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Enzymatic synthesis of low molecular weight amyloses with modified terminal groups

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ABSTRACT

Low molecular weight amyloses with modified terminal groups were synthesized by cyclomaltohexaose (α-cyclodextrin) transfer using (1→4)-α-D-glucan: 4-α-D-(1→4)-α-D-glucopyranosyltransferase (cyclising) (EC 2.4.1.19) from *Bacillus macerans*. 4-Nitrophenyl α-malto-oligosaccharides d.p. 2–7 served as acceptors, and cyclomaltohexaose served as the donor. The reaction was optimized to obtain a majority of species of definite chain lengths in a range of d.p. 10–20, depending upon the chain length of the acceptor.

The course of the coupling reactions, as well as the action of the enzyme in disproportionation, cyclisation, and hydrolysis of the products, were observed by h.p.l.c. analysis of the oligomer distributions. Using a 15-fold molar excess of cyclomaltohexaose and 0.5 units enzyme per μmol of acceptor at pH 5.2, the chromatograms revealed that the products of the coupling reaction were predominant during the first reaction period. By incubating the acceptors with the enzyme, but without the donor, the mechanism of disproportionation was elucidated as a transfer of malto-oligosaccharyl residues dependent upon the substrate chain length. The minimum chain length required for a direct cyclisation reaction was d.p. 7. The results were confirmed by separation and investigation of the products of hydrolysis and cyclisation, which were nonmodified α-malto-oligosaccharides and cyclomalto-oligosaccharides.

INTRODUCTION

Low molecular weight amyloses (LMWAs) with definite chain lengths in a range of 10–20 glucosyl units per molecule are potentially useful as model substances to answer open questions about the structure and behaviour of amylose and amylopectin. Their degree of polymerisation (d.p.) corresponds to the lengths of the outer chains of amylopectin[1], which are most probably responsible for starch crystallinity[2]. Furthermore, an obvious change in the structure of retrograded microcrystalline amyloses from the A-type to the B-type was observed in X-ray powder diffraction patterns of low molecular weight amyloses of d.p. 12 and 13, respectively[3].

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In order to investigate molecular structures of LMWAs in the solid state by single-crystal X-ray diffraction analysis or in solution by spectroscopic methods, larger amounts of compounds with definite chain lengths in a high degree of purity are necessary. Modification of the reducing end by an α-4-nitrophenyl group seems to facilitate the growth of single crystals[4]. On the other hand, a chromophore facilitates the detection of LMWAs during their chromatographic separation[5] and purification on a larger scale.[6].

To obtain LMWAs with modified terminal groups, enzymatic chain elongation of suitable primers or acceptors is the method of choice because introduction of a chromophore group after an acid hydrolysis of starch[7] or enzymatic degradation of amylose[8], amylopectin[9], or glycogen[10] is difficult and ineffective. The phosphorolytic synthesis of modified LMWAs has been described[11,12] using either potato phosphorylase (EC 2.4.1.1) or muscle phosphorylase b (EC 2.4.1.1) as the enzyme, 4-nitrophenyl α-malto-oligosaccharides of a d.p. of at least 5 as primers and glucose-1-phosphate as the monomer. Although the yields were very good, and the d.p. of the mixtures of LMWAs obtained with modified terminal groups were in the desired d.p. range, the separation of a single species was not effective because the reaction products contained more or less widespread distributions (up to 30 components per product).

An alternative is the chain elongation by a cyclomalto-oligosaccharide transfer reaction (coupling, see Eq. *1*) in reversion of the physiological reaction (cyclisation) of the cyclomalto-oligosaccharide glucosyltransferases employed. A method has been described by Wallenfels *et al.*[13] to prepare 4-nitrophenyl α-malto-oligosaccharides. Using the enzyme (1→4)-α-D-glucan: 4-α-D-(1→4)-α-D-glucopyranosyltransferase (cyclizing) (EC 2.4.1.19) from *Klebsiella pneumoniae* M 5, cyclomaltohexaose as a donor, and 4-nitrophenyl α-D-glucopyranoside as an acceptor, the reaction led to only low yields of oligomers in the range beyond d.p. 7 and remarkable amounts of nonsubstituted malto- and cyclomalto-oligosaccharides. These products result from the ability of the enzyme to cyclize, disproportionate, and hydrolyze the products of the coupling reaction[14,15] (Eqs. *1–3*). However, these 4-nitrophenyl α-malto-oligosaccharides are now commercially available and can serve as acceptors for further chain elongations and as substrates to reinvestigate the action mechanisms of cyclodextrin glucosyltransferases.

