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2-Chloro-4-nitrophenyl-β-*D*-maltoheptaoside: A New Substrate for the Determination of α-Amylase in Serum and Urine

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Summary: Tests for the determination of α-amylase activity based on oligosaccharides or their 4-nitrophenyl derivatives as substrates suffer from some disadvantages. Using a more acid indicator molecule and connecting this group in the β-form to maltoheptaose leads to a new substrate, 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside, for the determination of α-amylase activity.

Due to its pK value, only half of the 4-nitrophenol formed from 4-nitrophenyl-α-*D*-maltoheptaoside is spectrophotometrically detectable, whereas the total product from the new substrate is responsible for a measurable absorbance. In addition the molar lineic absorbance of the product is 1.8 times higher. Thus the new test is more sensitive than those employing 4-nitrophenyl derivatives.

Both P-type and S-type α-amylase isoenzymes have the same affinity for the new substrate; this is not the case for all the other substrates, i.e. oligosaccharides and their 4-nitrophenyl derivatives, used in α-amylase tests.

The sample volume may be reduced, thereby removing interference by substances like bilirubin, glucose and haemoglobin.

The test is insensitive to changes in temperature and pH and can be adapted to all mechanized analytical instruments.

2-Chlor-4-nitrophenyl-β-D-maltoheptaosid: Ein neues Substrat zur Bestimmung von α-Amylase in Serum und Harn

Zusammenfassung: Die Verfahren zur Bestimmung von α-Amylase auf der Basis von Oligosacchariden und den 4-Nitrophenolderivaten unterliegen einer Reihe von Störfaktoren, die zum Teil erhebliche praktische Bedeutung haben. Durch Einführung einer acideren chromophoren Gruppe und deren Kopplung in β-Form wird als neues Substrat für die α-Amylasebestimmung 2-Chlor-4-nitrophenyl-β-*D*-maltoheptaosid vorgestellt.

Aufgrund der höheren molaren linearen Absorbanz und aufgrund eines von den bisherigen Tests abweichenden Spaltmusters der Amylase weist der neue Test eine höhere Empfindlichkeit auf.

Auch die unterschiedliche Substrataffinität der Amylase-Isoenzyme, die von den bisher gebräuchlichen Tests bekannt ist, spielt bei dem neuen Substrat eine untergeordnete Rolle.

Das Probenvolumen kann reduziert werden und daraus resultiert eine geringere Empfindlichkeit gegenüber den Störfaktoren der Probe wie Bilirubin, Glucose und Hämoglobin.

Besonders unempfindlich ist das neue Testsystem gegenüber Schwankungen im pH- und Temperaturbereich.

Introduction

Oligosaccharides with a chain length of four to seven glucose units (maltotetraose to maltoheptaose) or the 4-nitrophenyl derivatives of these substrates are new substrates for the determination of the α-amylase (1,4-α-*D*-glucan 4-glucanohydrolase, EC 3.2.1.1) catalytic activity in serum and urine. Endogenous glucose in the sample causes interference, especially in the test kits with oligosaccharides as substrates (1).

Substrates with the 4-nitrophenol indicator molecule depend greatly on the pH stability of the test system, since the measurement is carried out at a pH equal to the pK of the indicator molecule due to the pH optimum of α-amylase. Under these conditions only 50% of the molecules are detectable as 4-nitrophenolate. Furthermore there is interference by glucose and bilirubin in the sample, and the salivary isoenzyme has a higher affinity for the substrates (2), including the unsubstituted oligosaccharide substrates.

Thus, 2-chloro-4-nitrophenol was selected as a more acid indicator molecule as the basis for a new test with a higher sensitivity and without the above mentioned disadvantages.

Materials and Methods

Among several possible indicator groups, 2-chloro-4-nitrophenol was selected as the new indicator system. The 2-chloro-4-nitrophenyl-β-*D*-glucoside was synthesized by the *Koenigs-Knorr* procedure (3). 2-Chloro-4-nitrophenyl-β-*D*-maltoheptaoside was synthesized by the procedure described by *Wallenfels* et al. (4) using cyclodextrin-glycosyltransferase (α-1,4-glucan 4-glycosyltransferase, EC 2.4.1.19) from *Bacillus macerans* or *Klebsiella pneumoniae* and α-cyclodextrin. The substrate was isolated by a preparative HPLC-Technique with a NH2-column and acetonitrile/water (72.5 + 27.5 by vol.) as eluent. The substrate fraction was detected at 313 nm, collected and lyophilised. A preparation of human pancreatic and salivary α-amylase with an activity of (Phadebas test) 1000 U/l was used for all the systematic studies.

