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ACTION PATTERN OF HUMAN PANCREATIC α-AMYLASE ON MALTOHEPTAOSE, A SUBSTRATE FOR DETERMINING α-AMYLASE IN SERUM

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SUMMARY

An enzymatic assay for the determination of α-amylase in serum was developed which employed a soluble substrate, maltoheptaose, and a coupled enzymatic indicator reaction consisting of α-glucosidase and the hexokinase—glucose-6-phosphate dehydrogenase system. We used high-performance liquid chromatography (HPLC) to establish the action pattern of maltoheptaose under the test conditions: (A) the action pattern of α-amylase alone, (B) that of the combined action of α-amylase and α-glucosidase. Conducive to this effort was: the availability of pure maltoheptaose and human pancreatic α-amylase; the development of an adequate procedure for sample pretreatment (partition chromatography on a mixed-bed ion exchanger) and of an HPLC system for separation of substrate and reaction products without interference from by-products of the assay (partition chromatography on a cation-exchange column with acetonitrile—water); and the use of a new, very sensitive refractometric detector revealing sugar amounts as low as 40 ng.

We derived the following stoichiometric equations:

$$\text{maltoheptaose} \xrightarrow{\alpha\text{-amylase}} 0.10\ \text{maltopentaose} + 0.79\ \text{maltotetraose} + 0.87\ \text{maltotriose} + 0.29\ \text{maltose}$$

$$\text{maltoheptaose} \xrightarrow[\alpha\text{-glucosidase}]{\alpha\text{-amylase}} 0.10\ \text{maltopentaose} + 0.77\ \text{maltotetraose} + 0.06\ \text{maltotriose} + 0.07\ \text{maltose} + 2.94\ \text{glucose}$$

The standard deviation of the rate coefficients is about 5%.

INTRODUCTION

The determination of serum α-amylase (1,4-α-D-glucaglucanohydrolase, EC

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3.2.1.1), a hydrolase which cleaves 1,4-α-glycosidic bonds, became a very important tool for the diagnosis of pancreatitis.

The basis of the most current procedures for the measurement of the enzymatic activity is the hydrolysis of starch or of chromogenic derivatives thereof [1, 2]. The reactions are followed by determining the formation of reducing groups or the release of the dye component. All these methods exhibit several drawbacks: (1) there is no exact stoichiometric relation between the reducing groups formed or the dye released and the number of hydrolyzed bonds; (2) the reactions are non-linear with time; (3) the rates observed are very much dependent on the kind and the quality of the polysaccharide used. We therefore developed a new method to determine α-amylase in serum by employing maltoheptaose as substrate (seven glucose residues in a linear 1,4-α-linkage) and a three-step NADH-forming indicator reaction: α-amylase acts on maltoheptaose yielding lower oligosaccharides which are partly hydrolyzed by α-glucosidase. The liberated glucose is determined by the hexokinase—glucose-6-phosphate dehydrogenase (NAD-dependent) method.

Since it was undecided what kind of reaction products originate from maltoheptaose and which of them enter the indicator reaction, the issue was to evaluate the action pattern of human pancreatic α-amylase on maltoheptaose under the reaction conditions envisaged for the enzymatic test. We had to establish the stoichiometry of the reaction, which is the basis for the calculation of amylase activity from the formation of NADH, i.e. from the photometric extinction in the enzymatic test. For this purpose two kinds of action patterns had to be investigated: that created by α-amylase alone and also the action pattern of the coupled α-amylase—α-glucosidase system to see how many mols of glucose result from 1 mol of degraded maltoheptaose.

EXPERIMENTAL

Chemicals

KH_2PO_4, NaCl, KOH, NaOH, methanol, conc. HCl, and acetonitrile were all reagent grade and purchased from E. Merck, Darmstadt, G.F.R. Bovine serum albumin and α-glucosidase were from Boehringer Mannheim GmbH, Mannheim, G.F.R. Maltoheptaose was synthesized by one of us (E. Schaich), and α-amylase was purified from human pancreas by H. Lenz (both Boehringer Mannheim GmbH, Biochemica Werk Tutzing, Tutzing, G.F.R.). The α-amylase had a specific activity of 230 units/mg lyophilisate, according to the amylochrome test (Hoffmann-La Roche, Basel, Switzerland).

Dowex cation-exchange resin 50W-X8 (H^+), 50—100 mesh, was reagent grade and the anion exchanger MWA-1 (OH^-), 20—50 mesh, was practical grade. Both were obtained from Serva, Heidelberg, G.F.R. The cation exchanger was preconditioned by washing with 1 *N* HCl, then with water, until the eluate was free from chloride, then with methanol, and again with water. The anion exchanger was treated with 1 *N* NaOH, then with water, until the eluate was neutral, then with methanol and again with water.

Chromatographic system

We used a Model 931 HSRI liquid chromatograph from Optilab, Stockholm,

Sweden. The system includes a thermostated column box, a Thermostir 941 to accommodate and thermostat the solvent reservoir, a refractometer Multiref 902 equipped with a measuring cell of 10 mm path length, a constant flow pump, Model 600/200, from Gynkotek, Munich, G.F.R., and a pulse dampener for Touzart-Matignon, Vitry-sur-Seine, France.

The detector works according to the interferometric principle [3]. Its sensitivity is about 100 times higher than that of conventional differential refractometers. However, utilization of this sensitivity requires a highly stable temperature and eluent flow-rate in the chromatographic system, since the smallest changes in these parameters become apparent as signal noise. To provide a temperature constancy of 0.01°C during each chromatographic run we employed a thermostat device consisting of two water-baths connected in series. The first bath was set at 27°C and countercooled with tap water. It tempered a second bath which thermostated the measuring cell of the detector, the column box and the solvent reservoir. This arrangement served to keep the heating pulses generated in the first water-bath from being passed on to the thermostated circuit.