Continuing the work of French[16] and Pazur and Marsh[17], the present study deals with the utilisation of (1→4)-α-D-glucan: 4-α-D-(1→4)-α-D-glucopyranosyltransferase (cyclizing) (EC 2.4.1.19) (CGTase) from *Bacillus macerans*, cyclomaltohexaose, and 4-nitrophenyl α-malto-oligosaccharides d.p. 2–7 for an enzymatic synthesis of LMWAs with modified terminal groups. The aim was to influence the course of reaction (coupling, Eq. *1*) to obtain mainly single species of LMWAs in a range of d.p. 10–20. At the same time the disproportionation, hydrolysis, and cyclisation should be limited to decrease the amount of undesired byproducts, nonsubstituted malto-oligosaccharides, and cyclomalto-oligosaccharides. These reactions were investigated by incubation of the various acceptors with the enzyme under synthesis conditions but without cyclomaltohexaose. A qualitative determination of unmodified byproducts of the coupling reaction was carried out.

$$p\text{NP-G}_m + \text{cG}_x \xrightleftharpoons{\text{coupling}} p\text{NP-G}_{m+x} \quad (1)$$

$$p\text{NP-G}_m + p\text{NP-G}_n \xrightleftharpoons{\text{disproportionation}} p\text{NP-G}_{m+y} + p\text{NP-G}_{n-y} \quad (2)$$

$$p\text{NP-G}_{m+x} \xrightarrow{\text{hydrolysis}} p\text{NP-G}_{m+x-z} + \text{G}_z \quad (3)$$

*p*NP = 4-nitrophenyl-
G = (1→4)-α-D-glucopyranosyl-, $m \geq 1, n \geq 2$
cG_x = cyclomalto-oligosaccharide, $x = 6\,(7,8); y \geq 1; z \geq 1$

RESULTS AND DISCUSSION

Optimum reaction conditions were determined for molar ratio of donor:acceptor, pH, temperature, and enzyme concentration by observing the course of reactions by h.p.l.c.. The decisive criterion was a maximum yield of products of the coupling reaction and a minimum of products of the disproportionation and cyclisation reactions. 4-Nitrophenyl α-maltopentaoside was used as an acceptor for all test reactions because it represents the first species of the homologous series able to form LMWAs with modified terminal groups in a range d.p. >10 by the coupling reaction.

Using a molar ratio of donor to acceptor of 1:1 at pH 6.4, the reaction yields mainly products of disproportionation and cyclization. The molar ratio of 4-nitrophenyl α-maltopentaoside to the primary product of coupling reaction (4-nitrophenyl α-D-maltoundecaoside) was 7.8:1 after 75 min; however, the conversion rate of the acceptor was already 60%. In order to force the reaction into the desired direction, a higher molar ratio of donor:acceptor was required.

Further experiments revealed that the disproportionation was more inhibited than the coupling and cyclization reactions at lower pH. At pH 4.5 the CGTase is almost inactive. The optimum pH range for the coupling reaction was found to be pH 5.0–5.5. An increase of temperature and enzyme concentration caused only an increase of all reaction velocities.

Figs. 1a and b illustrate the development of the distribution of the modified reaction products (detectable at 300 nm) dependent upon reaction time (50–250 min). The optimum reaction conditions were molar ratio donor:acceptor 15:1, 0.5 Kitahata units[18] of enzyme per μmol acceptor in 0.01M citrate buffer, containing 2mM $CaCl_2$, pH 5.2, and room temperature. At the beginning the coupling reaction is dominant. The preponderant products obtained were d.p. 11, 17, and 23. After 150 min, 72.5% of the acceptor was converted (chromatogram not shown). During the course of reaction the amounts of products of both disproportionation (d.p. 6–10, 12–16, and 19–22, respectively) and cyclization (d.p. 1) increased. Beyond 150 min the distribution became more