The optimal test conditions are:

Buffer: pH 7.2	Potassium phosphate	50 mmol/l
	KCl	50 mmol/l
Enzymes:	α-Glucosidase	>70 kU/l
	β-Glucosidase	>10 kU/l

Substrate: 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside		4 mmol/l
Sample/reagent ratio:		between 0.02 and 0.01
Detection wavelength:		405 nm
Measuring temperature:		25 °C, 30 °C, or 37 °C
Molar lineic absorbance of 2-chloro-4-nitrophenol at pH 7.2:	25 °C	1.66×10^3 m^2 × mol^{-1}
	30 °C	1.66×10^3 m^2 × mol^{-1}
	37 °C	1.67×10^3 m^2 × mol^{-1}

A reagent based on this specification was used for all measurements in this study.

Instruments

Photometer Eppendorf 1101 M with recorder 6511 and ACP 5040, Eppendorf Gerätebau, Hamburg.

For both instruments, the reaction was started with serum, and the first reading was made after 5 minutes.

Reaction products

From an incubation mixture (at 30 °C), aliquots were taken after 1, 5, 10, 20 and 30 minutes, and the reaction was immediatly terminated by centrifugation with the Amicon system MPS-1 (YMB membrane) for 3 minutes at 1800 *g* at 0–5 °C.

Filtrate (10 µl) was analysed with the HP Liquid Chromatograph 1084 B with a Hibar Lichrosorb NH2-Column 5 µ (Merck No. 50376) and acetonitrile/water (72.5 + 27.5 by vol.) as eluent. The signal was monitored at 313 nm.

Reagents

The glucosidases were products from Boehringer-Mannheim, β-glucosidase (β-*D*-glucoside-glucohydrolase, EC 3.2.1.21) Cat. No. 635332 (50 U/mg), α-glucosidase (α-*D*-glucoside-glucohydrolase, EC 3.2.1.21) Cat. No. 105422 (2 U/mg), α-cyclodextrin was a product from Sigma Chemical Co. St. Louis USA Cat. No. C 4642.

For quality control, Monitrol I (Lot. LTD 178) and Monitrol II (Lot. PTD 76) from American Hospital Supply, Deutschland GmbH, München were used. All other chemicals were analytical grade products from Merck Darmstadt.

Commercially available test kits for α-amylase determination:

α-Amylase PNP Enzymatischer Farbtest Boehringer Mannheim Cat. No. 568589.
M + D Amylase PNP American Hospital Supply, Deutschland GmbH, München, Cat. No. 140004.
Testomar®-Amylase Behring, Behringwerke Marburg, Cat. No. OSVV 10/11.

Results and Discussion

The difference between the dissociation of the two indicators is demonstrated in figure 1. The pK of 4-nitrophenol is 7.09, and that of 2-chloro-4-nitrophenol is 5.13. The time-reaction curve of the new

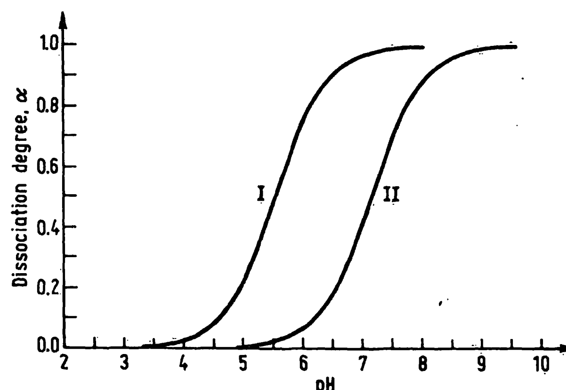

Fig. 1. Dissociation curve of the two indicators 2-chloro-4-nitrophenol (I, pK = 5.13) and 4-nitrophenol (II, pK = 7.09) measured at 405 nm.

test in comparison with tests using 4-nitrophenyl-α-*D*-maltoheptaoside and 2-chloro-4-nitrophenyl-α-*D*-maltoheptaoside is demonstrated in figure 2. The samples were salivary and pancreatic amylase each with a total activity (Phadebas test) of 1000 U/l. The new test is 3.6 times more sensitive than the other amylase tests. The increase in sensitivity is higher than calculated from the higher molar lineic absorbance.

This unexpected result was explained by the characterisation and determination of the reaction products by HPLC.

