Journal of Industrial Microbiology, 11 (1993) 199–204

Published by Elsevier

SIM 00469

The high maltose-forming α-amylase of *Saccharomonospora viridis*: mechanisms of action

William M. Fogarty, Bernadette S. Collins, Evelyn M. Doyle and Catherine T. Kelly

Department of Industrial Microbiology, University College Dublin, Dublin, Ireland

(Received 5 March 1992; revision received 30 October 1992; accepted 22 December 1992)

Key words: α-Amylase; Thermophilic; Mechanisms of action

SUMMARY

The thermophilic actinomycete, *Saccharomonospora viridis* produces a thermostable α-amylase which forms 63% (w/w) maltose on hydrolysis of starch. Maltotriose and maltotetraose are the only intermediate products observed during this reaction, with maltotriose accumulating to 40% (w/w). Both unimolecular and multimolecular mechanisms (transfers and condensation) have been shown to occur during the concentration-dependent degradation of maltotriose and maltotetraose. Such reactions result in the almost exclusive formation of maltose from maltotriose at high initial concentration. These mechanisms of action result in the production of the high levels of maltose obtained upon hydrolysis of starch and related substrates.

INTRODUCTION

Maltose-containing syrups have varied commercial and industrial applications, being used as bulking agents, carriers, crystallisation inhibitors, dietary D-glucose substitutes, moisture conditioners and stabilisers. There is an increasing demand for these maltose syrups worldwide. The enzymes currently used for the commercial production of high maltose syrups are the mould saccharifying α-amylases, mainly that of *Aspergillus oryzae*, resulting in syrups of 50–60% (w/w) maltose content [10]. Over half of the highly maltogenic α-amylases recorded to date, however, have been isolated from the actinomycetes, e.g., the thermophilic high maltose-forming α-amylase of *Thermoactinomyces* sp. No. 15 [11]. *Streptomyces praecox* NA273 produces over 80% (w/w) maltose from starch [15], this is the highest level of maltose attributed to an endo-acting amylolytic system.

The participation of nonhydrolytic transfer reactions has been demonstrated during maltooligosaccharide degradation by several α-amylases [5,12,15]. Condensation reactions have, however, been conclusively exhibited in only two amylolytic systems, those of Taka Amylase A [14] and porcine pancreatic amylase [13]. Evidence for both condensation and transfer events was observed in the case of the *S. viridis* α-amylase, maltose levels accumulating to 63% (w/w) as a result of these mechanisms of action.

Correspondence to: Dr. C.T. Kelly, Department of Industrial Microbiology, University College Dublin, Belfield, Dublin 4, Ireland.

MATERIALS AND METHODS

Materials

Bacillus macerans cyclodextrin glucanotransferase was purchased from Amano Biochemicals (Frankfurt, Germany), and maltotriose and maltotetraose from Boehringer (Mannheim, Germany). Aniline phthalate spray reagent was from Merck (Darmstadt, Germany); D-[U-^{14}C]glucose and barley β-amylase were purchased from Sigma (Dorset, UK).

Microorganism, enzyme production and purification

S. viridis was grown and its α-amylase produced and purified as previously described [3].

Enzyme assay

α-Amylase was assayed by the addition of 0.5 ml enzyme to 1% (w/v) soluble starch in 0.1 M Universal buffer (pH 6.5) and incubation of the reaction mixture at 40 °C for 30 min. The composition of Universal buffer is 0.2 M each of Na_2HPO_4, boric acid and glacial acetic acid. The reaction was stopped and reducing sugars determined using dinitrosalicylic acid (DNS) [2]. An enzyme unit is

defined as the amount of enzyme releasing 1 mg of glucose equivalents from the substrate, per 30 min at 40 °C.

Enzyme digests

All digests were prepared at 40 °C and pH 6.5 (Universal buffer, 0.1 M). The substrate concentrations used are outlined in the text. A uniform enzyme/substrate concentration of 1000 units/g of substrate was utilised for digests on starch (2%, w/v). At suitable time intervals 1 ml of the reaction mixture was removed and boiled for 10 min to inactivate the enzyme, prior to quantifying end-product formation.