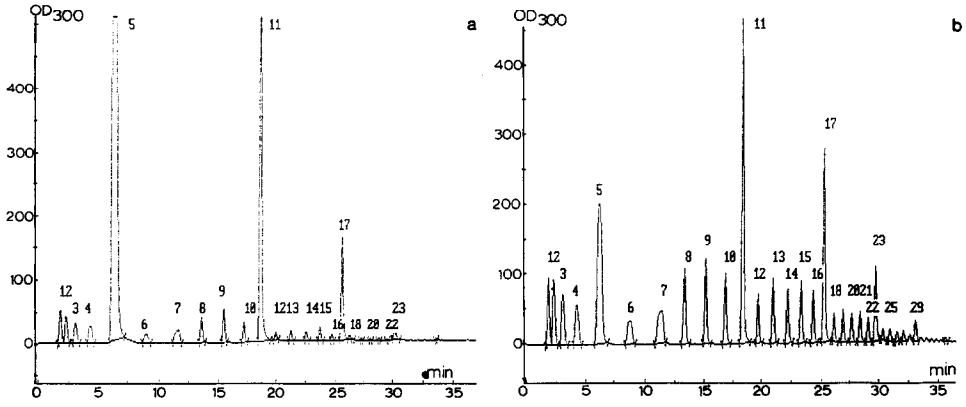


Fig. 1. Development of the chain-length distribution of 4-nitrophenyl α-malto-oligomers during the cyclomaltohexaose transfer reaction as a function of reaction time, after (a) 50 and (b) 250 min (h.p.l.c.[5], detection at 300 nm). Acceptor: 4-nitrophenyl α-maltopentaoside. The numbers indicate the d.p. of the species.

and more widespread, and the maxima of the desired compounds decreased. After 300 min (not shown) a white precipitate was observed that was shown to contain cyclomaltooligosaccharides and products of higher molecular weight. At the same time, peaks of all 4-nitrophenyl-modified species were reduced in size. Obviously, the side reactions, particularly hydrolysis and cyclisation, became more predominant producing unsubstituted byproducts after prolonged incubation times. Therefore, it was necessary to terminate the reaction after an appropriate time (120–150 min) to obtain maximum yields of coupling reaction products.

On preparative scale, LMWAs with modified terminal groups were synthesized using 4-nitrophenyl malto-oligosaccharides of d.p. 2–7 as acceptors under the condi-

TABLE I

Major products obtained by cyclomaltohexaose transfer reactions using 4-nitrophenyl α-malto-oligosaccharides d.p. 2–7 as acceptors after 100-min reaction time

Acceptor	*Products*									
d.p. c(%)[a]	*d.p.*	*c(%)*[a]	*d.p.*	*c(%)*[a]	*d.p.*	*c(%)*[a]	*d.p.*	*c(%)*[a]	*d.p.*	*c(%)*[a]
2 20.70	8	27.85	14	17.99	20	4.50	1	2.64	2	–
3 16.08	9	21.66	15	13.55	21	4.98	1	2.49	2	4.01
4 24.60	10	22.84	16	10.38	22	2.29	1	2.02	2	2.84
5 38.98	11	22.02	17	9.02	23	2.15	1	2.25	2	2.20
6 32.38	12	8.68	18	1.36	24	0.16	1	7.14	2	10.75
7 17.74	13	9.82	19	1.04	25	–	1	42.84	2	4.29

[a] Concentration (c) in mol.%.

tions determined above which are an optimum of all parameters with respect to reaction velocity, yield of desired products, and influences of undesirable reactions. The main components of the reaction mixtures after 100 min reaction time are given in Table I.