A serum with an elevated α-amylase activity was diluted with 9 g/l NaCl solution. The result of this test for linearity is shown in figure 3. The test system is linear up to at least 750 U/l at 25 °C.

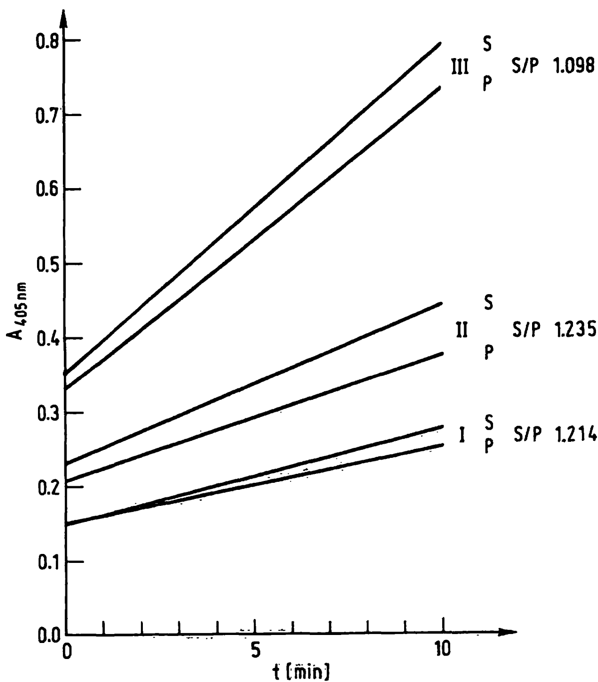

Fig. 2. Time-reaction-curves of the P- and S-type α-amylase isoenzymes (1000 U/l each, Phadebas test) for the different substrates 4-nitrophenyl-α-*D*-maltoheptaoside (I), 2-chloro-4-nitrophenyl-α-*D*-maltoheptaoside (II) and 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside (III). The quotient S/P expresses the affinity of the isoenzymes for the different substrates.

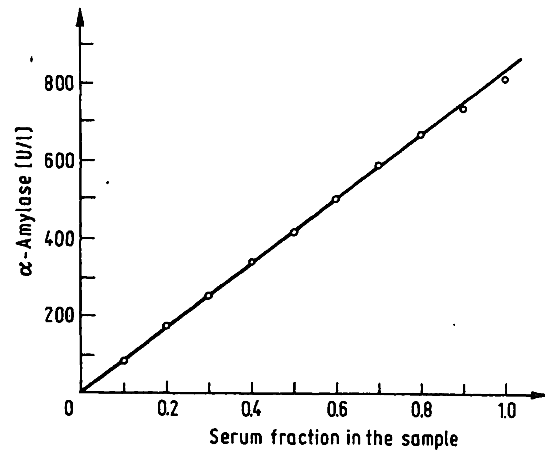

Fig. 3. Linearity of the new test, measurement at 25 °C. The sample was diluted with 9 g/l NaCl solution.

The stability of the reagent as listed above was tested under different storage conditions at 4 °C in the dark and 20 °C in the dark and in daylight. The results are shown in figure 4. The reagent is stable at 4 °C for at least 48 hours. At room temperature, the stability is 8 to 10 hours. There is practically no difference if stored in the dark or in daylight.

The imprecision within run and between runs using Monitrol I and Monitrol II and sera from patients with normal and elevated activities of α-amylase are demonstrated in table 1. The precision in the low level sample is much better than that for the other methods. Therefore this method gives better results in the range where decisions are made.

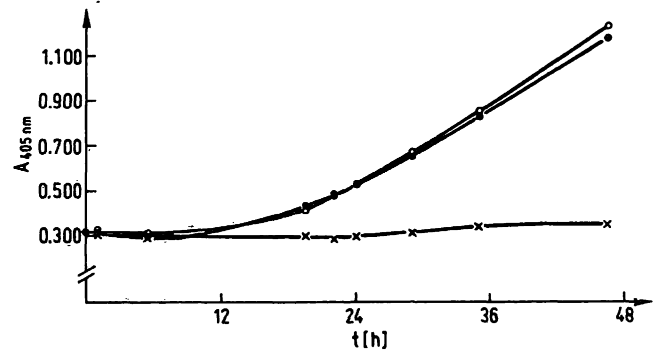

Fig. 4. Stability of the reagent at 4 °C in the dark (×——×), 20 °C in the daylight (●——●) and 20 °C in the dark (○——○).

Tab. 1. Imprecision within run and between runs of the α-amylase test with 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside. Manual test, Photometer Eppendorf 405 nm with recorder, 25 °C, 500 µl reagent and 10 µl serum.