HPLC analysis of hydrolysates

Identification and quantification of maltooligosaccharides was achieved by high-performance liquid chromatography (HPLC, Waters, Milford, MA, USA). A Waters 'Dextropak' and a BioRad (Richmond, VA, USA) oligosaccharide column were used for the detection of sugars in the maltotriose to maltooctaose range. Saccharides were detected by a differential refractometer and analysed by a Delsi Enica 10 (Argenteuil, France) integrator. Digests were filtered through 0.22 μm filters (Gelman, MI, USA) and 20 μl sample aliquots applied to the column using a Waters Marathon autosampler.

Preparation of reducing-end radiolabelled maltooligosaccharides

A modification of the *B. macerans* cyclodextrin glucanotransferase coupling procedure [6] was employed for the preparation of ^{14}C-reducing-end labelled α-1,4-linked maltotriose, maltotetraose and maltooctaose. 100 μl of *B. macerans* cyclodextrin glucanotransferase was added to 100 μl of nonlabelled glucose (0.1 M), 100 μl of α-cyclodextrin (0.1 M) and 100 μCi D-[U-^{14}C]glucose at pH 6.0 (phosphate buffer, 0.1 M) and allowed to react for 1 h at 40 °C.

Purification of radiolabelled sugars

The reducing-end radiolabelled maltooligosaccharides were separated by descending paper chromatography on Whatman No. 3 paper eluted with propan-1-ol (75%, v/v) at ambient temperature. Three consecutive descents were required.

Radiolabelled digest analysis

Enzyme was incubated with reducing-end radiolabelled substrate at 40 °C and pH 6.5 (Universal buffer, 0.1 M). The saccharide components of these digests were separated and purified by ascending paper chromatography at 85 °C [7]. Maltooligosaccharides were visualised by aniline phthalate spray reagent and excised from the paper. The radioactivity (counts per minute) of the sugars was analysed by an LKB 1211 Rackbeta liquid scintillation counter.

Frequency distribution of bond cleavage

The frequency with which specific bonds within a given substrate are cleaved was determined according to the method of Robyt and French [13].

Detection of maltotriose labelled at both ends

A 12-h digest on 100 mM reducing-end radiolabelled maltotetraose was prepared and the maltotriose formed was separated, purified and excised from the paper chromatogram as described earlier. The trisaccharide was subjected to limited hydrolysis by barley β-amylase. The detection of radiolabel in both products indicates the presence of radiolabel at both ends of the original maltotriose.

RESULTS AND DISCUSSION

The action pattern of the α-amylase of S. viridis

Degradation of 2% (w/v) starch by the α-amylase of *S. viridis* was monitored over a period of 32 h (Fig. 1). The initial, and only, intermediate hydrolytic products observed were maltotriose and maltotetraose. Maltotriose, the major intermediate, accumulated to 40% (w/w), a level comparable to that achieved by the maltotriose-forming α-amylase of *Bacillus* sp. 11-1S, 39% (w/w) [16]. As the reaction proceeded maltotetraose was completely degraded, maltotriose partially hydrolysed and levels of maltose and glucose accumulated. On completion of hydrolysis, the amylase of *S. viridis* produced higher levels of maltose (63%, w/w) and lower levels of maltotriose (9.4%, w/w) from starch than the *A. oryzae* enzyme [10]. This end-product profile of the *S. viridis* α-amylase resembles

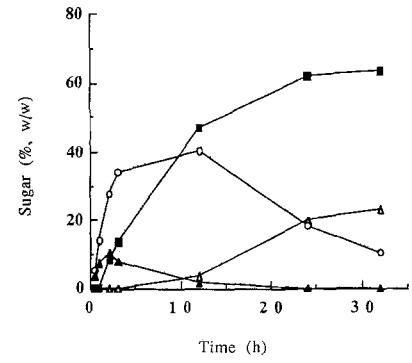

Fig. 1. The time-course of the action of the *S. viridis* α-amylase on 2% (w/v) starch: △, glucose; ■, maltose; ○, maltotriose; ▲, maltotetraose.

that exhibited by the α-amylases of the mesophilic actinomycetes, *Streptomyces limosus* [4] and *Streptomyces hygroscopicus* [8]. It is, however, a pattern which until now has not been observed amongst the thermophilic actinomycete α-amylases.