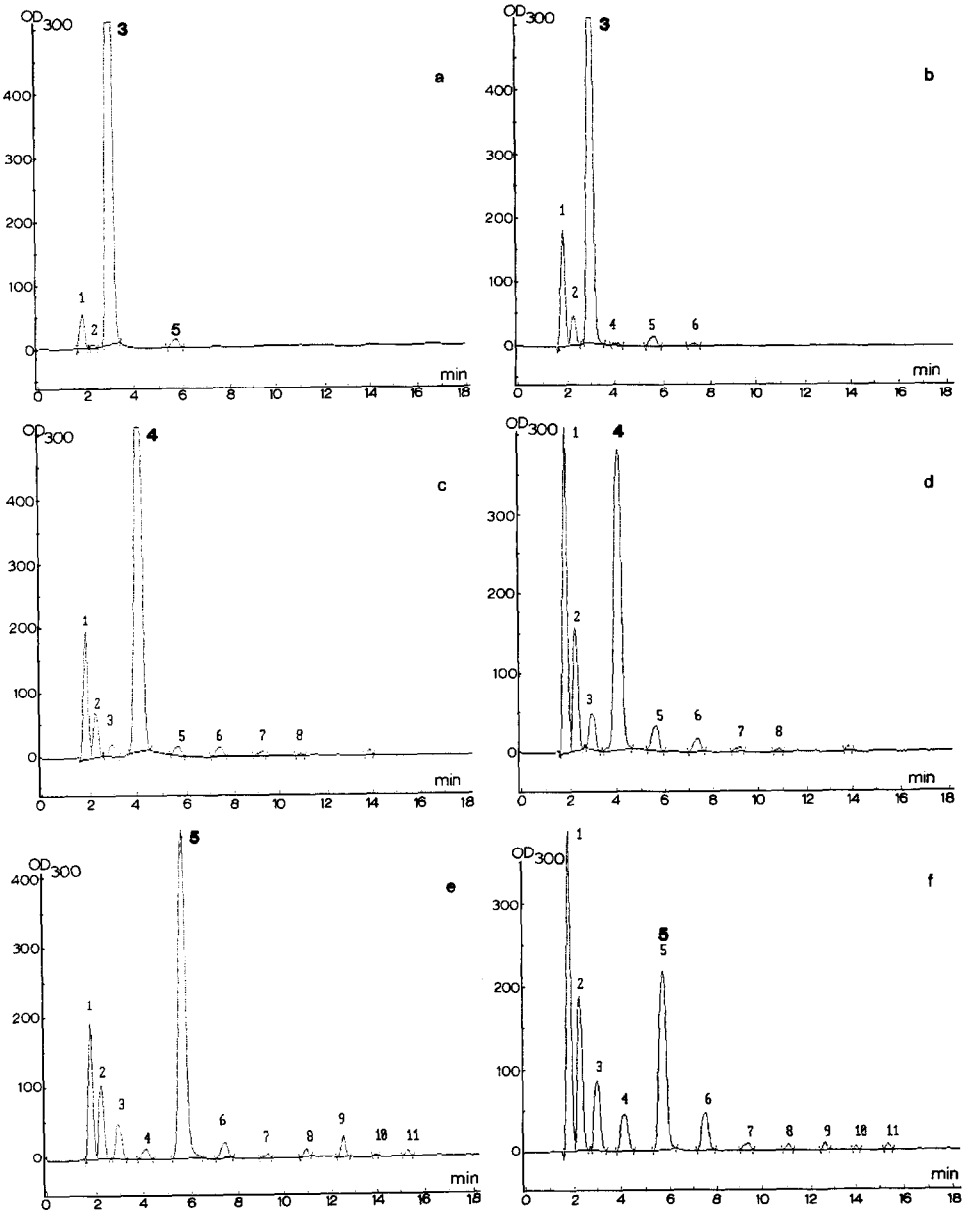
The differences in the action of the enzyme with the different acceptors were unexpected but rather obvious. During the first period of the reaction, the primary products of the coupling reaction of the maltoside, maltotrioside, and maltotetraoside were formed quickly, yielding considerable amounts of the octaoside, nonaoside, and decaoside, respectively. The secondary products were also present in significant amounts. By using the hexaoside and heptaoside as acceptors, yields of the dodecaoside and tridecaoside were essentially lower. On the other hand, the content of 4-nitrophenyl α-D-glucopyranoside and 4-nitrophenyl α-maltoside, respectively, was higher. Despite a 15-fold molar excess of cyclomaltohexaose, side reactions proceed at a higher rate than the coupling reaction with acceptors of d.p. >5.

These results prompted us to investigate the action of the CGTase impairing the yields of LMWAs with modified terminal groups by disproportionation, cyclisation, and hydrolysis of the acceptors.