Enzymatic procedure and subsequent sample treatment

The steps of sample preparation are listed in Fig. 1. The enzymatic assays were carried out in phosphate buffer (pH 7.0) at 30°C, with maltoheptaose as substrate. After certain time intervals aliquots were removed from the assay mixture and diluted in a slurry of the mixed-bed ion exchanger, thereby stopping the enzymatic reaction. The ion exchanger was poured into a column and washed with 300 ml of water. This amount was sufficient for quantitative elution of the oligosaccharides. The eluates were partly evaporated and lyophilized. The pre-column step was necessary to remove the protein and most of the buffer ions which would have interfered with the HPLC separation of the oligosaccharides.

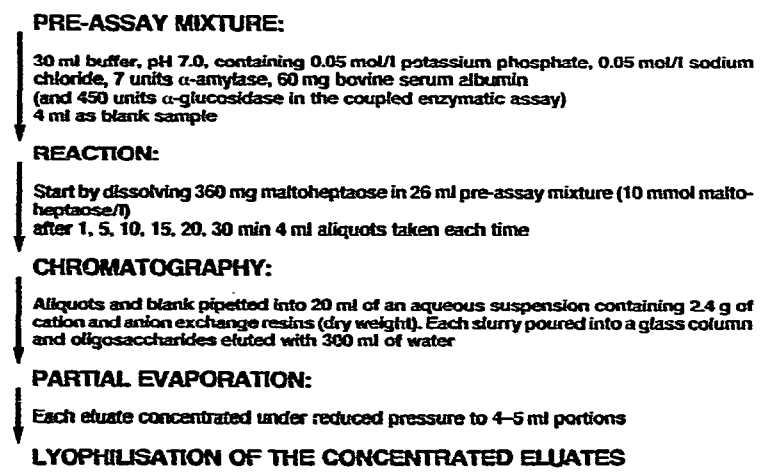


Fig. 1. Conditions of the kinetic assay and sample treatment.

Chromatographic analysis

Each lyophilized sample was dissolved in acetonitrile—water (1:1) at a concentration of 10 mg/ml for the quantification of the lower oligosacharides and at a concentration of 2 mg/ml for quantification of maltoheptaose. The lyophilized blank was dissolved in 5 ml of sample solvent. Twenty microlitres of each sample solution (containing 0.2 and 0.04 mg of oligosaccharides, respectively) were injected into the chromatographic system. For automatic sample injection we used the LC 7713 sampler from Kipp Analytica, Solingen, G.F.R.

The oligosaccharides were separated by partition chromatography on the cation-exchange column Nucleosil 10 SA (250 × 4 mm) from Macherey, Nagel & Co., Düren, G.F.R., with an eluent containing 72.5% acetonitrile in water, and at a flow-rate of 0.7 ml/min. The column back pressure was 5.1 MPa.

The column effluent was monitored by the Multiref differential refractometer and then routed back to the solvent reservoir. The Multiref was set at attenuation 20, i.e. full recorder scale was equivalent to 2×10^{-6} RI units.

A Hewlett-Packard (Böblingen, G.F.R.) automatic integrator, Type 3385A, recorded peak areas electronically from the detector signal.

Calculation

The areas of glucose to maltohexaose were taken from the chromatograms of the concentrated sample solutions. The maltoheptaose areas were obtained from the runs of the diluted sample solutions, since the amount of maltoheptaose contained in the concentrated sample solutions was above the linear detection range.

The factors relating peak area to weight are the same for all oligosaccharides. Therefore the weight per cent value of each oligosaccharide was equated to its area per cent value (100% being equal to the area sum of all oligosaccharide peaks in the sample). Dividing by the molecular weight and norming again to 100% gave the mol per cent values.

With α-amylase alone, four parallel kinetic assays were carried out; with the α-amylase—α-glucosidase system eight parallel assays were prepared and treated statistically.

RESULTS AND DISCUSSION

To investigate the action pattern of human pancreatic α-amylase we had to overcome two problems:

(1) To remove the so-called "buffer peak" in the chromatogram. This peak is produced by assay components that are not retained on the mixed-bed column. In conventional oligosaccharide separation by high-performance liquid chromatography (HPLC) on NH_2 columns with an acetonitrile—water eluent the buffer peak is eluted between maltose and maltotriose and interferes with the quantification of these two compounds.

(2) To achieve the sensitivity of detection necessary to quantify 0.2 mol % maltoheptaose fragments formed by the enzymatic hydrolysis, in the presence of a large excess of undegraded maltoheptaose. In preliminary experiments we had found that under assay conditions only about 20% of the substrate is

cleaved during an incubation period of 30 min. This surplus is necessary in order to keep a state of substrate saturation during the enzymatic assay.

First we tried to solve both problems by derivatizing the oligosaccharides with UV-absorbing tags. We worked with dansylhydrazine [4], dinitrophenylhydrazine [5], phenylhydrazine [6], and with 4-nitrobenzoyl chloride [7]. All those attempts to get a complete reaction and pure derivatives failed, since the higher oligosaccharides reacted sluggishly and isomers and side-products were formed.

Because we could not improve the procedure for the purification of the samples, we concentrated our efforts on developing an HPLC system in which the buffer peak was shifted to another part of the chromatogram where no interference with the oligosaccharide peaks did occur. Of the many methods we tested, chromatography on the Nucleosil SA cation-exchange column was the only one to yield the desired results. All oligosaccharides were separated in this system and the buffer peak was eluted together with the "solvent peak" at the beginning of the chromatogram.