	Imprecision within run				Imprecision between runs	
	Patient serum normal	Patient serum pathologic	Monitrol I control serum Lot. No. LTD 178	Monitrol II control serum Lot. No. PTD 76	Monitrol I control serum Lot. No. LTD 178	Monitrol II control serum Lot. No. PTD 76
n	10	10	10	10	10	10
x̄ · (U/l)	74.7	417.1	113.7	308.4	111.7	307.1
SD (U/l)	o.8105	2.9782	1.4252	3.0702	2.3937	4.5190
CV (%)	1.09	0.71	1.25	0.99	2.14	1.47

Most of the α-amylase methods with oligosaccharides as substrate detect the salivary (S) isoenzyme with greater sensitivity than the pancreatic (P) amylase (1, 2). With the new test this effect is reduced and can be neglected. In figure 2 this difference is listed as quotient S/P. For maltotetraose as substrate the quotient is 1.59 (5).

All methods using oligosaccharides or 4-nitrophenol substituted oligosaccharides depend on pH stability. If the molarity of the buffer is increased, the sensitivity of the test is altered. On the other hand the 50 mmol/l phosphate buffer is not suitable for measurements in urine and pancreatic juice. Slight changes in the pH have a remarkable and unpredictable influence on the absorbance signal. The new test using a 50 mmol/l buffer is practically unaffected by addition of HCl or NaOH. The results are shown in table 2.

The interference by glucose, ascorbic acid, haemoglobin and bilirubin is demonstrated in figures 5–8. Ascorbic acid has no influence, haemoglobin starts to show an influence by decreasing the signal at a concentration of 1.5 g/l. Glucose concentrations of more than 50 mmol/l give a slight inhibition of the test by affecting the α-glucosidase. This inhibition is independent of the level of the amylase activity and is also lower than that observed in the other tests. α-Glucosidase from another source may reduce this effect a little. Much better results are achieved with the new test when the influence of bilirubin is taken into account. In the test described here we find the signal slightly increased by about 2 to 3%. For the other tests the direction of the influence on the test is unpredictible and may amount to 5–8%. Hydroxyethyl-starch is split by the α-amylase to smaller branched fragments. The amylase is bound to these fragments to form complexes, which are not eliminated by the kidney, so in urine the amylase is decreased and in serum it is increased. This phenomenon is comparable with the macroamylasaemia where protein is the complexing agent.

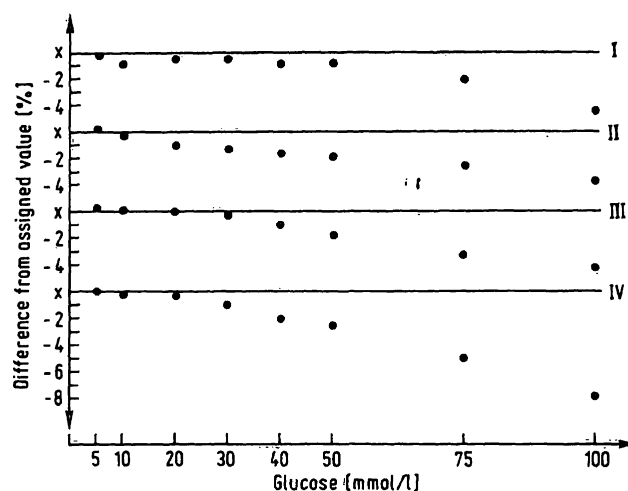

Fig. 5. Interference of glucose with the different α-amylase test systems. Serum with 300 U/l α-amylase at 25 °C and increasing glucose concentration.

I 2-Chloro-4-nitrophenyl-β-D-maltoheptaoside (new α-amylase test).
II 4-Nitrophenyl-α-D-maltoheptaoside, Boehringer Mannheim.
III 4-Nitrophenyl-α-D-maltoheptaoside, American Hospital Supply.
IV 4-Nitrophenyl-α-D-maltohexaoside and -pentaoside, Behring-Werke.

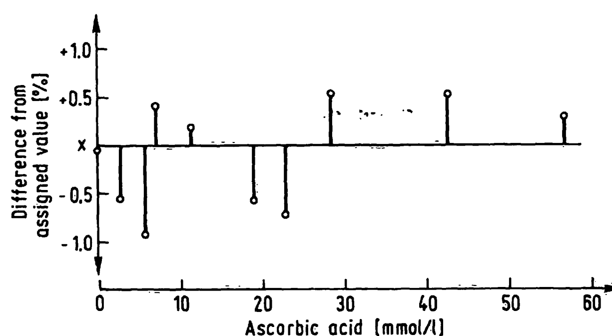

Fig. 6. Interference by ascorbic acid in the new α-amylase test. A serum with 380 U/l α-amylase at 25 °C and increasing concentrations of ascorbic acid.