The influence of disproportionation and cyclisation could be observed by h.p.l.c. analysis of the 4-nitrophenyl-modified products during incubation of the acceptors with CGTase under synthesis conditions but without cyclomaltohexaose. Figs. 2a–j show the distribution patterns of the 4-nitrophenyl α-malto-oligomers obtained after 10 and 30 min incubation time, respectively. Whereas the maltoside is not attacked (see also Fig. 3a), the maltotrioside is attacked rather slowly. The other oligomers are disproportionated in an increasing rate in order of their increasing chain length. It is a matter of fact that the patterns reflect a total of the different actions of the enzyme. Nonetheless, the compositions of the mixtures reveal that glucopyranosyl residues are not transferred in the initial step of the reaction; however, maltosyl, maltotriosyl, and maltotetraosyl residues are separated and transferred, depending upon the chain length of the substrate. A maltosyl unit is transferred preferentially from the maltotrioside, a maltotriosyl residue from the maltotetraoside, and a maltotetraosyl residue from the pentaoside and hexaoside (Figs. 2a, c, e, and g). Besides the preferred transfer units, other oligosaccharide residues are transferred as well during the course of reaction by cleavage of glucosidic bonds different from these preferred to create homologous series of 4-nitrophenyl α-malto-oligosaccharides.

Disproportionation of the maltoside was checked after prolonged incubation time. After 24 h a slight increase of the D-glucopyranoside peak was noted, and after 3 days a remarkable disproportionation was detected (Figs. 3a, and b). Consequently, a single glucopyranosyl residue is transferred with the slowest rate in comparison to longer malto-oligosaccharyl units.

The cyclisation reaction starts as soon as the minimum chain length is available. Because the heptaoside was attacked immediately and a large signal for the 4-nitrophenyl α-D-glucopyranoside was detected (Fig. 2i), the minimum chain length required for the direct cyclisation reaction is d.p. 7. The presence of the tridecaoside suggests even a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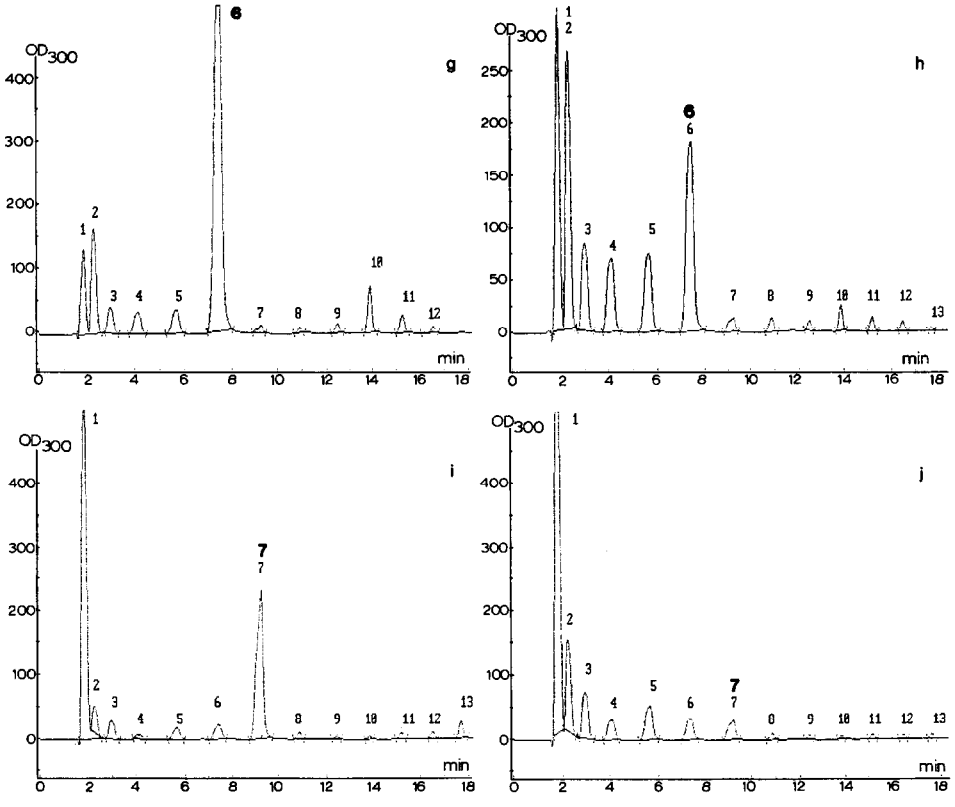

Fig. 2. Products of incubation of 4-nitrophenyl α-malto-oligomers d.p. 2–7 with CGTase under synthesis conditions, but without cyclomaltohexaose, after 10 (a, c, e, g, and i) and 30 min (b, d, f, h, and j), respectively. Bold numbers indicate the d.p. of the oligomers used.