The required sensitivity of detection was achieved by the Multiref differential refractometer. It had a quantitative detection limit of about 40 ng for glucose at a signal-to-noise ratio of 2:1, i.e. 0.02% when 0.2 mg of sample was injected. This enabled us to perform the amylase assay and to establish the action pattern under conditions close to the assay system employed in clinical chemistry. The amylase activity we used equalled about fifteen times the physiological α-amylase activity in human serum (7 units/30 ml according to the amylochrome test). To establish the action pattern the amylase concentration could not be lowered further, since during the elaborate sample preparation some spontaneous hydrolysis of maltoheptaose took place which interfered with the enzymatic hydrolysis.

Action pattern of α-amylase (without α-glucosidase)

Chromatograms C—H in Fig. 2 show the progress of enzymatic maltoheptaose hydrolysis in the amylase assay mixture after the indicated periods of incubation. Fig. 2A shows the chromatogram of the maltoheptaose lot which we used for our investigations. It is seen that it contains minor amounts of maltohexaose, maltopentaose, maltotetraose and of an unidentified impurity ("compound X"). Fig. 2B shows the pattern of the blank sample taken from the assay mixture before the start by addition of maltoheptaose. Since the eluate was routed back to the solvent reservoir, the solvent became enriched in maltoheptaose. The lack of maltoheptaose in the blank sample caused a negative peak, but because the peak area of maltoheptaose in the assay sample runs was more than 100 times higher, the error caused in the quantitative determination could be neglected.

The quantitative analysis of the chromatograms is presented in Table I. The concentrations of the oligosaccharides were calculated in mol per cent to display the relative interdependencies between the production of the respective oligosaccharides (100 mol % equals the total oligosaccharide content in each sample). As shown in Fig. 2E—H, maltopentaose and "compound X" were not fully separated in those samples that were incubated for 10 min or more. In

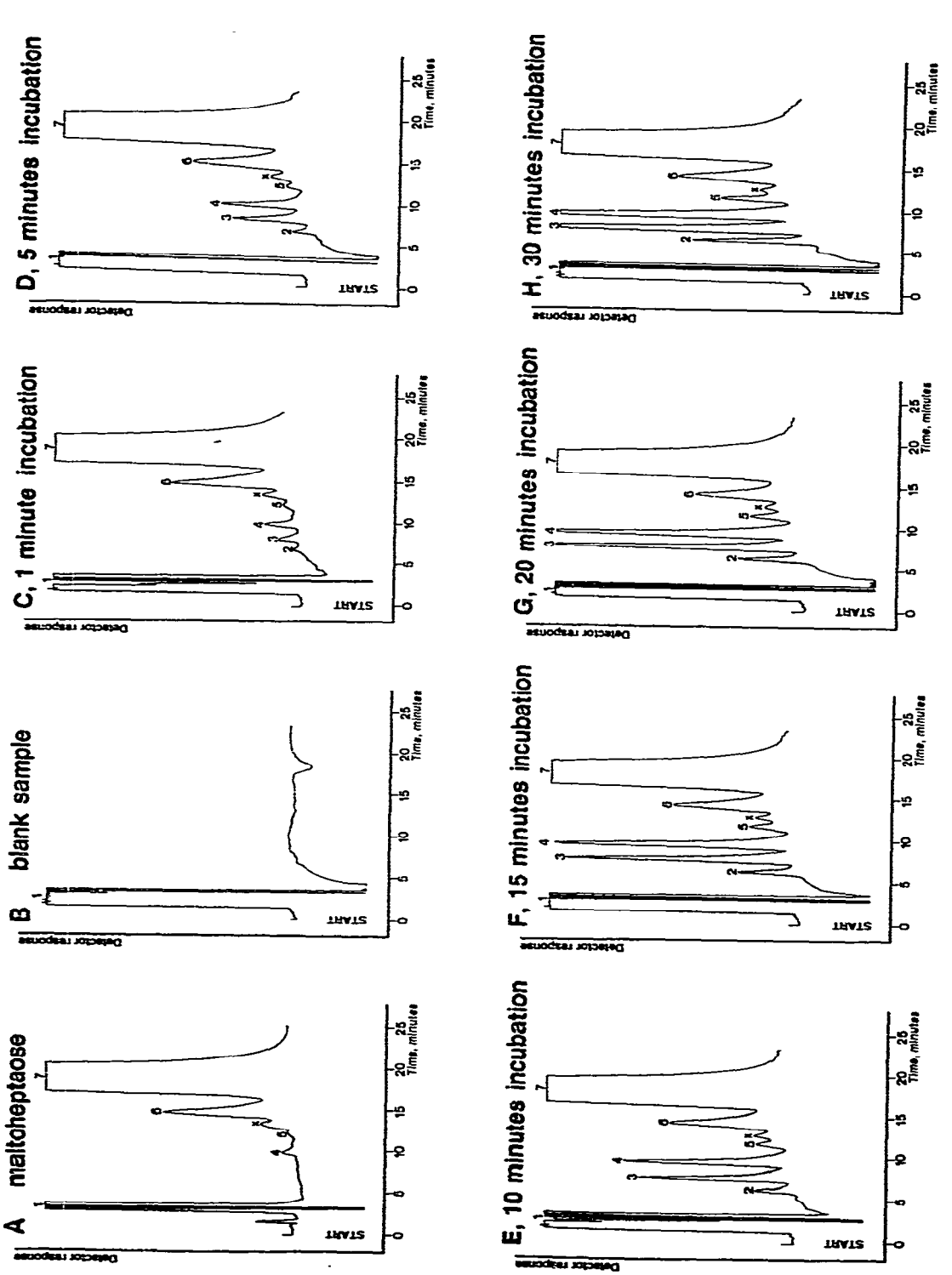

A maltoheptaose
B blank sample
C, 1 minute incubation
D, 5 minutes incubation
E, 10 minutes incubation
F, 15 minutes incubation
G, 20 minutes incubation
H, 30 minutes incubation
Detector response
START
Time, minutes
0
5
10
15
20
25