Tab. 2. Changes of the absorbance of the two indicators 2-chloro-4-nitrophenol and 4-nitrophenol after adding H^+ and OH^- ions.

		Addition of OH^- and H^+ ions			
		+ 10 µmol OH^-	+ 20 µmol OH^-	+ 10 µmol H^+	+ 20 µmol H^+
2-Chloro-4-nitrophenol in phosphate buffer 50 mmol/l pH 7.0 Absorbance (405 nm, 25 °C)	0.500	0.507 (+1.4%)	0.512 (+2.4%)	0.492 (−1.8%)	0.485 (−3.0%)
4-Nitrophenol in phosphate buffer 50 mmol/l pH 7.0 Absorbance (405 nm, 25 °C)	0.500	0.582 (+17.6%)	0.689 (+37.8%)	0.457 (−8.6%)	0.344 (−31.2%)

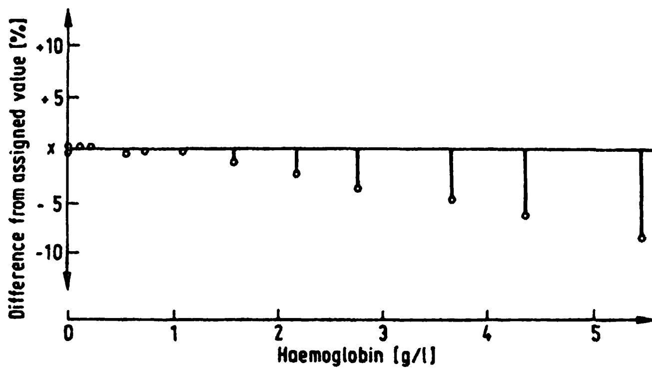

Fig. 7. Interference by haemoglobin in the new α-amylase test. A serum with 165 U/l α-amylase at 25 °C and increasing concentrations of haemoglobin.

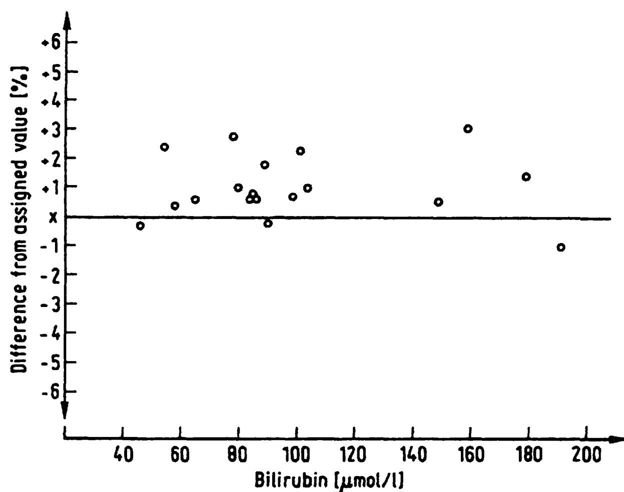

Fig. 8. Interference by bilirubin in the new α-amylase test. A serum with 300 U/l α-amylase at 25 °C and increasing concentrations of bilirubin.

Anticoagulants that complex calcium (e.g. oxalate, citrate) are not acceptable for sample preparation.

The new test was compared with the α-Amylase PNP Enzymatischer Farbtest Boehringer-Mannheim, and the M + D Amylase PNP test from American Hospital Supply both using 4-nitrophenyl-α-*D*-maltoheptaoside and Testomar®-Amylase Behring with a mixture of the substrates 4-nitrophenyl-α-*D*-maltopentaoside and -hexaoside.

Sera from 115 patients with amylase catalytic concentrations from 50 up to 840 U/l were included in the test. The results are given in table 3 and figure 9. The results with the new method are comparable with the results from other methods.