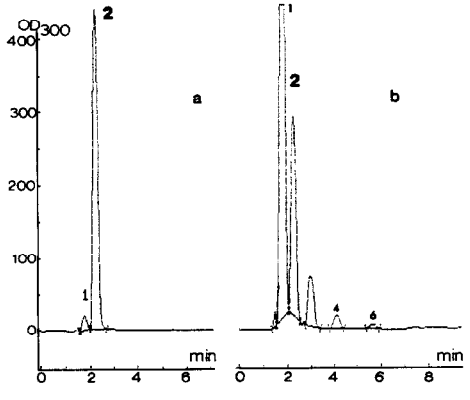

Fig. 3. Disproportionation of 4-nitrophenyl α-maltoside after (a) 24 h and (b) 3 days.

partial coupling reaction of the cyclomaltohexaose with remaining amounts of the acceptor.

Sufficient amounts of oligomers of d.p. 7 or longer have been produced by transfer of all possible malto-oligosaccharide residues (disproportionation) within 30 min to initiate cyclisation in every mixture, except that with the maltoside, as indicated by the high amounts of 4-nitrophenyl α-D-glucopyranoside (Figs. 2b, d, f, and h).

A considerable liberation of 4-nitrophenol could not be detected in any case. However, the disproportionation reaction is superimposed by hydrolysis.

The hydrolysis and cyclisation of the LMWAs with modified terminal groups resulting from the action of the CGTase was investigated by analysis of the unsubstituted byproducts of the synthesis. The malto- and cyclomalto-oligosaccharides without any chromophore that were obtained during the enzymatic synthesis were separated by inclusion chromatography on cyclomaltoheptaose polymer gel[6]. These compounds were not retarded by inclusion as are species that contain a 4-nitrophenyl group[19]. They are eluted first and were well separated into open-chain malto-oligosaccharides and cyclomalto-hexaoses, -heptaoses, and -octaoses. The latter were identified by h.p.t.l.c. according to a method described by Koizumi *et al.*[20]. The formation of cyclomalto-heptaose and -octaose was, as to be expected[21], rather slow, and appreciable amounts were detected only after reaction times $\geqslant 200$ min.

Malto-oligosaccharides were derivatized using 2-aminopyridine according to a method of Her *et al.*[22] in order to introduce a chromophore. These were subsequently analyzed by h.p.l.c., and the chromatograms of the malto-oligosaccharides originating from synthesis byproducts with 4-nitrophenyl α-maltotetraoside as an acceptor are shown in Fig. 4. The first mixture (Fig. 4a) contains predominantly glucose, maltose, and maltotriose (the peak splitting is due to the α and β anomers formed during the reaction with 2-aminopyridine). The distribution pattern confirms the assumption that the CGTase is able to hydrolyze and disproportionate 4-nitrophenyl α-malto-oligomers

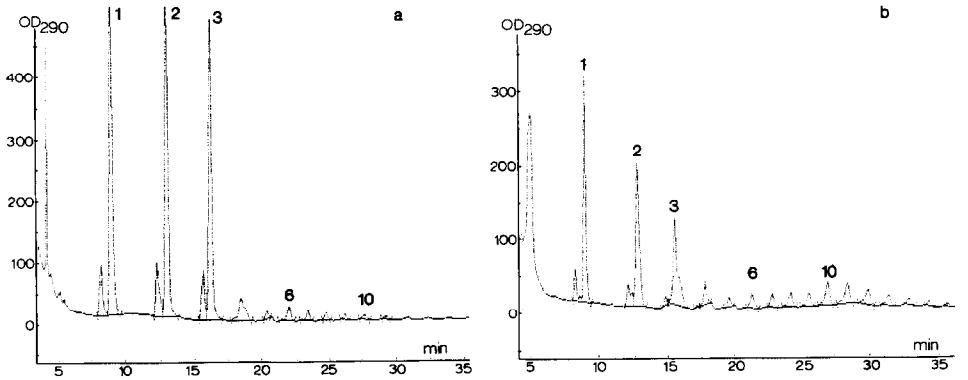

Fig. 4. H.p.l.c. elution profiles of unmodified malto-oligomer byproducts of the synthesis reaction on preparative scale after (a) 20 and (b) 100 min reaction time. Acceptor: 4-nitrophenyl α-maltotetraoside. The products were derivatized by 2-aminopyridine prior to h.p.l.c. analysis and detected at 290 nm.

depending upon the chain length of the substrate present. The malto-oligosaccharides formed are also treated as substrates by the enzyme. In the case of a tetraoside, a maltotriosyl residue is split and can be either transferred (see also Fig. 2c) or disproportionated to glucose, maltose, and small amounts of longer chain species. These are also used as acceptors to produce unsubstituted LMWAs by coupling with cyclomaltohexaose, which also undergo disproportionation and hydrolysis as indicated by Fig. 4b.

