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PHOSPHOROLYTIC SYNTHESIS OF LOW MOLECULAR WEIGHT AMYLOSES. A COMPARISON OF POTATO AND MUSCLE PHOSPHORYLASE

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In extension of previous studies the preparation of low molecular weight amyloses (LMWA) in the range of DP 5-40 was carried out by enzymatic synthesis with either potato phosphorylase or phosphorylase b from rabbit muscle. Interest was focused on the intermediate range of DP 10-20 and on compounds containing a p-nitrophenyl group at the reducing end. Therefore, p-nitrophenyl-α-D-maltopentaoside served as a primer. Investigation of the products by SEC/LALLS and HPLC showed characteristic differences between both phophorylases in the chain length distribution obtained. Whereas the synthesis by muscle phosphorylase leads to an expected Poisson distribution, the synthesis by potato phosphorylase is apparently superposed by a disproportionation reaction. Disproportionation is even stronger with a highly purified enzyme preparation. With both enzymes significant amounts of the desired p-nitrophenyl-α-D-malto-oligosaccharides DP 10-20 can be produced. These oligomers are of interest for X-ray single crystal diffraction analyses.

INTRODUCTION

As a step further in our work dealing with regularly shaped linear and branched model substances for starch we recently included low molecular weight amyloses (LMWA) of definite chain lenghts [1]. Compounds with chain length shorter than DP 25, particularly in the range of DP 10-20, appear to be most interesting because they represent an intermediate stage between the "low molecular" maltooligosaccharides and the "high molecular" amylose and amylopectin. More information about their properties in solution and in the solid state is strongly needed. In first studies with a series of pure maltooligomers DP 3-18, prepared from an α-amylolytic diegest of synthetic amylose by large-scale Bio-Gel P4 fractionation, a change of the X-ray powder diffraction pattern of

retrograded microcrystalline LMWA from the B-type to the A-type was observed with decreasing length, sharply between DP 13 and 12 [2]. The findings support observations by Hizukuri et al. [3], indicating that the average chain lenght of amylopectin and the X-ray type of starches are closely related.

The best method to elucidate details of molecular structure in the solid state is the single crystal X-ray diffraction analysis. However, the production of larger amounts of LMWA in a purity and quantity required for crystallization is still a problem. Recently we succeeded in the crystallization of a maltohexaose [4] and a maltooctaose with a p-nitrophenyl group in α-position at the reducing end as their polyiodide complexes. We assume that such a derivatization completed with complex formation facilitates the growth of single crystals, at least in the lower DP range. Therefore we wished to extend these studies to modified LMWA in the range of DP 10-20.

The usual methods to obtain LMWA, e.g. acid hydrolysis and amylolytic degradation of amylose or debranching of amylopectin and glycogen, cannot be applied, because a subsequent blocking of the reducing end in one configuration would be complicated. A much better way is the enzymatic chain elongation of a suitably modified acceptor by cyclodextrin glucosyltransferase and α-cyclodextrin or by phosphorylase and glucose-1-phosphate as the monomer donor (Equation 1). Both procedures have been investigated.

In this paper we will report on phosphorolytic syntheses and typical defferences observed between potato and muscle phosphorylase in the chain length distribution of the products and in the mechanism of growth.

MATERIALS AND METHODS

Potato phosphorylase [1] was isolated from preheated potato juice by fractionation with ammonium sulphate [5]. Further purification was carried out by hydrophobic interaction chromatography and subsequent gel filtration (II) [6]. For comparison a highly purified, almost crystalline potato phosphorylase (III) was used [7]. (II) and (III) appeared as a single band in SDS gel electrophoresis. An improved stability observed for (III) may result from its preparation in 0.1 M phosphate buffer pH 7.5, whereas (I) and (II) were prepared without addition of P_i. Rabbit muscle phosphorylase b (Sigma), containing small amount of P_i and 5'-AMP was used without further purification.

$$pNP\,G_n + G\text{-}1\text{-}p \rightleftharpoons pNP\,G_{n+m} + mP_i \tag{1}$$
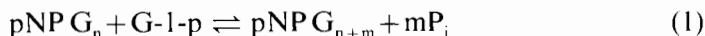
pNP = p-nitrophenyl, G-1-P = glucose-1-phosphate, P_i = inorganic phosphate, $n \geq 5$, $m = 11$