S. viridis α-amylase action on maltotriose

One of the most important aspects of maltose production by the amylolytic system of *S. viridis* is the formation of high levels of maltotriose and the subsequent degradation of the trisaccharide to yield maltose as the predominant end-product.

The products of maltotriose degradation (Table 1) depend upon substrate concentration. At the lower initial concentration examined (2 mM), the levels of maltose and glucose were approximately equimolar, indicating that unimolecular hydrolytic events are the most significant reactions occurring. As the starting concentration of maltotriose is raised, however, the G1/G2 value decreases and maltose predominates. At the higher concentration examined (200 mM), the very low G1/G2 values (0.13–0.47) and the appearance of maltotetraose provides evidence for the participation of multimolecular events.

These data could indicate that a straightforward transglucosylation reaction accounts for the observed deviation from unity in G1/G2 value. An investigation of the degradation pattern of reducing-end radiolabelled (^{14}C) maltotriose proved this to be an over-simplification of the facts (Table 2). Radiolabel was detected not only in maltotetraose but in maltopentaose and, to a minor extent, maltohexaose. The nondetection of the penta- and hexasaccharide during breakdown of nonlabelled maltotriose is postulated to arise as a result of the superior sensitivity of radioactivity scintillation counting over HPLC analysis. With increasing substrate concentration, the level of radiolabel present in maltose decreased and that found in glucose increased. Transglucosylation is thus, obviously, not the only multimolecular mechanism catalysed. An examination of the calculated frequency distribution of bond cleavage for this α-amylase (Table 3) showed preferential cleavage of reducing-end labelled maltotriose from radiolabelled maltooligosaccharides. By correlation of this data

TABLE 1

Products of *S. viridis* α-amylase action on maltotriose

S_0 (mM)	Time (h)	Product concentration (mM)				
		G1	G2	G3	G4	G1/G2
2	2	0.94	0.82	0.24	0	1.1
	3	0.97	0.93	0.10	0	1.04
200	8	2.6	20.9	63.8	2.4	0.13
	24	8.5	37.6	54.3	2.0	0.23
	48	24.8	52.5	20.8	0.9	0.47

S. viridis α-amylase was incubated with varying inititial concentrations of maltotriose in Universal buffer (0.1 M, pH 6.5) at 40 °C and the end-products analysed by HPLC. G1 to G4 are glucose to maltotetraose, respectively. S_0 is the initial substrate concentration.

TABLE 2

Products of *S. viridis* α-amylase action on radiolabelled maltotriose

S_0 (mM)	Time (h)	Distribution of radioactivity (%)					
		G1	G2	G3	G4	G5	G6
2	3	9.8	78.6	11.4	0	0	0
20	24	40.1	44.7	16.8	0	0	0
200	8	16.3	5.4	66.3	5.4	6.2	0.4
	24	20.5	8.1	53.8	14.1	3.6	0
	48	61.9	18.3	19.6	0	0	0

S. viridis α-amylase was incubated with varying initial concentrations of radiolabelled maltotriose in Universal buffer (0.1 M, pH 6.5) at 40 °C and the radioactivity levels of the end-products counted. G1 to G6 are glucose to maltohexaose, respectively. S_0 is the initial substrate concentration.

TABLE 3

The frequency distribution of bond cleavage of reducing-end labelled maltooligosaccharides by the α-amylase of *S. viridis*

Substrate	Concentration (mM)
○—○—●	
↑	
0.9 0.1	2
0.23 0.77	200
○—○—○—●	
↑	
0.8 0.2 0	2
○—○—○—○—○—○—○—●	
↑	
0 0 0 0 0.85 0.15 0	2

○, glucose residue; ● radiolabelled reducing glucose residue. The numbers indicate the frequency of hydrolysis at these points. A frequency of 1.0 indicates total specificity for that bond.

