.0, and dialysed against water for 8 h. This partially purified preparation was applied to a column of S-Sepharose (20×1.6 cm) pre-equilibrated with 0.02 M acetate buffer, pH 5.0. The enzyme was eluted from the column with 0.02 M acetate buffer, pH 5.0, containing 0.1 M NaCl at a flow rate of 2.0 mL/min. Active fractions were pooled and applied to a column of phenyl Sepharose CL-4B (20×1.6 cm) pre-equilibrated with Tris/HCl buffer containing 1.5 M KCl, pH 8.5. Enzyme elution was performed with the same buffer without salt at 4.0 mL/min. Active fractions were pooled and applied to a Pharmacia Superose 12 gel filtration column, connected to a Pharmacia fast protein liquid chromatography (FPLC) system, pre-equilibrated with 0.05 M phosphate buffer, pH 6.0, containing 0.15 M NaCl. The α-amylase was eluted as a single peak with the same buffer at 0.5 mL/min.

Preparation of reducing end radiolabeled maltooligosaccharides.—^{14}C-reducing end labeled α-(1→4)-linked maltooligosaccharides were prepared according to a modification of the procedure of French et al. [25]. 1 mL of *B. macerans* CGTase was added to a reaction mixture containing 1 mL D-glucose (100 mM), 1 mL α-cyclodextrin (100 mM) and 5 mL D-[U-^{14}C]glucose (100 μCi) in 100 mM phosphate buffer, pH 6.0. The reaction mixture was incubated for 1 h at 40 °C. The reaction was stopped by boiling for 10 min.

Purification of radiolabeled maltooligosaccharides.—Radiolabeled maltooligosaccharides were separated by descending paper chromatography on Whatman No.3 MM chromatography paper eluted with 3:1 propanol–H_2O at 30 °C. A second descent was performed. The sugars were eluted in 1 mL of deionised water, freeze-dried and resuspended in deionised water to the desired concentration.

Analysis of radiolabeled digests.—Aliquots of the purified enzyme solution were mixed with the substrate (20–500 μM) in 25 mM acetate buffer (pH 5.0), phosphate buffer (pH 6.0), or Universal buffer (pH 4.5–6.5). The reaction mixture was incubated at 40 °C and at various time intervals samples were removed and boiled to stop the reaction. The sugar products were fractionated by descending paper chromatography and the maltooligosaccharides were visualised by aniline phthalate reagent spray and excised from the paper. The radioactivity (cpm) of the sugars was analysed by an LKB 1211 Rackbeta liquid scintillation counter.

Frequency distribution of bond cleavage.—The calculated radioactivity levels (cpm) of the hydrolysis products were compared for each of the substrates examined as described by Robyt and French [26].

Determination of catalytic efficiency (k_{cat}/K_m).—The catalytic efficiency (k_{cat}/K_m) of the enzyme was determined for maltotriose to maltohexaose over the pH range pH 4.5–6.5 as per Ishikawa et al. [7]. In calculating each k_{cat}/K_m value from the slope of the plot of $\ln(G_i^*/G_n^*)$ against reaction time, six determinations were made for each point on the linear graph, with error bars computed using standard deviation.

Statistical analysis.—Where the F-test shows a significant treatment effect, means were significantly different from each other except where they have a letter in common. Statistical analyses of the bond cleavage patterns of each sugar were performed independently. For each frequency distribution value, the average of six determinations is shown. For the pH dependence of the bond cleavage pattern of G_4^*, the standard error for each average is listed in Table 1. The levels of significance are indicated as follows: $p < 0.05$ is signified by x and $p < 0.01$ is signified by xx.

Table 1
Standard errors for the pH dependency of the bond cleavage pattern of radiolabeled maltotetraose

Standard error				
pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5
2.887×10^{-3}	2.108×10^{-3}	2.472×10^{-3}	1.291×10^{-3}	2.309×10^{-3}

References

[1] E.M. Doyle, C.T. Kelly, and W.M. Fogarty, *Appl. Microbiol. Biotechnol.*, 30 (1989) 492–496.

