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Action Pattern of Human Pancreatic and Salivary α-Amylase on 1,4-α-*D*-Nitrophenylmaltooligosaccharides

1,4-α-D-Nitrophenylmaltooligosaccharides as Substrates of α-Amylase, I.

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Summary: High performance liquid chromatography (HPLC) was used to monitor the purity of the substrates and to establish the patterns of hydrolysis of *ortho-* and *para*-nitrophenylmaltooligosaccharides (2–7 glucose residues) catalysed by human pancreatic and salivary α-amylase. Separation of the reaction products from the remaining substrate was performed on a TSK-G-2000 PW or a RP18 column. By measuring the quantitative distribution of products, and assuming a 5-subsite model for the active site of α-amylase, differential activities for the hydrolysis of the different glycosidic bonds in the 2 series of substrates were deduced. A highly sensitive coupled continuous assay system is based on the formation of phenyloligosaccharides with 1–4 glucose residues by the action of the amylase unter test, coupled to hydrolysis of these products by yeast α-glucosidase. The most suitable test substrates were shown to be *para*-nitrophenyl-α-*D*-maltotetraoside and -pentaoside. Direct production of nitrophenol from *ortho*-nitrophenyl-α-*D*-maltotrioside is recommended for the measurement of the α-amylase activity of pancreatic and salivary gland secretions and extracts.

Wirkungsmuster von menschlicher Pankreas- und Speichelamylase bei der Hydrolyse von 1,4-α-D-Nitrophenylmaltooligosacchariden

1,4-α-D-Nitrophenylmaltooligosaccharide als Substrate der α-Amylase, I.

Zusammenfassung: Es wurde die Methode der Hochpräzisionschromatographie (HPLC) zur Kontrolle der Reinheit der Substrate sowie zur Ermittlung der Spaltungsmuster menschlicher Pankreas- und Speichelamylase angewendet. Als Substrate wurden 2- und 4-Nitrophenylmaltooligosaccharide mit 2–7 Glucoseresten benutzt. Die Trennung der Reaktionsprodukte von dem restlichen Substrat erfolgte unter Verwendung von TSK-G-2000 PW oder RP 18-Säulen. Aufgrund der quantitativen Produktverteilung einerseits und einem 5-Unterbindungsstellen-Modell des aktiven Zentrums der α-Amylase andererseits, konnten differenzierte Aktivitäten für die Hydrolyse der verschiedenen Glucosidbindungen in den beiden genannten Serien von Substraten bestimmt werden. Auf Basis der katalytischen Aktivität von Hefe-α-Glucosidase für die Hydrolyse von Nitrophenyloligosacchariden mit 1–4 Glucoseresten und der Geschwindigkeit von deren Bildung durch α-Amylase aus den ursprünglichen Substraten werden 4-Nitrophenyl-α-*D*-maltotetraosid und -pentaosid als am besten geeignete Substrate für ein hochempfindliches gekoppeltes kontinuierliches Testsystem empfohlen. Die direkte Bildung von Nitrophenol aus 2-Nitrophenyl-α-*D*-maltotriosid wird für die kontinuierliche Messung der Amylaseaktivität von Pankreas- und Speicheldrüsensekreten und -extrakten empfohlen.

Introduction

Determination of α-amylase (1,4-α-*D*-glucan 4-glucanohydrolase, EC 3.2.1.1) activity in biological fluids was studied, because there is a growing interest in this enzyme, and the measurement of its serum and urinary levels in humans is clinically important. The most widely used methods have hitherto been based on the hydrolysis of soluble or dyed starch, monitoring the formation of reducing groups or the release of coloured components (1, 2, 3). They are all time consuming and have several drawbacks:

(a) Since the substrate is a mixture of molecular species with possibly different degrees and rates of hydrolysis, reactions may be non-linear with time;

[1]) This paper includes results from the thesis of *B. Meltzer*.

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(b) there may be no exact stoichiometric relationship between the reducing groups formed or the dye released and the number of hydrolysed bonds;

(c) the quality and kind of polysaccharide used which may differ from lot to lot greatly influence the observed rates (4).

In the course of our investigations on the development of an enzymatic assay for the determination of α-amylase, two series of 1,4-α-*D*-nitrophenylmaltooligosaccharides (*ortho* and *para*) were used as substrates (5). In order to determine the most suitable substrate to use in a continuous assay system, it was necessary to fractionate these two series of oligosaccharides and to identify the amylase reaction products. A TSK-G-2000 PW column (6) was used in the HPLC system in order to separate free sugars and nitrophenylated products simultaneously, thus making it possible to determine product distribution, and to determine the extent to which liberated oligosaccharides are further split by amylase during the test.