As known before, an effective primer in synthesis with potato phosphorylase must have a minimum lenght of four unsubstituted α-1,4-linked glucose residues [8]. Thus p-nitrophenyl-α-D-maltopentaoside (Boehringer) and maltotetraose served as primers in syntheses of modified and unmodified LMWA, respectively. As elaborated in previous studies [9] synthesis was carried out at pH 6.0 and 40°C

using a molar ratio of primer to monomer of 1:11. Digests contained: 2.68 g (6.3 mmol) G-1-P, Na$_2$ salt (Boehringer) and 500 mg (0.53 mmol) p-nitro-phenyl-α-D-maltopentaoside (353 mg maltotetraose) in a total volume of 35 ml. All phosphorylases were used in a ratio of 0.1 U per μmol of primer. The conversion of G-1-P was followed by colorimetric determination of the liberated P$_i$.

RESULTS AND DISCUSSION

PHOSPHOROLYTIC SYNTHESES

As seen from Table 1 equilibrium is reached after 3-4 hours. Whereas potato phosphorylase shows a more rapid initial rate of reaction, the muscle phosphorylase leads to a somewhat higher conversion at the end. The difference in the initial rate may be ascribed to both enzymes differing in the topography of their saccharide binding sites and affinities [10]. The effect obviously disappears with further progress of the reaction when oligomers grow longer.

Characterization of the synthetic products was carried out by HPLC and by size exclusion chromatography combined with low angle laser light scattering (SEC/LALLS), a technique usually applied to higher molecular weight compounds. The simultaneous detection of laser scattering intensity (LALLS-detector) and concentration (DRI-detector) provides information about molecular weight averages M$_w$, M$_n$, M$_z$, polydispersities M$_w$/M$_n$, M$_z$/M$_n$ and molecular weight distribution (MWD) without any external calibration required.

Table 1. Conversion of glucose-1-phosphate in phosphorolytic synthesis

Reaction time (min)	% conversion of total G-1-P by	
	potato phosphorylase	muscle phosphorylase
5	14.1	7.5
10	24.4	15.0
20	38.6	26.4
40	58.0	43.8
60	67.2	57.5
120	77.7	78.8
180	83.3	88.8
240	85.3	91.3

Data are summarized in Table 2 and typical MWD curves shown in Fig. 1. Molecular weight averages calculated from the maximum DP of the HPLC chromatograms are generally somewhat lower than those determined by LALLS, but they follow the same trend. Polydispersity data already indicate that the products, modified and unmodified LMWA, obtained with the muscle

Table 2. Molecular weight averages and polydispersities from SEC/LALLS of products obtained with potato and muscle phosphorylase from p-nitrophenyl-α-D-maltopentaoside (pNP-G5) and maltotetraose (G4) primer

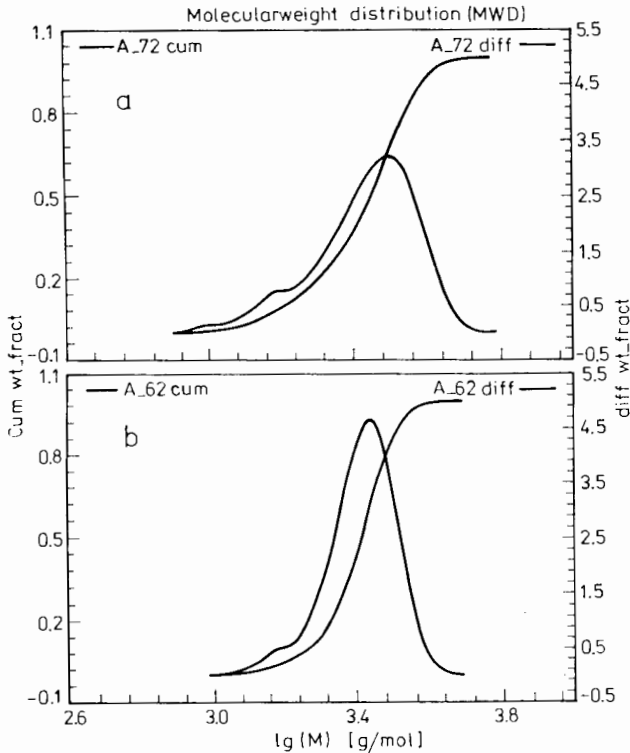| Enzyme	Primer	DP max. (HPLC)	M_{av} exp. g/mol	SEC/LALLS				
				M_w g/mol	M_n g/mol	M_z g/mol	M_w/M_n	M_z/M_n
potato (II)	pNP-G5	10	1800	2400	2100	2700	1.14	1.13
potato (III)	pNP-G5	11	1950	2800	2500	3000	1.11	1.07
potato (III)	G4	14	2250	3700	3300	4000	1.12	1.08
muscle	pNP-G5	14	2400	2600	2400	2700	1.08	1.04
muscle	G4	15/16	2500	3400	3250	3600	1.06	1.06