TABLE 4

S. viridis α-amylase action on maltotetraose

S_0 (mM)	Time (h)	Product concentration (mM)								
		G1	G2	G3	G4	G5	G6	G7	G8	G1/G3
2	0.25	1.1	0.07	0.06	0.6	0	0	0	0	18.3
	1.5	2.7	0.4	0.2	0.2	0	0	0	0	13.5
200	5.0	5.7	3.3	8.3	129	0	0.4	0	0	0.69
	24.0	18.5	17.4	20.1	82.1	1.3	0.3	1.1	0.7	0.92
	48.0	30.1	36.4	18.6	19.7	0	0	0	0	1.6

S. viridis α-amylase was incubated with varying initial concentrations of maltotetraose in Universal buffer (0.1 M, pH 6.5) at 40 °C and the end-products analysed by HPLC. G1 to G8 are glucose to maltooctaose, respectively. S_0 is the initial substrate concentration.

with the observed shift in product ratio seen upon maltotriose hydrolysis, a mixture of transglucosylation, transmaltosylation and condensation events were proposed to be responsible for the results obtained. Transglucosylation would explain the label distribution to maltotetraose, whereas transmaltosylation would give rise to increased label in glucose and account for the label observed in maltopentaose. Both mechanisms may thus be postulated to play a significant contributory role in maltotriose degradation at elevated initial concentration. Condensation would give rise to increased label occurring in maltotriose (no net change) and, to a lesser degree, in maltose and, hence, may be precluded as being of major importance. The detection of labelled maltohexaose, however, indicates that the latter mechanism does occur to some extent.

In conclusion, multimolecular mechanisms are involved during the degradation of maltotriose by the α-amylase of *S. viridis*, the result being a preponderance of maltose in the final saccharide mix. A similar result was observed with the α-amylases of *S. praecox* [15] and *S. hygroscopicus* [8]. Interestingly, the α-amylase of *S. viridis* catalyses the preferential cleavage of radiolabelled maltose from low initial concentrations of labelled maltotriose whereas porcine pancreatic α-amylase [13] and Taka-Amylase A [14] release radiolabelled glucose in excess of radiolabelled maltose from the trisaccharide under similar conditions.

S. viridis α-amylase action on maltotetraose

The second, and minor, intermediate of starch hydrolysis by the α-amylase of *S. viridis* is maltotetraose, with levels reaching 11% (w/w). The most significant change occurring during degradation of higher levels of maltotetraose (Table 4) was the alteration in the glucose to maltotriose value (G1/G3) observed. A G1/G3 value of 13.5–

TABLE 5

Products of *S. viridis* α-amylase action on radiolabelled maltotetraose

S_0 (mM)	Time (h)	Distribution of radioactivity (%)							
		G1	G2	G3	G4	G5	G6	G7	G8
2	1.5	0	28.3	54.1	17.6	0	0	0	0
20	24	7.3	24.9	46.5	21.1	0.1	0.01	0	0
200	5.0	1.6	7.1	10.2	80.4	0.9	0.3	0	0
	24.0	1.8	13.6	22.3	55.6	3.8	2.6	0.95	0.3
	48.0	9.6	20.1	42.0	29.3	1.2	0.3	0.14	0

S. viridis α-amylase was incubated with varying initial concentrations of radiolabelled maltotetraose in Universal buffer (0.1 M, pH 6.5) at 40 °C and the radioactivity levels of the end-products counted. G1 to G8 are glucose to maltooctaose, respectively. S_0 is the initial substrate concentration.

18.3 was recorded at 2 mM concentration. Whereas, much lower values, 0.69–1.6, were detected at 200 mM. A change in the G1/G2 (glucose to maltose) value was also noted. These changes may be explained by the contribution of multimolecular nonhydrolytic reactions. The presence of maltooligosaccharides higher than maltotetraose (maltopentaose to maltooctaose) support this conclusion. An investigation of the end-products obtained upon degradation of radiolabelled (^{14}C) maltotetraose (Table 5) gave 3.8% radiolabel in the maltopentaose moiety and radiolabel was also detected in the sugars maltohexaose to maltooctaose. The amount of radiolabel present in the final end-products, however, remained almost unchanged with increasing substrate concentration – except for the levels of radioactivity being detected in the glucose moiety. An examination of the plausible multimolecular mechanisms involved in maltotetraose degradation by this system (Fig. 2) may help explain this.