Materials and Methods

Chemicals

The 1,4-α-*D*-nitrophenylmaltooligosaccharides (*ortho* and *para*) were synthesized in our laboratory according to the methods described earlier (5); α-glucosidase, 50 kU/g, 2 g/l (cat. no. 105406), Boehringer Mannheim, Mannheim, G.F.R.; α-amylase was purified from human pancreas and saliva following the procedure of *Sky-Peck & Thuvasethakul* (7). Both enzymes were diluted to an catalytic activity concentration of 2058 nkat/l; according to the Phadebas test (Pharmacia Diagnostics, Sweden)[2]). Other chemicals were reagent grade and purchased from E. Merck, Darmstadt, G.F.R.

Apparatus

A HPLC system from Waters Associates Inc., Milford, Massachusetts was used, consisting of a constant flow pump model 6000A, the UV detection model 440, the differential refractometer R401, and a TSK-G-2000 PW column (7.5 X 600 mm) supplied by Toyo Soda Co., Tokyo and/or a RP18 column, (8 X 250 mm, 10 μm), supplied by Knauer, Oberursel, G.F.R. UV and refractive index peaks in the effluent were registered with a LS 44 recorder from Linseis, Selb, G.F.R.

A Waters Data Module M 730 recorded peak areas electronically from the detector signal.

Experimental

Each substrate was dissolved in triethanolamine/HCl buffer (0.05 mol/l; pH 7,6) to a concentration of 16.1 mmol/l. A quantity of 0.1 ml pancreatic or salivary α-amylase was added to 0.5 ml of every substrate, followed by incubation of the reaction mixtures at 37 °C for 20 min. To stop the reaction, the samples were heated to 90 °C for 5 min. Twenty microliters of each substrate solution were injected first into the chromatographic system to give the blank, followed by injections of the corresponding reaction mixtures with pancreatic (second injection) and salivary (third injection) α-amylase. Each of the 24 hydrolyses were analysed seven times. The two values with the greatest absolute deviations were discarded. From the remaining five data the mean values and standard deviations were calculated. These ranged from 2.7% (*o*-nitrophenylmaltotrioside + salivary α-amylase) to 7.4% (*p*-nitrophenyl-α-*D*-maltoheptaoside + salivary α-amylase), the maximal deviations being 3.3% and 8.8% respectively. Separation on the TSK-G-2000 PW column was performed with quartz distilled water at a flow rate of 0.8 ml/min. The column back pressure was 2.7 MPa. For the RP18 column, a water/methanol mixture (volumes, 70 ml + 30 ml) was used (1.5 ml/min).

Sugars were monitored by the differential refractometer set at attenuation 4, and the nitrophenylated compounds were measured at 313 nm with the UV detector set at a sensitivity of 0.5 absorbance units.

[2]) A highly purified cristallized human pancreatic α-amylase used as standard in all experiments was a gift from Prof. *Takehiko Yamomoto*, Osaka City University, Japan.

Results

As shown in figure 1, the TSK-G-2000 PW-gel column clearly separates the free sugars from the nitrophenylated compounds, and even the different sugars and nitrophenylated products are fractionated simultaneously into characteristic peaks. The RP18 column is most practical for the separation of the nitrophenylated compounds. The experimental product distribution curves of the substrates with 2—7 glucose residues were quite similar to theoretical predictions, made on the basis of the subsite theory (8) which took into account "simple hydrolysis", disregarding condensation and multiple attack.