Fig. 1. Molecular weight distribution (SEC/LALLS) of p-nitrophenylated products obtained by synthesis with (a) potato phosphorylase (III) and (b) muscle phosphorylase

enzyme exhibit a more narrow distribution than those obtained with the potato enzyme. This is clearly seen from Fig. 1. Independent of the purity of the potato phosphorylase used a relatively broad distribution is found.

Fig. 2 shows the chain length distribution derived from the HPLC chromatograms (Table 3 and 4, 180 min) in comparison to a corresponding theoretical Poisson distribution. For muscle phosphorylase there is an excellent agreement with a Poisson distribution. The serious deviation shown by potato phosphorylase, yet unknown from previous syntheses of high molecular weight amyloses, prompted us to investigate the development of chain length distribution with both enzymes as a function of reaction time.

Aliquots were removed at time intervals and the reaction stopped by treatment with a mixed bed ion exchanger to remove salts and enzymes. The products were analyzed by HPLC.

The time dependent changes in the molar composition of the oligomer mixtures calculated by integration of the HPLC chromatograms (Fig. 3) are listed in Table 3 and 4. Results give clear evidence that synthesis with both enzymes proceeds in a different way. In the case of the muscle phosphorylase
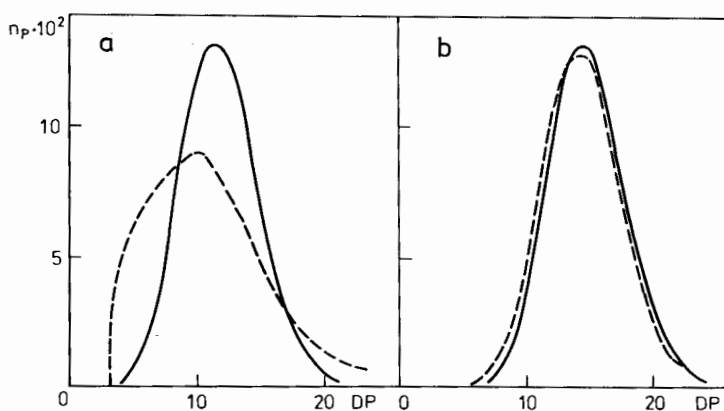
Fig. 2. Chain length distribution from HPLC (% molar ratio, see Table 3 and 4) (- - -) and Poisson distribution (—) in synthesis with (a) potato and (b) muscle phosphorylase

chain growth takes place normally with a continuous shift of the maximum from the pentaoside primer up to DP 14 in the equilibrium state (Fig. 3 e-h). In the case of the potato phosphorylase the reaction is more complicated (Fig. 3 e-d). Already after five minutes the formation of p-nitrophenyl-α-D-maltotetraoside can be detected. Two distinct maxima develop within about forty minutes. This can be correlated with two simultaneous independent enzymic activities. After two hours the distribution shows a single maximum in the region of DP 12. Even later, the maximum is shifted to DP 10 and traces of the maltotrioside are detected. The flattening of the distribution continues, indicating that a disproportionation reaction influences the chain length distribution while synthesis is reduced or even stopped under equilibrium conditions.

DISPROPORTIONATION REACTION

These phenomena, when first observed in preliminary studies [9], seemed to indicate that the less purified potato phosphorylase (I) is contaminated by a disproportionating enzyme, most probably D-enzyme or a special kind of α-amylase [11, 12]. The use of carefully purified potato enzyme in the present studies did not confirm this assumption. On the contrary, the effect was even more pronounced. In order to examine the disproportionation separately, p-nitrophenyl-α-D-maltopentaoside was incubated with the enzymes under the same conditions as in synthesis but in the absence of G-1-P. The results from HPLC analysis are shown in Fig. 4.