The net result of transglucosylation with this enzyme would be an increase in the levels of both maltose and maltotriose achieved (Fig. 2B). Similarly with transmaltosylation (Fig. 2C), the yield of the di- and trisaccharide would be seen to rise, hence this mechanism may be presumed to occur. A maltotriosyl transfer (Fig. 2D) would give rise to increased label in maltotriose. In addition, it is the only nonhydrolytic mechanism which would explain the observed label redistribution to glucose and must therefore be presumed to contribute significantly to the overall reaction. The condensation of two molecules of maltotetraose would result in the formation of one molecule of doubly-labelled maltooctaose (Fig. 2E) which would be subsequently hydrolysed to yield maltotriose and interiorly-labelled maltopentaose (predominantly). 15% of the maltooctaose obtained as a result of maltotetraose condensation would, however, be cleaved at the bond between glucose units 6 and 7 (Table 3) to yield maltohexaose labelled in the interior. Subsequent degradation of this saccharide would give rise to a maltotriose moiety labelled at the nonreducing-end. Degradation of maltotetraose by the *S. viridis* amylase may, thus, be postulated to give rise to the formation of maltotriose moieties labelled at the reducing- and nonreducing-ends. The presence of radiolabel at the reducing-end would occur as a result of both simple hydrolysis (Fig. 2A) and transfer events, whereas label would be distributed to the nonreducing-end due to the participation of condensation. Detection of radiolabel specifically at the nonreducing-end cannot easily be determined. Detection of such trisaccharide moieties with label at either end, however, would provide evidence for the catalysation of condensation by this enzyme. In order to investigate the position of the radiolabel in the maltotriose formed, it was subjected to hydrolysis by barley β-amylase, yielding two products, glucose and maltose.

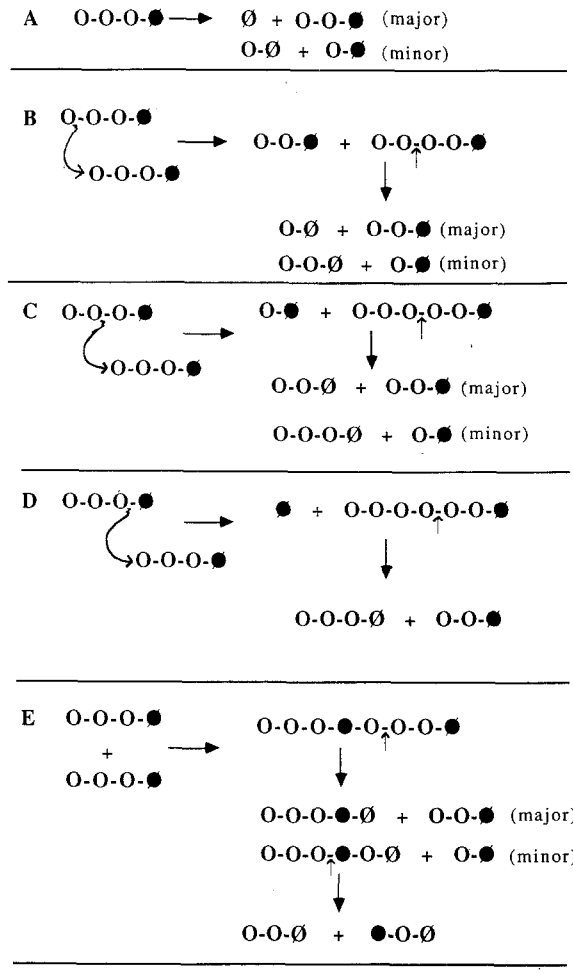

Fig. 2. Possible maltotetraose degradation pathways by the α-amylase of *S. viridis*. A, simple hydrolysis; B, transglucosylation; C, transmaltosylation; D, transmaltotriosylation; E, condensation. ○, glucose residue; ◊ reducing glucose residue; ●, radiolabelled glucose residue; ◆ radiolabelled reducing glucose residue.

The presence of label in both products would indicate the presence of label at both ends of the original trisaccharide molecule. The detection of radioactivity in both the glucose (42 cpm) and maltose (97 cpm) moieties thus indicates the catalysation of condensation by this enzyme.