[2] K. Ishikawa, I. Matsui, S. Kobayashi, H. Nakatani, and K. Honda, *Biochemistry*, 32 (1993) 6259–6265.

[3] J.A. Thoma, C. Brothers, and J. Spradlin, *Biochemistry*, 9 (1970) 1768–1775.

[4] K. Hiromi, *Biochem. Biophys. Res. Commun.*, 40 (1970) 1–6.

[5] T. Suganuma, R. Matsuno, M. Ohnishi, and K. Hiromi, *J. Biochem.*, 84 (1978) 293–316.

[6] E. Prodanov, C. Seigner, and G. Marchis-Mouren, *Biochem. Biophys. Res. Commun.*, 122 (1984) 75–81.

[7] K. Ishikawa, I. Matsui, K. Honda, S. Kobayashi, and H. Nakatani, *Arch. Biochem. Biophys.*, 289 (1991) 124–129.

[8] L. Keating, C. Kelly, and W. Fogarty, *Biochem. Soc. Trans.*, 24 (1996) 44S.

[9] M.P. Brosnan, C.T. Kelly, and W.M. Fogarty, *Eur. J. Biochem.*, 203 (1992) 225–231.

[10] B.S. Collins, C.T. Kelly, W.M. Fogarty, and E.M. Doyle, *Appl. Microbiol. Biotechnol.*, 39 (1993) 31–35.

[11] J.D. Allen and J.A. Thoma, *Biochem. J.*, 159 (1976) 121–132.

[12] Y. Sakano, Y. Kashiwagi, and T. Kobayashi, *Agric. Biol. Chem.*, 46 (1982) 639–646.

[13] T. Suganuma, M. Ohnishi, K. Hiromi, and T. Nagahama, *Carbohydr. Res.*, 282 (1996) 171–180.

[14] I. Matsui, K. Ishikawa, E. Matsui, S. Miyairi, S. Fukui, and K. Honda, *J. Biochem.*, 109 (1991) 566–569.

[15] W.M. Fogarty, B.S. Collins, E.M. Doyle, and C.T. Kelly, *J. Ind. Microbiol.*, 11 (1993) 199–204.

[16] M. Sano, Y. Sakano, and T. Kobayashi, *Agric. Biol. Chem.*, 49 (1985) 2843–2846.

[17] I. Matsui, K. Ishikawa, S. Miyairi, S. Fukui, and K. Honda, *Biochemistry*, 31 (1992) 5232–5236.

[18] M. Kobayashi, M. Miura, and E. Ichishima, *Biochem. Biophys. Res. Commun.*, 183 (1992) 321–326.

[19] M. Dixon, E. Webb, C.J.R. Thorne, and K.F. Tipton, *Enzyme Kinetics*, in M. Dixon and E. Webb (Eds.), *Enzymes*, 3rd ed., Academic Press, New York, 1979, pp 47–206.

[20] K. Ishikawa, I. Matsui, and K. Honda, *Biochemistry*, 29 (1990) 7119–7123.

[21] E.H. Ajandouz and G.J. Marchis-Mouren, *Carbohydr. Res.*, 268 (1995) 267–277.

[22] K. Ishikawa, I. Matsui, and K. Honda, *Biosci. Biotechnol. Biochem.*, 59 (1995) 1175–1176.

[23] P. Bernfeld, *Methods Enzymol.*, 1 (1955) 149–158.

[24] A.S.G. Huggett and D.A. Nixon, *Biochem. J.*, 66 (1957) 12–19.

[25] D. French, M.L. Levin, E. Norberg, P. Nordin, J.H. Pazur, and G.M. Wild, *J. Am. Chem. Soc.*, 76 (1954) 2387–2390.

[26] J.F. Robyt and D. French, *J. Biol. Chem.*, 245 (1970) 3917–3927.