After four hours of incubation with the phosphate-free prepared potato phosphorylase (II) at pH 6.0 (0.1 M citrate) a slow disproportionation is observed. After 24 hours the amounts of p-nitrophenyl-α-D-maltotetraoside

Table 3.Changes of chain length distribution in synthesis with potato phosphorylase (III)
Concentration in mol % calculated by integration of HPLC chromatograms

DP	0	5	10	20	40	60	120	180	240 min
3								0.04	0.04
4		2.81	1.91	2.18	2.76	2.48	3.70	4.89	4.39
5	100	22.25	10.90	7.65	5.69	5.06	5.47	6.37	6.09
6		40.42	35.11	20.04	11.38	8.58	6.74	7.24	6.73
7		21.45	25.95	19.09	11.70	8.64	6.92	7.87	7.55
8		6.62	9.68	9.19	7.13	6.08	7.06	8.33	8.13
9		2.08	4.16	6.02	6.09	6.08	7.34	8.80	8.21
10		2.72	5.06	8.55	8.54	7.92	8.39	9.05	8.05
11		1.55	3.14	7.36	8.68	8.52	8.60	8.62	7.61
12		0.07	2.46	7.39	9.24	9.11	8.70	8.01	7.30
13			1.55	5.22	7.94	8.40	7.91	7.05	6.97
14			0.05	3.29	6.17	7.20	6.70	6.17	6.02
15				2.24	4.55	5.86	5.50	4.73	5.31
16				1.65	3.75	5.05	4.41	3.77	4.90
17				0.09	2.82	4.08	3.37	2.90	3.77
18					2.12	3.07	2.70	2.30	2.90
19					1.26	2.19	2.38	2.05	2.45
20					0.09	1.41	1.60	1.51	1.97
21					0.05	0.08	1.18	0.09	1.38
22						0.07	1.07	0.08	0.09
23						0.04	0.09	0.07	0.08

Table 4. Changes of chain length distribution in synthesis with muscle phosphorylase

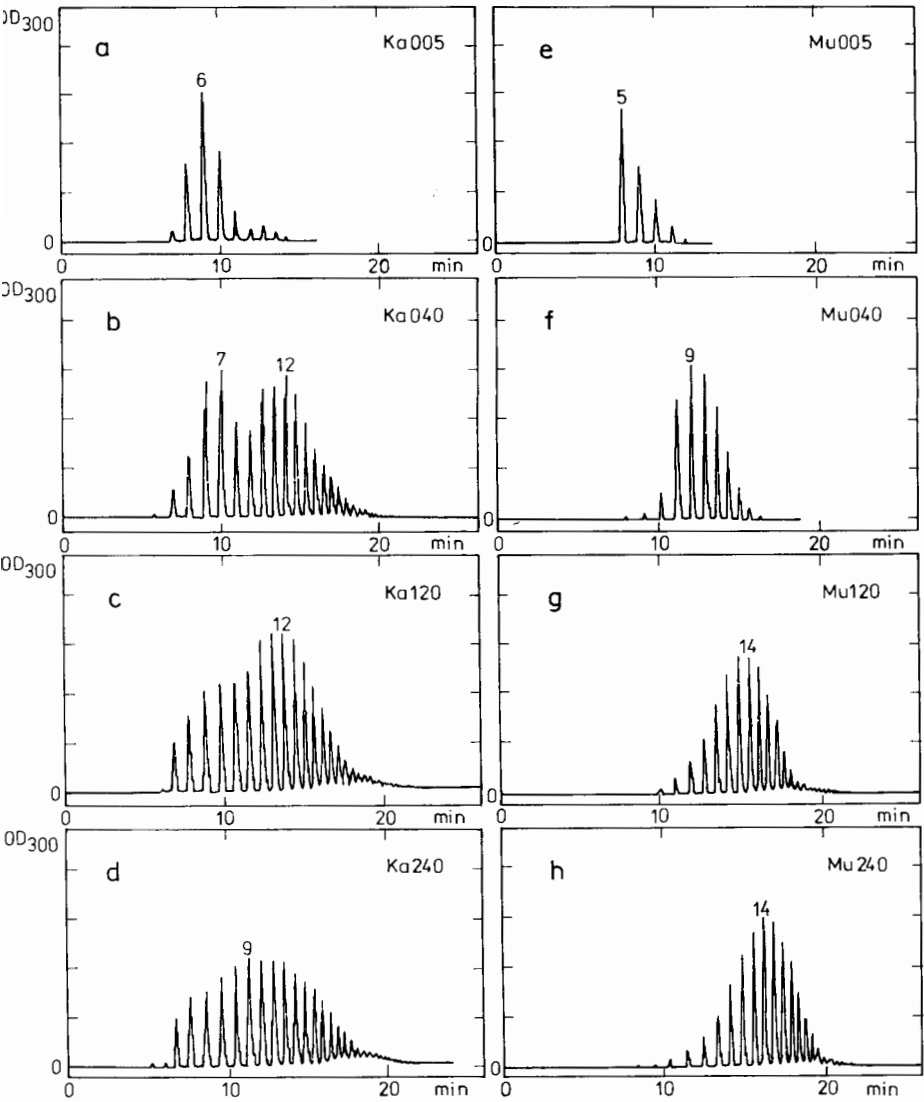| DP	0	5	10	20	40	60	120	180	240 min
5	100	51.55	27.42	5.76	0.18	0.04			
6		28.28	25.21	8.49	0.72	0.08		0.13	0.23
7		14.86	25.84	19.43	3.85	1.09	0.53	0.51	0.64
8		4.47	18.05	37.66	18.55	5.89	1.45	0.98	1.35
9		0.30	3.11	19.21	22.89	11.26	3.11	2.68	2.84
10			0.36	7.32	21.05	15.48	5.36	4.76	4.69
11				2.11	16.35	18.92	8.59	7.37	6.94
12				0.60	9.94	18.25	11.84	10.08	9.22
13					4.39	13.59	13.77	12.21	11.13
14					1.64	8.43	14.23	13.25	12.17
15					0.04	4.57	12.37	12.66	12.08
16						2.05	10.18	11.06	11.08
17						0.89	6.97	8.67	9.13
18						0.23	4.67	6.55	6.88
19							2.75	4.13	4.66
20							1.67	2.68	2.95
21							1.17	1.38	1.63
22							0.87	0.93	1.08
23									0.77
24									0.50