TABLE I

HYDROLYSIS PRODUCTS FROM MALTOHEPTAOSE BY HUMAN PANCREATIC α-AMYLASE

Incubation time (min)	Amount of hydrolysis compounds* in assay mixture (mol %)						
	G_2	G_3	G_4	G_5	Compound X	G_6	G_7
1	0.25	0.66	0.91	0.11	0.31	2.86	94.90
5	0.77	2.48	2.40	0.17	0.29	2.56	91.32
10	1.32	4.58	4.38	0.46		2.45	86.52
15	2.03	6.67	6.28	0.66		2.39	81.66
20	2.61	8.74	8.17	0.75		2.25	77.17
30	4.10	12.34	11.34	1.38		2.56	68.00

*G_2 = maltose; G_3 = maltotriose; G_4 = maltotetraose; G_5 = maltopentaose; G_6 = maltohexaose; G_7 = maltoheptaose.

this case the sum of their areas was determined since "compound X" does not seem to be a substrate of α-amylase and its concentration remains constant throughout the incubation period.

In Fig. 3 these data are presented graphically. The diagram shows that during the 30 min of incubation maltoheptaose decreases linearly to 68 mol % (equal to 80 weight per cent). As main products of the enzymatic action maltotriose and maltotetraose are produced in about equimolar amounts. Both compounds increase linearly up to 20 min. Then their production rates diminish slightly. The concentrations of maltohexaose and "compound X" which are contaminants of maltoheptaose do not change significantly. The amount of glucose remains below 0.15 mol % after 30 min of incubation. So we can safely assume that glucose is not a product of α-amylase action itself. These results can be explained by the reaction scheme in Fig. 4. The main reaction pathway is the cleavage of maltoheptaose into maltotetraose and maltotriose.

Only 10% of maltoheptaose, degraded enzymatically, is hydrolyzed to maltopentaose and maltose. A small amount of maltose is derived from the enzymatic hydrolysis of maltotetraose, since from our experiments it seems very likely that maltopentaose and maltotetraose themselves can act as substrates for α-amylase, competing with maltoheptaose. This explains the steeper increase in maltose compared to maltopentaose in Fig. 3 and the slower increase in maltotetraose compared with maltotriose. The values that are underlined in the reaction scheme were directly calculated from the chromatographic runs, i.e. from the concentration changes during incubation. The values in

Fig. 2. Chromatograms of the reaction products of maltoheptaose after various periods of incubation with α-amylase. Peaks: 1 = solvent front; 2 = maltose; 3 = maltotriose; 4 = maltotetraose; 5 = maltopentaose; x = compound X; 6 = maltohexaose; 7 = maltoheptaose. (A) Pure maltoheptaose as used for the assay. (B) Blank sample before the addition of substrate. (C—H) Chromatograms after 1, 5, 10, 15, 20, and 30 min, respectively, of incubation. Incubation conditions: 7 units of α-amylase in 30 ml of 0.05 mol phosphate buffer (pH 7.0) at 30°C. Chromatographic conditions: column, 10 μm Nucleosil SA (250 × 4 mm); solvent, acetonitrile—water (72.5 : 27.5); flow-rate, 0.7 ml/min; temperature, 27°C; detection, differential refractometer Multiref 902, full scale = 2×10^{-6} RI units.

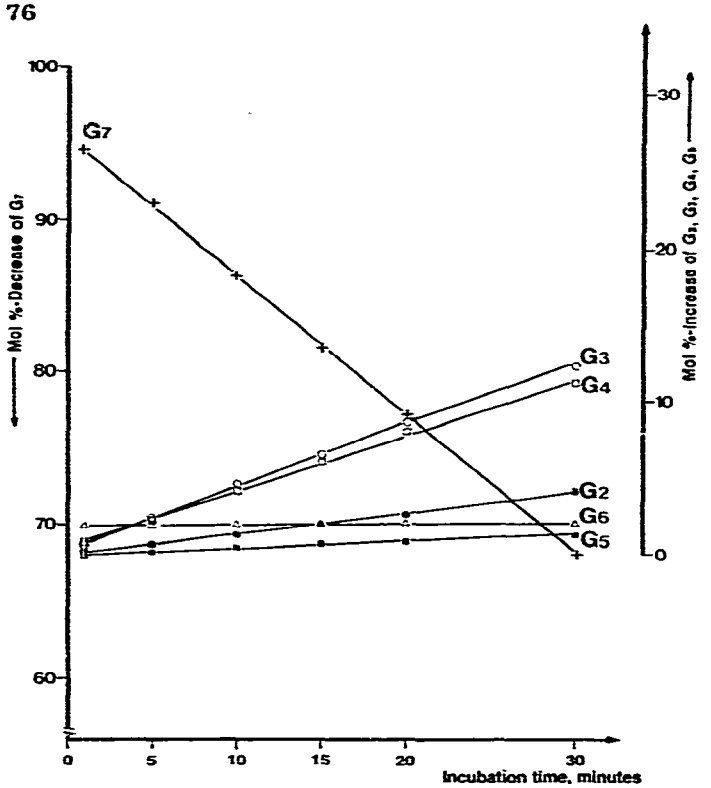


Fig. 3. Kinetics of α-amylase with maltoheptaose as substrate. For abbreviations see Table I.

parentheses relate to unobservable reaction steps and are derived from the underlined values. Since we measured that 0.87 mol of maltotriose (stable end-product) originated from 1 mol of maltoheptaose, also 0.87 mol of maltotetraose must have originated primarily in the assay. From the fact that only 0.79 mol was found it can be concluded that the missing 0.08 mol was hydrolyzed to maltose. The second source of maltose is the side-reaction of maltoheptaose (cleavage to maltopentaose and maltose).

