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Action Pattern of Serum Amylase Using *p*-Nitrophenyl-maltoheptaoside as Substrate

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Summary: Selected serum samples with different isoamylase patterns, as revealed by agarose gel electrophoresis, were analysed by the Phadebas amylase test, and by using *p*-nitrophenyl-maltoheptaoside as the substrate. The products with the latter substrate were examined by high performance liquid chromatography (HPLC). For some samples, different catalytic activities were registered by the two amylase methods. In these cases, electrophoresis showed a double pancreatic band associated with increased pancreatic amylase catalytic activity, or an abnormal, broad salivary band associated with increased salivary enzyme catalytic activity. The stoichiometric factors for most of these samples, calculated from the amount of *p*-nitrophenol detected after HPLC, differed from the mean values.

Wirkungs-Muster von Amylase im Serum bei Anwendung von p-Nitrophenyl-maltoheptaosid als Substrat

Zusammenfassung: Ausgewählte Proben mit verschiedenen Isoamylasemustern, untersucht mit der Agarose-Elektrophorese, wurden sowohl mit dem Phadebas-Amylase-Test als auch mit *p*-Nitrophenyl-maltoheptaosid als Substrat untersucht. Die mit dem letzteren Substrat erhaltenen Produkte wurden mit der HPLC-Methode geprüft. Wir fanden bei einigen Proben Abweichungen in den Amylaseaktivitäten zwischen den beiden Amylase-Methoden. In diesen Fällen war entweder ein doppeltes Band des pankreatischen Isoenzyms mit erhöhter Pankreasamylaseaktivität vorhanden oder ein abnorm breites Speichelamylaseband wurde bei erhöhter Speichelamylaseaktivität gesehen. Die stöchiometrischen Faktoren für diese Proben, berechnet nach der Menge *p*-Nitrophenol, die bei der Analyse mit HPLC gefunden wurde, unterschieden sich von den Mittelwerten der meisten Proben.

Introduction

Several authors have studied the reaction mechanism of purified human pancreatic or salivary amylase (1,4-α-*D*-glucan glucanohydrolase, EC 3.2.1.1) using *p*-nitrophenyl-maltoheptaoside as substrate (1→5). Using this substrate and amylase in the absence and presence of α-glucosidase (α-*D*-glucoside glucohydrolase, EC 3.2.1.20), *Hägele* et al. quantitated the amount of *p*-nitrophenol produced and proposed that a stoichiometric factor of 3 (calculated as the reciprocal of *p*-nitrophenol) should be used in the calculation of amylase catalytic activity (5). We have examined the reaction mechanism of amylase with *p*-nitrophenyl-maltoheptaoside using authentic patient samples with different isoamylase patterns.

Materials and Methods

All the chemicals and column material required for high performance liquid chromatography (HPLC) were purchased from Merck (Darmstadt, West Germany). Purified human serum albumin was obtained from Behringwerke (Calbiochem-Behring Corp. La Jolla CA 92037). *p*-Nitrophenyl-maltoheptaoside and α-glucosidase were purchased from Boehringer Mannheim (Stockholm, Sweden). *p*-Nitrophenyl derivates of maltotriose, maltotetraose, maltopentaose, and purified human pancreatic and salivary amylases were gifts from Boehringer Mannheim (Tutzing, West Germany). Phadebas amylase test was purchased from Pharmacia Diagnostica (Uppsala, Sweden).

Serum samples were selected on the basis of the isoamylase patterns determined by the electrophoretic method of *Skude* (6). Total amylase catalytic activities were determined with the Phadebas amylase method and *p*-nitrophenyl-maltoheptaoside colorimetric method (Boehringer Mannheim) adapted to Greiner Selective Analyzer (GSA II, Greiner Electronics AG, Langenthal, Switzerland) (7). The GSA II analyzer was calibrated with a serum pool

whose amylase activity had been determined by the Phadebas method. This procedure simplified method comparisons.

For HPLC, 25 µl sample were incubated at 37 °C with 500 µl of 5 mmol/l *p*-nitrophenyl-maltoheptaoside in buffer (50 mmol/l NaH_2PO_4 and 50 mmol/l NaCl, pH 7.1). When α-glucosidase was included in the incubations, a catalytic concentration of approximately 30 kU/l in the reaction mixture was used. The amylase reaction was stopped by adding 500 µl acetonitrile, 75 µl of 1 mol/l H_3PO_4 and 1 ml of a solution containing equal volumes of acetonitrile and KH_2PO_4, adjusted to pH 7.1. The HPLC system was as described by *Hägele* et al. (5) except that no guard column was used. Peak areas were integrated electronically. Some of the samples were also incubated with *p*-nitrophenyl-maltotetraoside and -maltotrioside.