Fig. 3. Chain length distribution of LMWA in syntheses with pNP-G5 after 5, 40, 120 and 240 min by potato (a-d) and muscle phosphorylase (e-h)

and-hexaoside are considerably increased. At pH 7.0 (0.1 M Tris/HCl) the disproportionation is already strong after four hours. In contrast, the muscle phosphorylase does not attack the primer at pH 6.0 even after 24 hours. At pH 7.0, however, disproportionation takes place, but is considerably slower than with the potato enzyme.

Incubation of the pentaoside with highly purified potato phosphorylase (III) containing 0.03 μmol P_i per ml of the digest gave a more rapid reaction than (II)
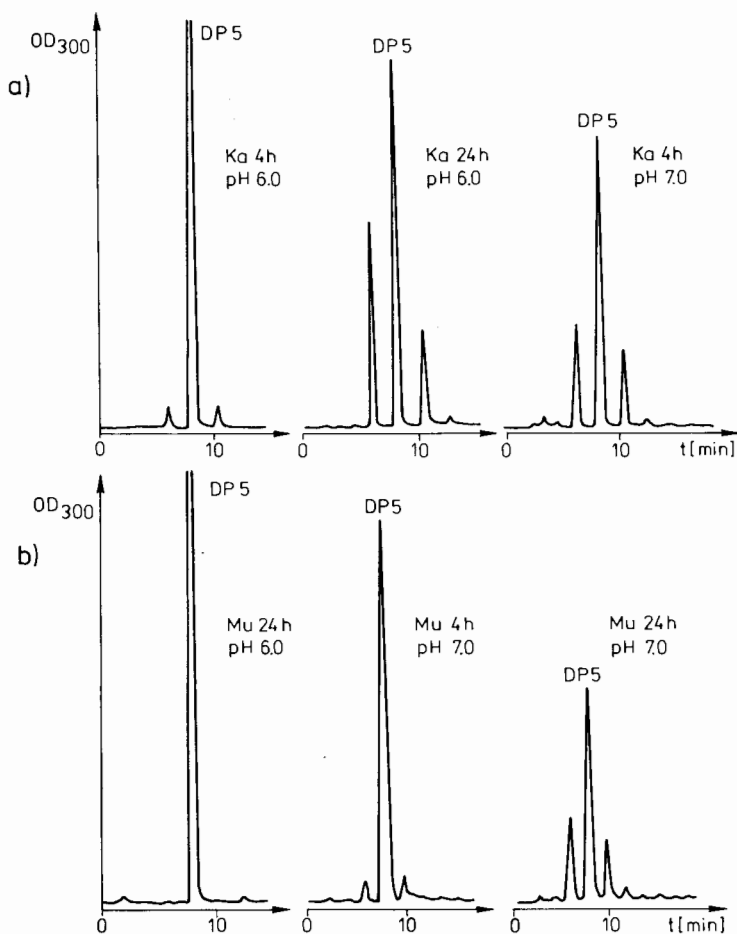
Fig. 4. Disproportionation of p-nitrophenyl-α-D-maltopentaoside (DP 5) on treatment with (a) potato phosphorylase (II) and (b) muscle phosphorylase at pH 6.0 and 7.0 for 4 h and 24 h.