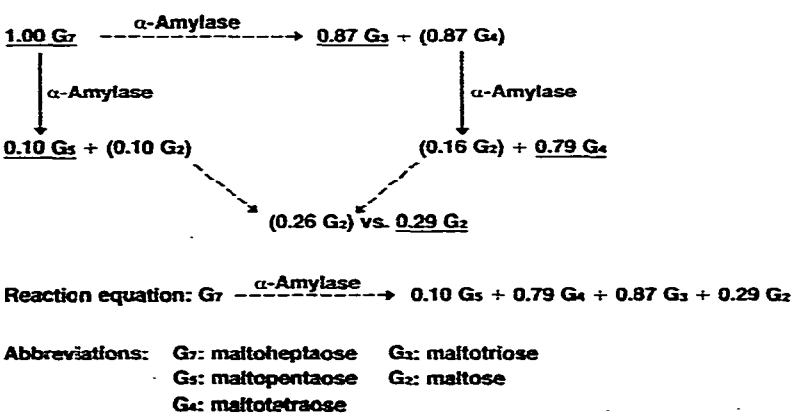


Fig. 4. Reaction scheme for the degradation of maltoheptaose by the action of α-amylase.

The measurable liberation of 0.1 mol of maltopentaose from 1 mol of maltoheptaose must be accompanied by the occurrence of an equimolar amount of maltose. Altogether 0.29 mol of maltose should result from 1 mol of reacted maltoheptaose. This inferred value is in good agreement with the experimental value of 0.26 mol. To show that the α-amylase action pattern does not change with lower enzyme concentrations we performed the kinetic assay with one-tenth of the enzyme activity used for the previous experiments. Fig. 5 presents the resulting patterns in analogy to Fig. 2. It is obvious that there is no qualitative change in the appearance of the patterns although the chromatographic runs cannot be evaluated quantitatively.

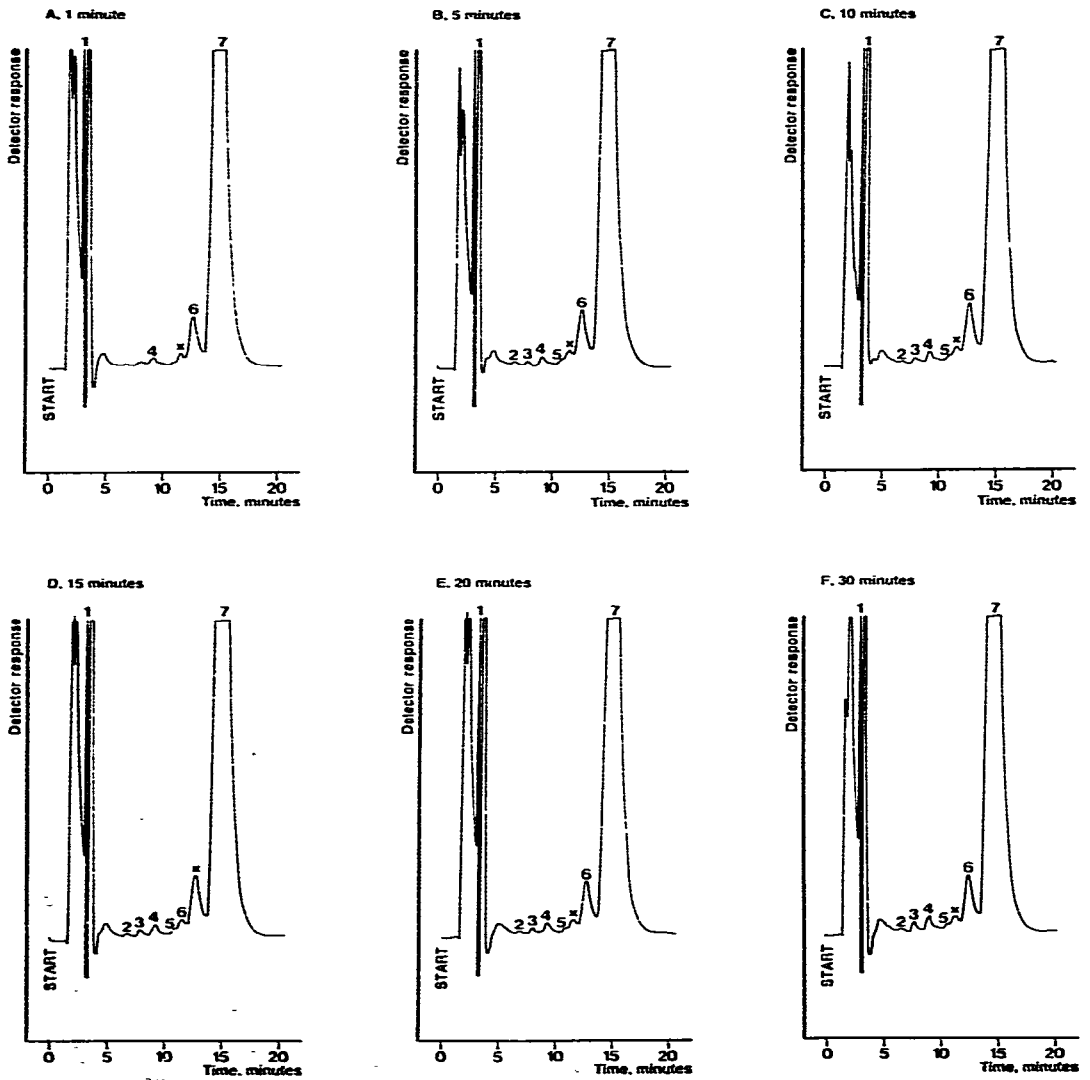


Fig. 5. Chromatograms of the reaction products from maltoheptaose by human pancreatic α-amylase at physiological activity after various periods of incubation. Peak identification and chromatographic conditions as in Fig. 2.