The results obtained correspond to the action mechanism of the (1→4)-α-D-glucan: 4-α-D-(1→4)-α-D-glucopyranosyltransferase (cyclizing) (EC 2.4.1.19) from *Klebsiella pneumoniae* described by Bender[23]. The enzyme, a cyclomaltohexaose glucanotransferase, as well as the CGTase from *Bacillus macerans*[15], transfers maltosyl residues from maltotetraose and D-glucopyranosyl residues from maltotriose after prolonged incubation, but maltose is almost unreactive. The lowest membered malto-oligosaccharide suitable for cyclisation is maltooctaose. Bender[24] also observed maltose as a primary product of CGTase actions at unsubstituted malto-oligosaccharides.

According to our investigations, CGTase from *Bacillus macerans* does not liberate 4-nitrophenol from 4-nitrophenyl α-malto-oligosaccharides. The minimum chain length for direct cyclisation of these modified malto-oligosaccharides is already d.p. 7. Thus the enzyme seems to act by cyclizing substrates containing seven or more α-D-glucosidic bonds, regardless of the nature of residue located at the reducing end. Using a modified α-D-maltoheptaoside, the penultimate glucosidic bond to the reducing end of the molecule is split to initiate ring closure, and a product containing the ultimate glucosidic bond, the glucopyranoside, remains.

Disproportionation and hydrolysis require at least two α-D-glucosidic bonds per molecule, and again the penultimate glucosidic bond is split. Evidently a split and transfer of longer malto-oligosaccharyl residues is preferred in comparison to glucosyl residues. The preferred transfer of a maltotetraosyl unit from the 4-nitrophenyl α-maltopentaoside and -hexaoside (see Figs. 2e, and g) suggests a catalytic site of the enzyme consisting of four subsites[25]. A glucopyranosyl residue fits in each subsite. The residues which form the ultimate glucosidic bond at the reducing end of the molecule remain either in regions of the enzyme where a glucosidic bond is not cleaved or on the outside (which is the most probable explanation). The other glucosyl residues of the substrates fill in the subsites dependent upon the chain length until all subsites are occupied; however, a complete fill-up is not necessary. Cleavage of the penultimate or of a foregoing glucosidic bond of the substrate takes place without cyclisation if the chain length of the substrate is not sufficient. It is interesting, however, that a maltotetraosyl residue seems to be the largest unit transferred as an open-chain unit, because it might be expected that a maltopentaosyl residue is transferred from 4-nitrophenyl α-maltohexaoside. The products containing the modified reducing end or at least the ultimate glucosidic bond withdraw. The unmodified malto-oligosaccharide residue formed either remains in the active site and is transferred to another acceptor molecule (disproportionation), or it is removed from the enzyme without further reaction (hydrolysis).

Therefore, in the process of substrate recognition, the substituent at the reducing end is not a decisive element. Even the configuration of the ultimate glucosidic bond has little importance, for it is not involved at all. Only the chain length, and, of course, the configuration of the penultimate and preceding glucosidic bonds, determine whether or not a compound is recognized as a suitable substrate by cyclomaltohexaose glucanotransferases.

The coupling reaction used to obtain the desired 4-nitrophenyl-modified LMWAs is superimposed by disproportionation and cyclisation, as well as by hydrolysis of the modified acceptors and products. The latter reaction initiates disproportionation and chain elongation of unsubstituted malto-oligomers. The yields of LMWAs with modified terminal groups are considerably decreased by these reactions, although yields of three single species up to 20% each, calculated on a basis of the amount of acceptor used, could be isolated as the main products from one batch by preparative chromatography[6]. A quantitative determination of the relative proportions of all products is in progress, and the isolation of LMWAs with modified terminal groups on preparative-scale chromatography will be described elsewhere[26].