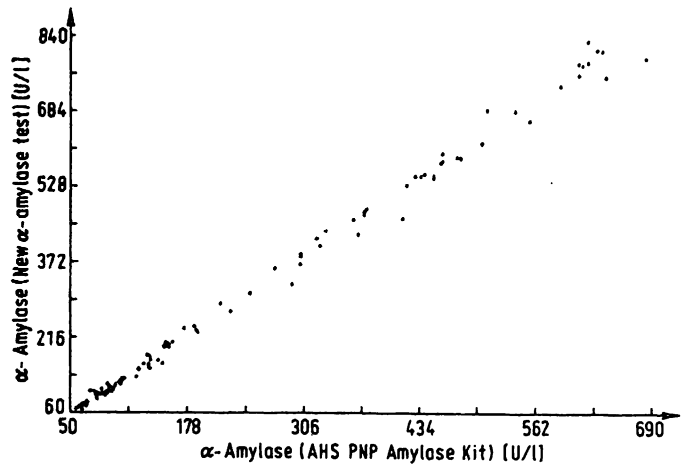

Fig. 9. Scattergram as an example of the comparison of methods, T = 25 °C.
x = M + D Amylase PNP (American Hospital Supply).
y = 2-chloro-4-nitrophenyl-β-*D*-malthoheptaoside α-amylase test (n= 115).

Reaction products

The determination of reaction products of the α-amylase with the different substrates has already been published by different groups (6–9).

These publications dealing with determination of reaction products of 4-nitrophenyl-α-oligosaccharides are based on different conditions and the method of sample preparation (e.g. reaction was stopped by boiling or adding acid or alkaline reagents). The time interval was up to 70 minutes. We used the same time schedule as *Hägele* (8) to make sure that there is a linear increase of the reaction products. Therefore it is appropriate to compare our results with those from this group. No results have been published by other groups on the influence of sample preparation (hydrolysis by boiling or treatment with

Tab. 3. Comparison of methods. The new test compared with three commercial tests, measuring at 25 °C, preincubation time 5 minutes for all tests. n = 115, range 50–840 U/l.

y \ x	α-Amylase PNP Enzymatischer Farbtest Boehringer Mannheim	M + D Amylase PNP American Hospital Supply	Testomar®-Amylase Behring
2-Chloro-4-nitrophenyl-β-*D*-maltoheptaoside	y = 1.0964 x − 10.9 r = 0.996	y = 1.2684 x − 8.3054 r= 0.995	y = 2.2640 x − 7.1463 r = 0.995

acid or alkaline reagents). From the reaction products the reaction equation is derived and the stoichiometric coefficient is calculated. The result is shown in table 4. In contrast to the reaction products of the 4-nitrophenyl-α-*D*-maltoheptaoside (8), where about 30% of the reaction products are used to produce a signal, the new substrate gave an increased amount of about 70%. This result was primarily not expected. This increase in sensitivity depends on steric effects by binding the indicator group in the β-form.

The release of the different reaction products from the substrate 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside by the salivary and pancreatic α-amylase is listed in tables 5 and 6. The results demonstrate the action of the α-amylase (tab. 5) and of the complete test system with the α- and β-glucosidase on the substrate (tab. 6). *Wallenfels* (9) found that the linkage of 4-nitrophenyl (α- or β-form) to the oligosaccharides has no effect on the reaction velocity.

These findings are not confirmed in our study for the 2-chloro-4-nitrophenyl α- and β-*D*-maltoheptaosides.

The preliminary reference values at the 97.5 percentile (40 healthy males and 30 healthy females) for 25 °C in serum and urine are:

serum up to 120 U/l,
urine up to 650 U/l.

Tab. 4. Calculation of the stoichiometric coefficient from the reaction products of 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside with α-amylase and α- and β-glucosidases giving the indicator signal. The stoichiometric coefficient is the increase in moles of the reaction products divided by the decrease of the substrate.
Cl-4-NP = 2-chloro-4-nitrophenol
Cl-4-NP-β-G_2 = 2-chloro-4-nitrophenyl-β-*D*-maltoside
Cl-4-NP-β-G_3 = 2-chloro-4-nitrophenyl-β-*D*-maltotrioside
Cl-4-NP-β-G_4 = 2-chloro-4-nitrophenyl-β-*D*-maltotetraoside
Cl-4-NP-β-G_7 = 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside