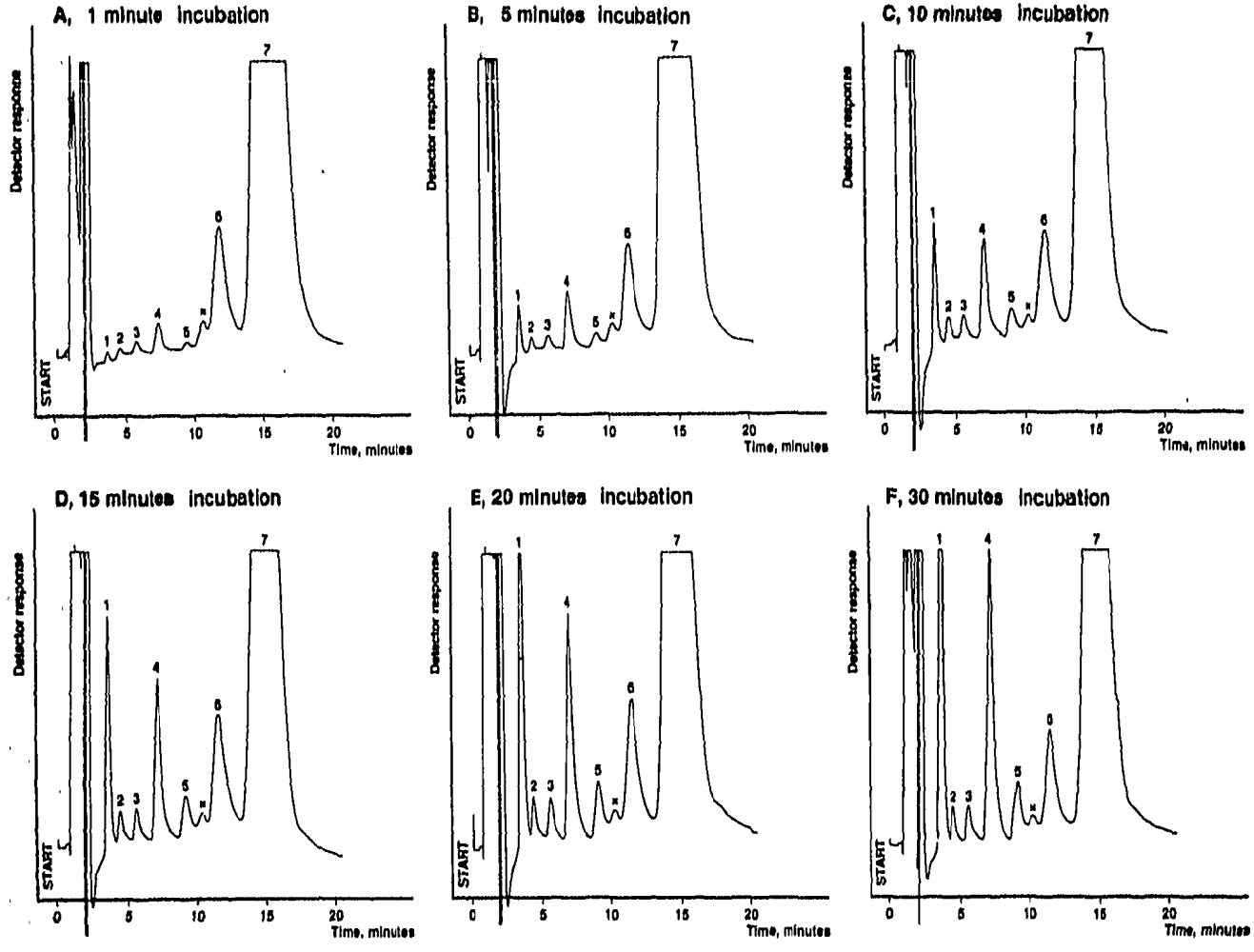


Fig. 6. Chromatograms of the action patterns of maltoheptaose after the indicated periods of incubation with α-amylase and α-glucosidase. Peak identification and conditions as in Fig. 2, except that here peak 1 is the glucose peak.

TABLE II

HYDROLYSIS PRODUCTS FROM MALTOHEPTAOSE BY THE COUPLED ENZYME SYSTEM α-AMYLASE–α-GLUCOSIDASE

Incubation time (min)	Hydrolysis compounds in the assay mixture							
	G_1	G_2	G_3	G_4	G_5	Compound X	G_6	G_7
	Weight per cent (± S.D.)							
1	0.16 (± 0.10)	0.28 (± 0.15)	0.40 (± 0.16)	0.77 (± 0.24)	0.55 (± 0.35)	0.37 (± 0.06)	4.13 (± 0.80)	93.33 (± 1.89)
5	0.83 (± 0.14)	0.42 (± 0.15)	0.54 (± 0.18)	1.62 (± 0.24)	0.79 (± 0.36)	0.37 (± 0.05)	4.34 (± 0.89)	91.12 (± 1.84)
10	1.76 (± 0.23)	0.43 (± 0.13)	0.55 (± 0.15)	2.52 (± 0.28)	0.88 (± 0.28)	0.36 (± 0.07)	4.11 (± 0.75)	89.38 (± 1.61)
15	3.03 (± 0.34)	0.53 (± 0.15)	0.71 (± 0.16)	3.83 (± 0.38)	1.22 (± 0.29)	0.36 (± 0.05)	4.53 (± 0.80)	85.80 (± 1.66)
20	4.09 (± 0.44)	0.56 (± 0.10)	0.74 (± 0.13)	4.84 (± 0.40)	1.34 (± 0.19)	0.36 (± 0.05)	4.34 (± 0.58)	83.73 (± 1.31)
30	6.27 (± 0.75)	0.55 (± 0.16)	0.74 (± 0.16)	6.70 (± 0.75)	1.55 (± 0.26)	0.34 (± 0.06)	3.83 (± 0.66)	80.02 (± 2.36)
	Mol per cent							
1	1.0	0.9	0.9	1.3	1.1	–	4.7	90.4
5	4.9	1.3	1.2	2.6	1.1	–	4.7	84.3
10	9.9	1.3	1.1	3.8	1.1	–	4.2	78.5
15	15.8	1.4	1.3	5.5	1.4	–	4.3	70.2
20	20.3	1.4	1.3	6.5	1.4	–	3.9	65.0
30	28.2	1.3	1.2	8.2	1.5	–	3.2	56.4