even at pH 5.5 and 6.0. After two hours more than 50% of the primer were disproportionated to the tetraoside and higher oligomers up to DP 10.

Overlooking the present results it appears more likely that the disproportionating activity is intimately associated with the phosphorylase itself rather than due to a contaminating enzyme. On the one hand, with the potato and muscle enzyme the effect is sensitive to the pH. The disproportionation when based on a synthesis/degradation equilibrium would then favorably occur at pH 7.0. On the other hand, it should be discussed whether P_i is actually involved in the glucosyl transfer reaction. Klein et al. (13) postulated a catalytic mechanism of phosphorylase action in which a "mobile" phosphate anion plays a versatile role. It could serve as a proton carrier for substrate activation, stabilizing the intermediate and acting as a nucleophile which can accept a glucosyl residue

reversibly. However, muscle phosphorylase was not able to disproportionate the pentaoside at pH 6.0 even in the presence of 5.5μmol P_i. Furthermore, the high concentration of P_i liberated in synthesis, 90 μmol/ml at equilibrium, did obviously not lead to a broadening of the narrow distribution pattern of the products. The lower rate of disproportionation by the P_i free potato phosphorylase (II) and the considerable increase of rate with phosphorylase (III) containing small amounts of P_i suggest that P_i could have a still unknown function in the case of the non-regulated plant phosphorylase.

CONCLUSIONS

Significant differences were observed between the chain length distribution of LMWA obtained by either potato phosphorylase or muscle phosphorylase b. Under the conditions applied synthesis by muscle phosphorylase leads to a more narrow chain length distribution within the desired range of DP 10-20. The synthesis by potato phosphorylase is superposed by a disproportionation of the primer and maltooligomers formed. Broadening of the distribution pattern decreases the yield of DP 10-20.

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FOSFOROLITYCZNA SYNTEZA AMYLOZY O NISKIEJ MASIE CZĄSTECZKOWEJ.
PORÓWNANIE FOSFORYLAZY ZIEMNIACZANEJ I MIĘŚNIOWEJ

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Streszczenie

W kontynuacji poprzednich badań nad otrzymaniem niskomolekularnych cząsteczek amylozy w zakresie DP 5-40 przeprowadzono enzymatyczne syntezy przy użyciu dwu fosforylaz: ziemniaczanej i fosforylazy ,,b" z mięśnia królika. Zainteresowanie skierowano na przejściowy zakres DP 10-20 i na składniki zawierające grupę p-nitrofenylową przy końcu redukującym. Dlatego jako starter służył p-nitrofenyl-α-D-maltopentaozyd.

Badanie produktów za pomocą chromatografii żelowej (SEC) i metody niskokątowego rozpraszania światła laserowego (LALLS) oraz chromatografii HPLC wykazało charakterystyczne różnice pomiędzy dwoma fosforylazami w podziale długości łańcucha. Zważywszy, że synteza przy użyciu fosforylazy mięśnia prowadzi do spodziewanej dystrybucji Poissona'a, to synteza przy użyciu fosforylazy ziemniaczanej najwidoczniej nałożyła się na reakcję dysproporcjonowania. Znaczące ilości pożądanego p-nitrofenyl-α-D-malto-oligosacharydu o DP 10-20 mogą być produkowane za pomocą obu enzymów. Oligomery te są interesujące z punktu widzenia analizy dyfrakcji promieni X przez monokryształy.