EXPERIMENTAL

Enzyme. — (1→4)-α-D-Glucan: 4-α-D-(1→4)-α-D-glucopyranosyltransferase (cyclizing) (EC 2.4.1.19), a lyophilizate from *Bacillus macerans* was a gift from Prof. J. Szejtli, Chinoin Budapest (Hungary) and was used without further purification. For the reactions, 1 mg (2000 Kitahata units[18]) was dissolved in 0.01M citrate buffer pH 6.4–2mM $CaCl_2$ (1 mL).

Substrates. — The acceptors, 4-nitrophenyl α-malto-oligosaccharides d.p. 2–7, (Boehringer Mannheim, F.R.G.) and the donor, cyclomaltohexaose (Toshin, Japan), were checked by h.p.t.l.c.[21] for purity and were also used without purification.

Cyclomalto-oligosaccharide transfer reactions. — (A). Test reactions to determine optimum reaction conditions. 4-Nitrophenyl α-D-maltopentaoside (10 mg, 10 μmol), cyclomaltohexaose (10–150 mg, 10–150 μmol) and enzyme (1.25–6.25 Kitahata units, 5–25 μg) were dissolved in 0.01M citrate buffer–2mM $CaCl_2$, pH 6.4 (1 mL). For temperature and pH optimization, donor and acceptor were mixed in a molar ratio of 15:1 (150:10 mmol) in 0.01M citrate buffer–2mM $CaCl_2$ (1 mL), pH 4.5–6.5 (steps of 0.5 pH units) at 15, 25, and 37°. Enzyme (2 μg, 0.5 units per μmol acceptor) were added to start the reactions. The course of all reactions was observed by h.p.l.c. (see below). For this purpose, aliquots were injected at the start and every 50 min thereafter up to 10 h. *(B). Enzymatic synthesis on preparative scale.* Each 4-nitrophenyl α-malto-oligosaccharide d.p. 2–7 (0.1–0.2 mmol) was dissolved at room temperature in 0.01M citrate buffer–2mM $CaCl_2$, pH 5.2 (10–25 mL). Cyclomaltohexaose (1.5–3 mmol) and enzyme (200–400 μg, 50–100 units) were added. After 2–2.5 h, the reaction was terminated by addition of BioRad AG50W-X12 [H^+] cation exchanger (2 mL, wet vol). The cyclomaltohexaose was precipitated with tetrachloroethane (0.5 μL/mg of cyclomaltohexaose), and filtered off, and the filtrate was lyophilized.

Disproportionation reactions. — Each 4-nitrophenyl α-malto-oligosaccharide d.p. 2–7 (10 μmol) was dissolved in 0.01M citrate buffer–2mM $CaCl_2$, pH 5.2 (1 mL) and incubated with 20 μg (5 units) enzyme at room temperature. Aliquots were removed after 10 and 30 min, respectively, and analyzed by h.p.l.c..

Chromatographic methods. — All reactions and their products were investigated by normal-phase h.p.l.c. as described[5] using an amino-bonded silica gel (Hypersil APS-2, Shandon, UK, column dimension 4 × 250 mm) as a stationary phase and acetonitrile–water (linear gradient 75:25, developed over a period of 60 min, flow rate 1.5 mL/min) as a mobile phase. Injection volume was 20 μL. 4-Nitrophenyl-modified malto-oligosaccharides and LMWAs were detected at 300 nm (h.p.l.c. equipment: Knauer, F.R.G.). Unsubstituted byproducts were seperated by inclusion chromatography[6] using a cyclomaltoheptaose polymer gel (Wacker, F.R.G.) as a column packing material (bed size 25 × 1500 mm) and degassed and deionized water as an eluent. Optical dispersion at 280 nm (Dual-path monitor UV-2, Pharmacia, Sweden) and refractive index (Differential refractometer, Knauer, F.R.G.) were detected in a series configuration. Cyclomalto-oligosaccharides were identified by h.p.t.l.c.[20] (h.p.t.l.c. plates Si 50000, Merck, F.R.G.) using 30:25:20 1-butanol–pyridine–water as a mobile phase. Malto-oligosaccharides were derivatized with 2-aminopyridine[22] and analyzed by h.p.l.c. (90:10 acetonitrile–water, linear gradient, developed over a period of 120 min, and detection at 290 nm; for column, see above).

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