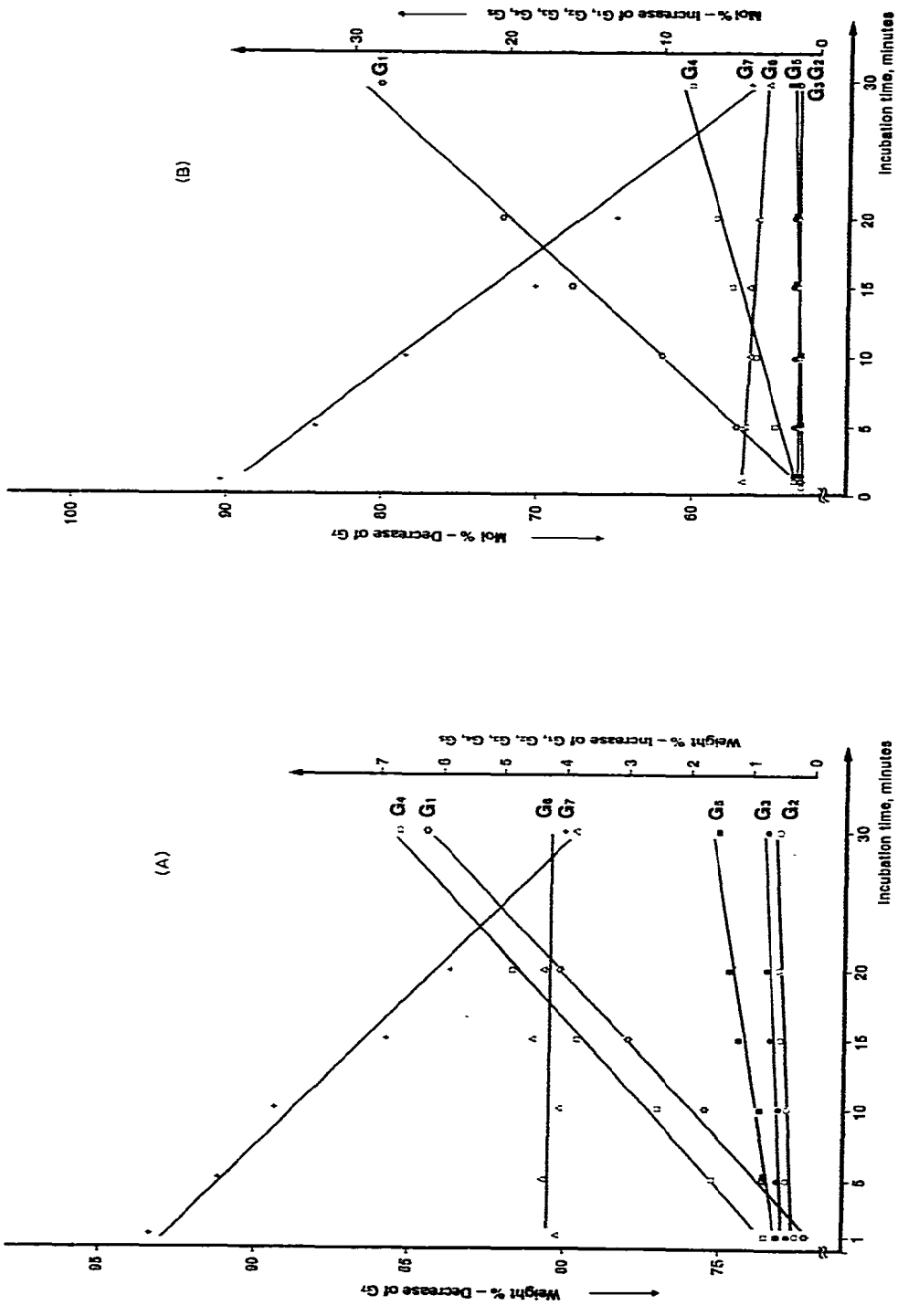


Fig. 7. Kinetics of the coupled enzyme system α-amylase—α-glucosidase with maltoheptaose in weight per cent (A) and in mol per cent (B). For abbreviations see Table I.

Action pattern of α-amylase—α-glucosidase

The second series of incubation assays contained in addition to α-amylase also α-glucosidase with the same enzymatic activity as used in the photometric test for determination of α-amylase activity in body fluids. Enzymatic procedure, sample pre-treatment and chromatographic conditions were the same as before.

Fig. 6 shows the chromatograms of assay samples after consecutive periods of incubation. Table II and Fig. 7A and B give the quantitative evaluation of the chromatographic runs from eight independent assays by weight and mol per cent.

In the combined assay glucose and maltotetraose increase rapidly and linearly. The maltopentaose concentration also increases significantly during the incubation period, whereas maltose and maltotriose reach only small steady state concentrations.

The large standard deviations (10—25%) displayed by the weight per cent mean values of the hydrolysis products can be explained to a large extent by differences in the α-amylase activities among the eight parallel assays (amylase is the rate-limiting enzyme). Whereas the extent of maltoheptaose hydrolysis, i.e. the weight percentages of the hydrolysis products, depends on α-amylase activity in the test, the relative distribution of the hydrolysis products should be independent. Therefore, for example, the quotient weight % (glucose)/ weight % (maltotetraose) should be independent of amylase activity and should have smaller standard deviations than the weight per cent values (see Table III). Since the statistical error is greater for smaller peaks, the coefficient of variation (C.V.) of this quotient becomes lower with increasing time of incubation, and is around 5% for incubation periods of 15 min and more. The rate equation was derived from the net change of the weight per cent values for each oligomer during the whole incubation period. From these differences the rate coefficients for each oligosaccharide were calculated (Table IV).