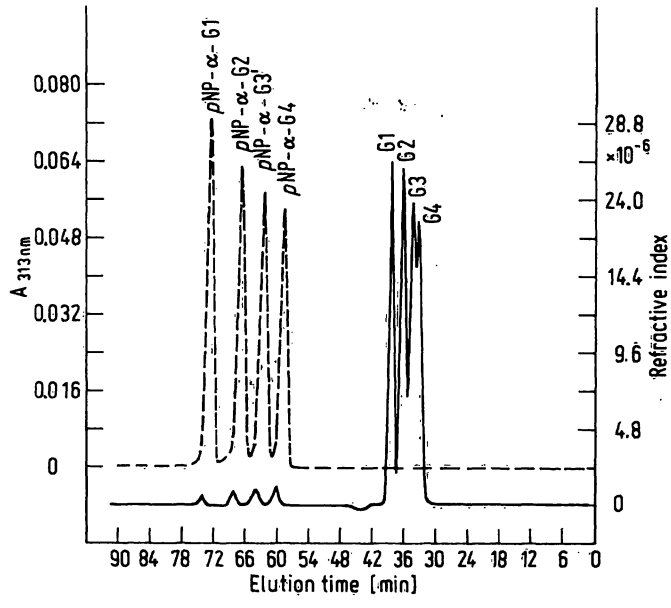

Fig. 1. Simultaneous separation by high performance liquid chromatography of free sugars with 1—4 glucose residues and the corresponding nitrophenylated compounds on a TSK-gel column. For conditions of separation see "Experimental". G1, G2, G3 and G4 are glucose, maltose, maltotriose and maltoetraose, respectively.
Abbreviations for the nitrophenylated compounds: *para*-1,4-α-*D*-nitrophenylglucoside = *p*NP-α-G1, *para*-1,4-α-*D*-nitrophenylmaltoside = *p*NP-α-G2, . . . and so on for the higher homologues.
— = Refractive index; - - - = UV spectrometry (A_{313nm}).

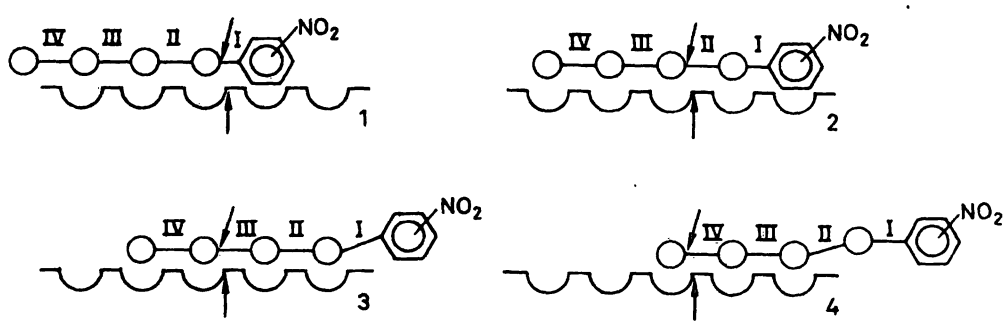

Fig. 2. Possible productive enzyme-substrate complexes of α-amylase and *para*-1,4-α-*D*-nitrophenyl-maltotetraoside (*p*NP-α-G4). The indentations in the active centre of the enzyme represent glucosyl binding sites; ↑↓, catalytic groups of the enzyme; —, an α-1→4 glycosidic bond; the glycosidic bonds are designated as I, II, III and IV; the substrate chain is so orientated that the nitrophenylated end is at its right.

Figure 2 shows a model of the enzyme-substrate complexes for different cleavage patterns of α-amylase with α-*D*-maltotetraoside as substrate. Analogous binding types may be assumed for the other substrate molecules with different chain lengths. Note that in binding patterns 1 and 2 (if nitrophenol or nitrophenyl-α-*D*-glucoside are produced) the nitrophenyl residue occupies one of the two subsites provided for aglyconic glucose residues.

All substrates were submitted to digestion with the same amount of α-amylase (according to activity determination with Phadebas substrate) under standard conditions. The quantities of the reaction products are listed in tables 1 and 2, reflecting the extent of the different types of binding by the subsites as represented in figure 2.

Binding of type 1 was found only with *o*- and *p*-nitrophenyl-α-*D*-maltoside and -maltotrioside. It was used earlier in a direct spectrophotometric test of human saliva using *p*-nitrophenyl-α-*D*-maltoside (9). The direct splitting of this compound to nitrophenol and maltose can be used to test only very high α-amylase activity, like that present in undiluted saliva. This activity was not observed at our enzyme concentration.

The *ortho* isomers are much better for direct assay, *o*-nitrophenyl-α-*D*-maltotrioside being the most suitable. Yet for human amylases, in the low concentrations present in urine and blood samples, the sensitivity with this substrate is still too low.

We never observed production of glucose corresponding to type 4 splitting; if maltotrioside is produced from a

Tab. 1. Hydrolysis of 4-nitrophenyl-α-*D*-maltodextrins with human pancreatic (p) and salivary (s) α-amylase; experimental conditions given under methods.