Substrate	Enzyme	Product	Coefficient		Enzymes	Product	Coefficient	
Cl-4-NP-β-G_7	α-P-Amylase, H_2O	Cl-4-NP-β-G_2	0.16	(±0.02)	α-Glucosidase, β-Glucosidase, H_2O	Cl-4-NP	0.67	(÷0.04)
		Cl-4-NP-β-G_3	0.57	(±0.02)		Cl-4-NP-β-G_3	0.03	(÷0.02)
		Cl-4-NP-β-G_4	0.27	(±0.02)		Cl-4-NP-β-G_4	0.30	(±0.03)
Cl-4-NP-β-G_7	α-S-Amylase, H_2O	Cl-4-NP-β-G_2	0.15	(±0.02)	α-Glucosidase, β-Glucosidase, H_2O	Cl-4-NP	0.64	(±0.01)
		Cl-4-NP-β-G_3	0.53	(±0.01)		Cl-4-NP-β-G_3	0.03	(±0.01)
		Cl-4-NP-β-G_4	0.32	(±0.02)		Cl-4-NP-β-G_4	0.33	(±0.00)

$$3\ \text{Cl-4-NP-}\beta\text{-G}_7 + 3\ H_2O \xrightarrow{\alpha\text{-Amylase}} 2\ \text{Cl-4-NP-}\beta\text{-G}_3 + \text{Cl-4-NP-}\beta\text{-G}_4\ (+\ \text{Oligosaccharides})$$

$$2\ \text{Cl-4-NP-}\beta\text{-G}_3 + 6\ H_2O \xrightarrow[\beta\text{-Glucosidase}]{\alpha\text{-Glucosidase}} 2\ \text{Cl-4-NP}\ (+\ 6\ \text{Glucoses})$$

Stoichiometric coefficient: 1:1.5 = 0.67

Tab. 5. Reaction products of 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside during different incubation periods with pancreatic and salivary α-amylase at 30 °C, expressed as stoichiometric coefficients (± SD).
Cl-4-NP-β-G_2 = 2-chloro-4-nitrophenyl-β-*D*-maltoside
Cl-4-NP-β-G_3 = 2-chloro-4-nitrophenyl-β-*D*-maltotrioside
Cl-4-NP-β-G_4 = 2-chloro-4-nitrophenyl-β-*D*-maltotetraoside

Incubation period		Pancreatic α-amylase			Salivary α-amylase		
Start	End	Cl-4-NP-β-G_2	Cl-4-NP-β-G_3	Cl-4-NP-β-G_4	Cl-4-NP-β-G_2	Cl-4-NP-β-G_3	Cl-4-NP-β-G_4
1 min	5 min	0.14 ± 0.05	0.60 ± 0.05	0.26 ± 0.07	0.20 ± 0.03	0.53 ± 0.04	0.27 ± 0.02
	10 min	0.16 ± 0.02	0.58 ± 0.01	0.27 ± 0.02	0.17 ± 0.01	0.53 ± 0.01	0.30 ± 0.01
	20 min	0.15 ± 0.01	0.58 ± 0.01	0.28 ± 0.01	0.16 ± 0.00	0.54 ± 0.01	0.30 ± 0.01
	30 min	0.15 ± 0.01	0.58 ± 0.01	0.27 ± 0.01	0.16 ± 0.00	0.53 ± 0.00	0.31 ± 0.00
5 min	10 min	0.16 ± 0.02	0.57 ± 0.02	0.27 ± 0.02	0.15 ± 0.02	0.53 ± 0.01	0.32 ± 0.02
	20 min	0.15 ± 0.00	0.58 ± 0.02	0.28 ± 0.02	0.15 ± 0.02	0.54 ± 0.00	0.31 ± 0.02
	30 min	0.16 ± 0.01	0.57 ± 0.01	0.27 ± 0.01	0.15 ± 0.01	0.54 ± 0.01	0.31 ± 0.00
10 min	20 min	0.16 ± 0.01	0.57 ± 0.02	0.28 ± 0.01	0.15 ± 0.01	0.55 ± 0.00	0.30 ± 0.01
	30 min	0.15 ± 0.01	0.58 ± 0.01	0.27 ± 0.01	0.16 ± 0.01	0.54 ± 0.01	0.30 ± 0.01
20 min	30 min	0.16 ± 0.03	0.58 ± 0.03	0.26 ± 0.02	0.16 ± 0.00	0.54 ± 0.02	0.30 ± 0.03

Tab. 6. Reaction products of 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside during different incubation periods at 30 °C with the α-amylase isoenzymes in the complete test system (α-glucosidase and β-glucosidase), expressed as stoichiometric coefficients.
Cl-4-NP = 2-chloro-4-nitrophenol,
Cl-4-NP-β-G_3 = 2-chloro-4-nitrophenyl-β-*D*-maltotrioside,
Cl-4-NP-β-G_4 = 2-chloro-4-nitrophenyl-β-*D*-maltotetraoside.