As the stoichiometric equation is independent of the amylase activity in the test, the statistical error of the coefficients should be around 5%. From the stoichiometric coefficients the reaction scheme in Fig. 8 can be constructed. The coefficients of the end-products are calculated from the chromatograms and are underlined. These values and those obtained from the action pattern of amylase alone (Fig. 4) were used to derive the coefficients of the reaction inter-

TABLE III

STATISTICAL ERROR INFLUENCING THE RATE COEFFICIENTS

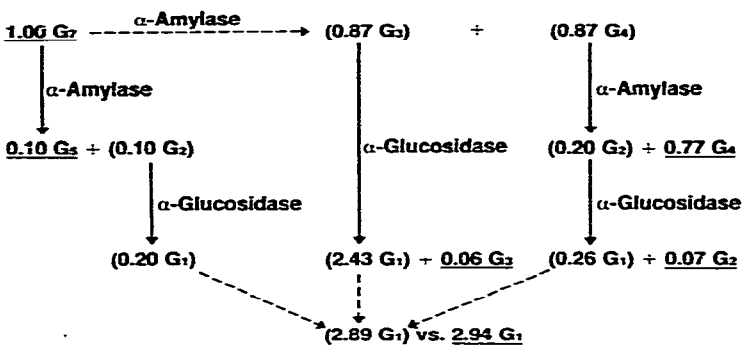
Period of incubation (min)	C.V. (%) of the quotient weight % (glucose)/weight % (maltotetraose)
1	34
5	12
10	8
15	6
20	4
30	5

TABLE IV

RATE COEFFICIENTS FOR HYDROLYSIS OF THE MALTOHEPTAOSE

Oligomer	Concentration changes during 29 min of incubation	Stoichiometric coefficients
Glucose	+ 6.11	+2.94
Maltose	+ 0.27	+0.07
Maltotriose	+ 0.34	+0.06
Maltotetraose	+ 5.94	+0.77
Maltopentaose	+ 1.00	+0.10
Maltohexaose	+ 0.30	+0.03
Maltoheptaose	−13.31	−1.00

mediates which could not be measured directly. In the scheme they are placed in parentheses. Consideration of both reaction schemes leads to the following interpretation. Again maltotriose and maltotetraose are the most important primary reaction products of the amylase-dependent hydrolysis of maltoheptaose. Whereas maltotriose is cleaved by the auxiliary enzyme α-glucosidase almost quantitatively into three glucose molecules, maltotetraose remains largely stable in the assay. Only 11% is cleaved by amylase into two maltose units which are further hydrolyzed to glucose by α-glucosidase. The third pathway that provides glucose runs via the side-reaction of α-amylase, namely the cleavage of maltoheptaose into maltose and maltopentaose. A test for the consistency of the data is the coefficient of glucose which can be obtained either directly from the chromatograms or calculated from the data pool. Both values differ by 2%, showing that the data pool is self-consistent. The stoichiometric coefficients of comparable reaction steps in both schemes are in agreement. The enzymatic indicator system has therefore no impact on the



Rate equation: $G_7 \xrightarrow[\alpha\text{-Glucosidase}]{\alpha\text{-Amylase}} 0.10\ G_5 + 0.77\ G_4 + 0.06\ G_3 + 0.07\ G_2 + 2.94\ G_1$

Abbreviations:
G_7: maltoheptaose
G_5: maltopentaose
G_4: maltotetraose
G_3: maltotriose
G_2: maltose
G_1: glucose

Fig. 8. Reaction scheme for the degradation of maltoheptaose by the combined action of α-amylase and α-glucosidase.

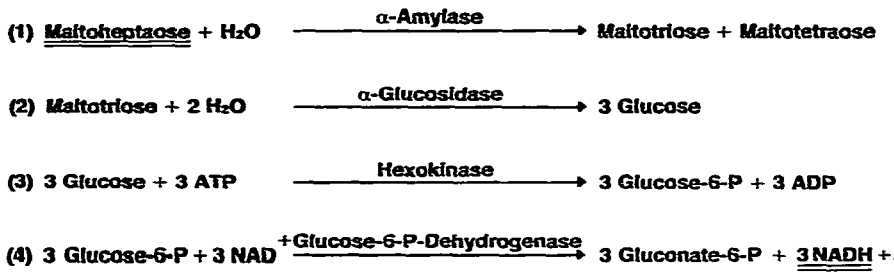


Fig. 9. Assay principle (simplified).

primary amylase-dependent reaction steps, even if there are several common intermediates. Secondly, the results show that the α-glucosidase content in the assay is high enough to cleave nearly all maltose and maltotriose resulting from amylase action on maltoheptaose. Only steady-state concentrations of these two intermediates remain in the assay, reflecting the K_M values of α-glucosidase.

Knowing the mechanism and the stoichiometric coefficients of the enzymatic cleavage of maltoheptaose we could set up the reaction equations pertaining to the α-amylase test (Fig. 9). Each cleavage of maltoheptaose by α-amylase effects the occurrence of three glucose molecules which lead to the formation of three molecules of NADH, i.e. the indicator reaction amplifies the primary reaction by a factor of 3, a feature which adds to the sensitivity of the test.

CONCLUSION

This work shows that HPLC can be used in clinical chemistry to investigate the mechanisms of multiple-step enzymatic reactions. By applying chromatography to the reaction mixtures the concentrations of various reaction intermediates and end-products can be obtained, i.e. more parameters than a photometric test can deliver. In this way complex reaction cascades can be split up into a set of individual steps making clear the stoichiometry of the overall reaction.

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