Substrate[1])	Mol hydrolysed from 1.0 mol substrate	Mol produced from 1.0 mol substrate								
		*p*NP-α-G1	*p*NP-α-G2	*p*NP-α-G3	*p*NP-α-G4	G1	G2	G3	G4	G5
*p*NP-α-G2	p 0.002	–	–	–	–	–	–	–	–	–
	s 0.001	–	–	–	–	–	–	–	–	–
*p*NP-α-G3	p 0.063	0.049	0.014	–	–	–	0.045	–	–	–
	s 0.044	0.037	0.007	–	–	–	0.032	–	–	–
*p*NP-α-G4	p 0.129	0.059	0.055	0.015	–	–	0.054	0.060	–	–
	s 0.115	0.033	0.072	0.010	–	–	0.065	0.028	–	–
*p*NP-α-G5	p 0.164	0.021	0.114	0.019	0.010	–	0.016	0.100	0.018	–
	s 0.133	0.006	0.116	0.011	–	–	0.010	0.099	0.004	–
*p*NP-α-G6	p 0.239	–	0.110	0.100	0.029	–	0.030	0.080	0.105	–
	s 0.243	–	0.107	0.101	0.035	–	0.030	0.101	0.092	–
*p*NP-α-G7	p 0.270	–	0.014	0.075	0.181	–	–	0.161	0.060	0.009
	s 0.285	–	0.021	0.068	0.191	–	–	0.199	0.060	0.018

[1]) *p*NP-α-G1 . . . 2 . . . 3 . . . 7 = 4-nitrophenyl-α-*D*-glucoside, -maltoside, -maltotrioside, -maltoheptaoside; G1 . . . 2 . . . 3 . . . 5 = glucose, maltose, maltotriose, maltopentaose.

Tab. 2. Hydrolysis of 2-nitrophenyl-α-D-maltodextrins with human pancreatic (p) and salivary (s) α-amylases; experimental conditions given under methods.

Substrate[1])	Mol hydrolysed from 1.0 mol substrate	Mol produced from 1.0 mol substrate								
		oNP-α-G1	oNP-α-G2	oNP-α-G3	oNP-α-G4	G1	G2	G3	G4	G5
oNP-α-G2	p 0.010	–	–	–	–	–	0.010	–	–	–
	s 0.020	–	–	–	–	–	0.020	–	–	–
oNP-α-G3	p 0.053	–	0.033	–	–	–	–	0.020	–	–
	s 0.063	0.003	0.033	–	–	–	0.002	0.030	–	–
oNP-α-G4	p 0.070	0.017	0.045	0.008	–	–	0.037	0.015	–	–
	s 0.080	0.016	0.058	0.006	–	–	0.055	0.010	–	–
oNP-α-G5	p 0.079	0.012	0.056	0.008	0.003	–	0.004	0.057	0.010	–
	s 0.091	0.003	0.064	0.016	0.008	–	0.011	0.062	0.011	–
oNP-α-G6	p 0.013	0.004	0.068	0.026	0.015	–	0.010	0.023	0.067	0.002
	s 0.124	0.010	0.065	0.036	0.013	–	0.009	0.035	0.066	0.008
oNP-α-G7	p 0.143	–	0.029	0.068	0.046	–	–	0.040	0.065	0.029
	s 0.162	–	0.044	0.069	0.049	–	–	0.042	0.070	0.039

[1]) oNP-α-G1 . . . 2 . . . 3 . . . 7 = 2-nitrophenyl-α-D-glucoside, -maltoside, -maltotrioside, -maltoheptaoside; G1 . . . 2 . . . 3 . . . 5 = glucose, maltose, maltotriose, maltopentaose.

tetraoside or tetraoside from pentaoside, this is due to transglycosylation[3]). Nitrophenyl-α-D-glucoside production according to 2 is important only for the short substrates with 3 and 4 glucose residues. Binding type 3 has equal or higher importance with the tetraoside and pentaoside. With 6 and 7 glucose residues in the chain the competition of the nitrophenyl residue for an aglyconic glucose vanishes completely and the nitrophenylglucosides with 3 and 4 glucose residues become predominant products.

If the rate of hydrolysis of nitrophenylmaltodextrins by α-amylase is measured in an assay system using α-glucosidase as indicator enzyme, the rate of nitrophenol production by this enzyme must be considered. Table 3 gives these data for α-glucosidase from yeast measured with *o*- and *p*-nitrophenyl-α-D-glucoside, -maltoside, -maltotrioside and maltotetraoside.