A mixture of multimolecular events, therefore, including glucosyl, maltosyl and maltotriosyl transfers plus condensation may be postulated to account for the alteration in end-product distribution observed on hydrolysis of increasing concentrations of maltotetraose.

In conclusion, *S. viridis* produces a thermostable α-amylase which forms high levels of maltose as follows:

(i) Hydrolysis of the α-1,4 linkages in starch to yield, eventually, high levels of maltotriose (major) and maltotet-

raose, followed by the conversion of maltotriose to maltose (predominantly) and maltotetraose to glucose, maltose and maltotriose by a combination of multimolecular events, transfers and condensation;

(ii) The overall result of this mechanism of action is the accumulation of maltose to 63% (w/w) in the final saccharide mixture.

REFERENCES

1 Allen, J.D. and J.A. Thoma. 1978. Model for carbohydrase action. *Aspergillus oryzae* α-amylase degradation of maltotriose. Biochem. J. 17: 2345–2350.

2 Bernfeld, P. 1955. Amylases, α- and β-. Methods Enzymol. 1: 149–158.

3 Collins, B.S., C.T. Kelly and W.M. Fogarty. 1992. The maltogenic α-amylase of *Saccharomonospora viridis*. Biochem. Soc. Trans. 20 (1): 818–819.

4 Fairbairn, D.A., F.G. Priest and J.R. Stark. 1986. Extracellular amylase synthesis by *Streptomyces limosus*. Enzyme Microb. Tech. 8 (2): 89–92.

5 French, D. and M. Abdullah. 1966. Transferase activity in malt amylase preparations. Cereal Chem. 43: 555–562.

6 French, D., M.L. Levine, E. Norberg, P. Nordin, J.H. Pazur and G.M. Wild. 1954. Studies on the Schardinger dextrins. VII. Co-substrate specificity in coupling reactions of *Bacillus macerans* amylases. J. Am. Chem. Soc. 76: 2387–2390.

7 French, D., J. Mancusi, M. Abdullah and G. Brammer. 1965. Separation of starch oligosaccharides by high temperature paper chromatography. J. Chromatogr. 19: 445–447.

8 Hidaka, H. and T. Adachi. 1980. Studies on the α-amylase from *Streptomyces hygroscopicus* SF 1084. In: Mechanisms of Saccharide Polymerisation and Depolymerisation (Marshall, J.J., ed.), pp. 101–108, Academic Press, New York.

9 Hyslop, P. and B.P. Sleeper. 1964. α-Amylase of *Streptomyces albus*. Bacteriological Proceedings, Microbial Physiology, P13, p. 89.

10 Norman, B.E. 1979. The application of polymerisation degrading enzymes in the starch industry. In: Microbial Polysaccharides and Polysaccharidases (Berkeley, R.C.W., G.W. Gooday and D.E. Ellwood, eds.), pp. 339–376, Academic Press, London.

11 Obi, S.K.C. and F.J.C. Odibo. 1984. Some properties of a highly thermostable α-amylase from *Thermoactinomyces* sp. Can. J. Microbiol. 30: 780–785.

12 Robyt, J.F. and D. French. 1967. Multiple attack hypothesis of α-amylase action – action of porcine pancreatic, human salivary and *Aspergillus oryzae* α-amylases. Arch. Biochem. Biophys. 122: 8–16.

13 Robyt, J.F. and D. French. 1970. Action pattern of porcine pancreatic α-amylase in relationship to the substrate binding site of the enzyme. J. Biol. Chem. 245 (15): 3917–3927.

14 Suganuma, T., M. Ohnishi, R. Matsuno and K. Hiromi. 1976. Quantitative analysis of the action of Taka-Amylase A on maltotriose. J. Biochem. 80: 645–648.

15 Suganuma, T., T. Mizukami, K. Moori, H. Ohnishi and K. Hiromi. 1980. Studies on the action pattern of an α-amylase from *Streptomyces praecox* NA-273. J. Biochem. 88: 131–138.

16 Uchino, F. 1982. A thermophilic and unusually acidophilic amylase produced by a thermophilic acidophilic *Bacillus* sp. Agric. Biol. Chem. 46: 7–13.