Incubation period		Pancreatic α-amylase			Salivary α-amylase		
Start	End	Cl-4-NP	Cl-4-NP-β-G_3	Cl-4-NP-β-G_4	Cl-4-NP	Cl-4-NP-β-G_3	Cl-4-NP-β-G_4
1 min	5 min	0.64 ± 0.07	0.05 ± 0.05	0.31 ± 0.04	0.57 ± 0.04	0.04 ± 0.03	0.39 ± 0.03
	10 min	0.65 ± 0.04	0.03 ± 0.01	0.31 ± 0.03	0.60 ± 0.01	0.04 ± 0.01	0.36 ± 0.02
	20 min	0.68 ± 0.02	0.03 ± 0.01	0.29 ± 0.01	0.65 ± 0.01	0.02 ± 0.01	0.33 ± 0.00
	30 min	0.70 ± 0.01	0.02 ± 0.00	0.28 ± 0.01	0.67 ± 0.01	0.02 ± 0.00	0.31 ± 0.00
5 min	10 min	0.67 ± 0.04	0.03 ± 0.02	0.30 ± 0.03	0.64 ± 0.01	0.03 ± 0.01	0.33 ± 0.00
	20 min	0.70 ± 0.01	0.02 ± 0.02	0.28 ± 0.02	0.68 ± 0.01	0.02 ± 0.00	0.30 ± 0.01
	30 min	0.71 ± 0.00	0.02 ± 0.00	0.27 ± 0.00	0.69 ± 0.00	0.02 ± 0.01	0.29 ± 0.01
10 min	20 min	0.71 ± 0.02	0.01 ± 0.01	0.28 ± 0.02	0.69 ± 0.01	0.01 ± 0.00	0.30 ± 0.02
	30 min	0.72 ± 0.01	0.01 ± 0.01	0.27 ± 0.01	0.70 ± 0.02	0.01 ± 0.01	0.29 ± 0.01
30 min	30 min	0.74 ± 0.01	0.01 ± 0.00	0.25 ± 0.01	0.72 ± 0.02	0.01 ± 0.00	0.27 ± 0.00

The reference values do not differ from those obtained with the 4-nitrophenyl substituted substrate (10). The reference value seems to be determined mainly by the chain-length of the substrate.

Conclusion

The main advantages of the new α-amylase test with 2-chloro-4-nitrophenyl-β-*D*-maltoheptaoside as substrate are:

1. The test is insensitive to changes in pH caused by the specimen.
2. The new indicator system has a higher molar lineic absorbance.
3. The signal of the new indicator 2-chloro-4-nitrophenol is insensitive to changes in temperature, F 37 °C/25 °C = 1.01 (for 4-nitrophenol F = 1.18).
4. Reduction of the sample volume effects a reduction of the interfering substances.
5. The two-fold increased production of reaction products producing a signal increases the sensitivity of the new test.
6. The cleavage pattern of the new test has been characterised and the reaction products have been estimated quantitatively. The rate of reaction shows no dependence on reaction time over a long reaction period.
7. The new test has a better precision in the low activity range.
8. The test has a wide linear range.
9. With the new substrate there is only a slight difference in the substrate affinity for the P- and S-type amylase isoenzymes.
10. The new test easy to run on all automated analytical instruments.

References

1. Lorentz, K. (1983) J. Clin. Chem. Clin. Biochem. *21*, 463–471.
2. Henkel, E. (1981) III. Int. Congress of Enzymol. Salzburg, Ber. Oesterr. Ges. Klin. Chem. *4*, 128.
3. Koenigs, W. & Knorr, E. (1901) Ber. d. Chem. Ges. Berlin *34*, 957–981.
4. Wallenfels, K., Földi, P., Niermann, H., Bender, H. & Linder, D. (1978) Carbohydrate Res. *61*, 359–368.
5. Meier, H., Henkel, E. & Dankert, H. (1979) J. Clin. Chem. Clin. Biochem. *17*, 709–716.
6. Davis, H. (1982) Clin. Chem. *28*, 1485–1489.
7. Wallenfels, K., Laule, G. & Meltzer, Barbara (1982) J. Clin. Chem. Biochem. *20*, 581–586.
8. Hägele, E. O., Schaich, E., Rauscher, Elli, Lehmann, P., Bürk, H. & Wahlefeld, A. W. (1982) Clin. Chem. *28*, 2201–2205.
9. Wallenfels, K. & Janatsch, G. (1983) Ärztl. Lab. *29*, 339–348.
10. Hohenwallner, W. (1983) Ber. Oesterr. Ges. Klin. Chem. *6*, 31–33.

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