As can be seen the rate of hydrolysis decreases by a factor of nearly 10 for every glucose unit added by an α-1,4-linkage to nitrophenyl-α-D-glucoside. Accordingly 50–1000 times more α-glucosidase is needed as indicator enzyme if nitrophenyl-α-D-maltotrioside and -tetraoside are the products in the α-amylase reaction instead of the glucoside and maltoside. Therefore exceedingly high amounts of α-glucosidase must be added to the system to make sure that α-amylase remains rate determining if nitrophenyl-α-D-maltotetraoside is a major part of its products. On the other hand in our experience with several hundreds of serum and urine specimens tested with *p*-nitrophenyl-α-D-maltotetraoside, the moderate amount of α-glucosidase needed to hydrolyze the amylase products in a fast reaction never produced a significant blank value with the substrate.

[3]) unpublished results from our laboratory.

Discussion

In choosing the most suitable substrate in a highly sensitive coupled assay system the cleavage rates of the original substrates as well as of the products must be considered. It is interesting to note that the longest members of the series are the best substrates for α-amylase but produce the highest amounts of the worst substrates for α-glucosidase. In contrast, the lower members, which give reasonable amounts of good glucosidase substrates, are themselves increasingly susceptible to cleavage by glucosidase. The compounds with 4 and 5 glucose residues seem to be a good compromise as substrates for an assay system for low activity α-amylase samples.

The pentaoside needs much higher amounts of the indicator enzyme since the best substrate of glucosidase is produced only in very small amounts. Of the *ortho*-nitrophenol derivatives only the pentaoside seems to be a good choice considering its rate of hydrolysis and that of its products. But generally *o*-nitrophenolate-

Tab. 3. Catalytic activity of α-glucosidase (EC 3.4.1.20) with ortho and para nitrophenylated substrates (16.1 mmol/l). Catalytic concentration of the enzyme in the assay system was 0.50 kU/l. Triethanolamine/HCl (0.05 mol/l; pH 7.6). 3 min incubation time at 30 °C.

Substrate[1])	nkat	Substrate[2])	nkat
pNP-α-G1	382.4	oNP-α-G1	783.5
pNP-α-G2	52.3	oNP-α-G2	73.3
pNP-α-G3	5.3	oNP-α-G3	2.5
pNP-α-G4	1.5	oNP-α-G4	1.0

[1]) pNP-α-G1 . . . 2 . . . 3 . . . 4 = 4-nitrophenyl-α-D-glucoside, -maltoside, -maltotrioside, -maltotetraoside.

[2]) oNP-α-G1 . . . 2 . . . 3 . . . 4 = 2-nitrophenyl-α-D-glucoside, -maltoside, -maltrotrioside, -maltotetraoside.

producing tests have much lower sensitivity since the absorption coefficient of the *ortho* isomer is about four times smaller than that of the *para* isomer. The results summarized in tables 1 and 2 also relate to the issue of whether amylases from different sources display different action patterns. The direct liberation of nitrophenol from *o*-nitrophenyl-α-*D*-maltotrioside is higher with amylase of salivary than of pancreatic origin. With *p*-nitrophenyl-α-*D*-maltotetraoside salivary amylase produces generally much more *p*-nitrophenyl-α-*D*-maltoside than -glucoside, whereas with the pancreas enzyme the amounts of the two main hydrolysis products are about equal. It will be a task for future work to see if these findings with enzyme samples purified from human glands also apply to the salivary- and pancreatic-derived activity in serum and urine.

When using the "fastest" substrates with 6 and 7 glucose units it may be argued that many more α-1,4-glucosidic bonds are split than molecules of nitrophenol liberated, because the primary products of hydrolysis, nitrophenyl-α-*D*-maltotetraoside, maltotetraose and possibly maltotriose are split "gratuitously". Inspection of figure 3 and 4 may answer this question.

They show the complete product distribution when nearly all of the original substrate is hydrolysed, in contrast to the numbers of tables 1 and 2, which represent the product distribution after less than 30% splitting. Even when nearly all of the original maltohexa- and heptaose derivatives are split the liberated free sugars maltotetraose, maltotriose and maltose show up in nearly quantitative correspondence to their nitrophenylated counterparts with 2,3,4 and 3,4,5 glucose residues respectively. This is good evidence for the fact that secondary splitting by α-amylase of primary amylolytic products does not contribute appreciably to the production of latent α-glucosidase substrates. This explains our observation that the middle members of our substrate series in the coupled assay with α-glucosidase

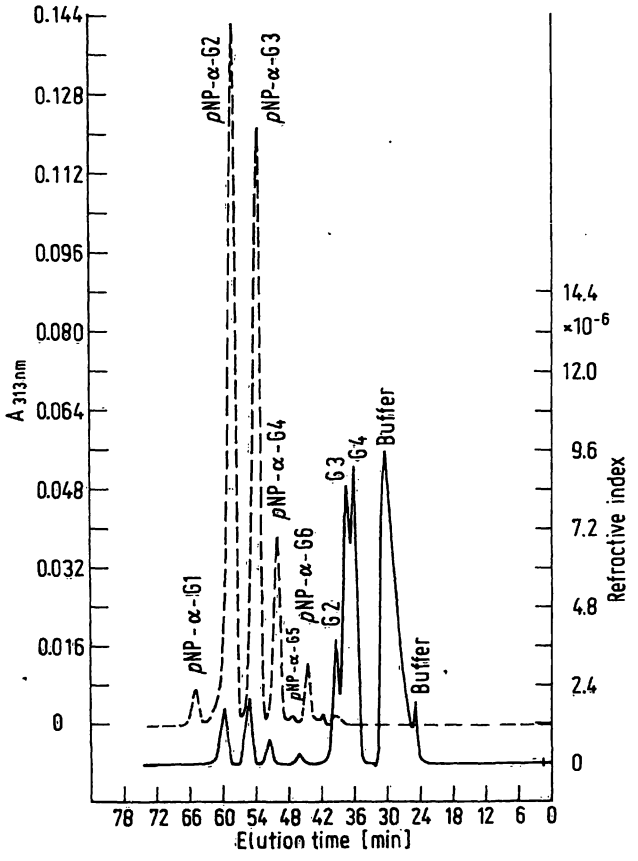

Fig. 3. Product distribution of *p*NP-α-G6 (16.1 mmol/l) after hydrolysis with salivary α-amylase fractionated on the TKS-gel column. For conditions of separation see "Experimental".
— = Refractive index; - - - = UV spectrometry (A_{313nm}).

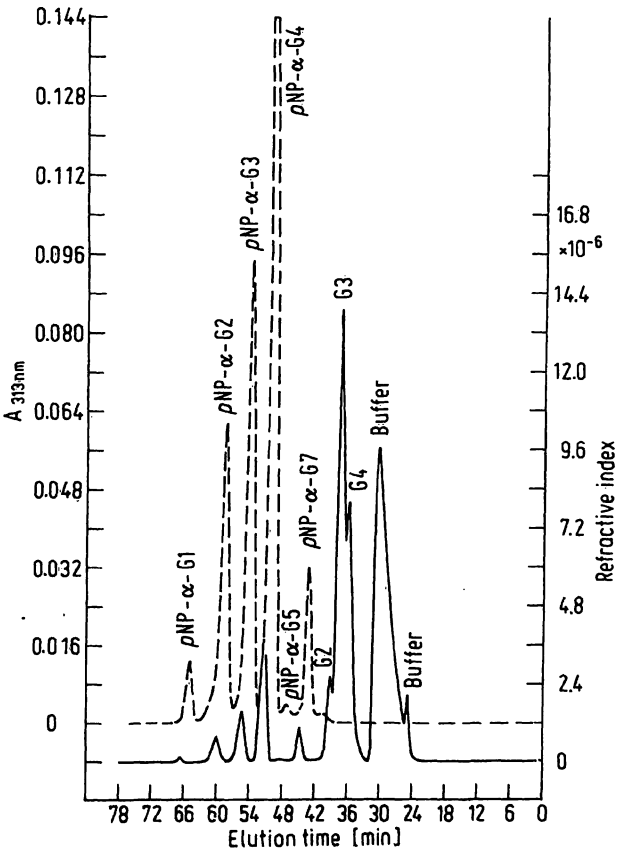

Fig. 4. Product distribution of *p*NP-α-G7 (16.1 mmol/l) after hydrolysis with salivary α-amylase fractionated on the TSK-gel column. For conditions of separation see "Experimental".
— = Refractive index; - - - = UV spectrometry (A_{313nm}).

give higher rates of nitrophenol liberation than the higher ones, although these are the better substrates of α-amylase alone. In addition to being nonproductive, the primary amylolytic cleavage products in the indicator reaction of the coupled system may even be counterproductive, because bad substrates generally act as competitive inhibitors for better substrates.

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