The effect of salivary amylase on the reaction mechanism was tested by adding increasing quantities of saliva to a constant pancreatic amylase catalytic activity. All the samples were diluted once more to the same final amylase catalytic activity.

Least square regression analyses and *Wilcoxon* test for pair differences were used to compare amylase activities obtained by the Phadebas and the *p*-nitrophenyl-maltoheptaoside colorimetric test (Boehringer Mannheim). The data from HPLC studies (in the absence of α-glucosidase) were used to study the relationships between the reaction products *p*-nitrophenyl-maltoside, -maltotrioside and -maltotetraoside. The stoichiometric factors were calculated for the samples incubated with *p*-nitrophenyl-maltoheptaoside in the presence of α-glucosidase. Mathematically, this is the reciprocal of the amount of *p*-nitrophenol detected after HPLC.

Results and Discussion

All the analyses were performed in duplicate. The comparison of amylase activities obtained with the Phadebas method and with the *p*-nitrophenyl-maltoheptaoside colorimetric method are shown in table 1. A good correlation between the two methods was obtained for samples with normal isoamylase pattern. In the case of samples with pancreatic amylase dominance, the catalytic activities with the *p*-nitrophenyl-maltoheptaoside method were slightly lower (by approximately 5%) for most samples, with the exception of four samples where the catalytic activities were between 15 and 30% lower than corresponding catalytic activities with the Phadebas method. Double pancreatic band (fig. 1) were seen in three of these samples. With increased salivary amylase, the catalytic activities with the *p*-nitrophenyl-maltoheptaoside colorimetric method were generally slightly higher (by approximately 4%), with the exception of five samples where the activities with the *p*-nitrophenyl-maltoheptaoside colorimetric method were between 50 and 70% higher than the corresponding catalytic activities with the Phadebas method. These samples had abnormal, broad salivary bands (fig. 1). The regression equation for all the samples in table 1 was $y = 0.95\ x + 0.52$ µkat/l ($r = 0.983$). This equation reverted to $y = 0.99\ x - 0.08$ µkat/l ($n = 0.996$) when the samples with the large discrepancies mentioned above were excluded from the statistical analysis.

Tab. 1. Comparison of amylase catalytic activities determined by the Phadebas and by the *p*-nitrophenyl-maltoheptaoside colorimetric methods.

	Normal P and S	P > 0.65[a])	S > 0.55[b])
n	25	28	32
$\bar{x}$, µkat/l[c])	4.78	16.44	7.81
$\bar{y}$, µkat/l[c])	4.80	15.12	8.96
y-intercept	0.14	−0.36	0.16
Slope	0.98	0.94	1.13
Correlation coefficient r	0.996	0.994	0.967

[a]) P denotes pancreatic amylase.
[b]) S denotes salivary amylase.
$\bar{x}$ is the reference method (Phadebas) and
$\bar{y}$ is the *p*-nitrophenyl-maltoheptaoside colorimetric method.
[c]) The pair difference tests were not significant for normal P and S, but significant for P > 0.65 and S > 0.55 ($p < 0.01$).
1 µkat is the enzyme catalytic activity which converts 1 µmol of substrate per second under defined conditions.

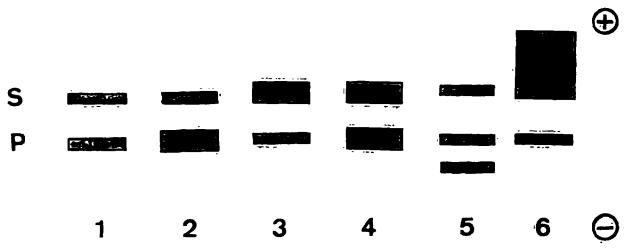

Fig. 1. Electrophoretogram of amylase isoenzymes. The electrophoresis and detection of the amylase isoenzymes (P = pancreatic; S = salivary) isoenzymes was performed according to *Skude* (6).
1) Normal sample
2) Increased P
3) Increased S
4) Increased P and S
5) Double P band
6) Broad S band

For HPLC studies, the retention times of the *p*-nitrophenylated products obtained after the reaction of amylase were standardized with authentic *p*-nitrophenyl-maltotrioside, -maltotetraoside, -maltopentaoside and -maltoheptaoside. A deviation of ± 1 min was accepted. The column was repacked when deviations in retention times were greater than the tolerance levels stated. The coefficient of variation for peak integration was not allowed to exceed 5% for duplicate analyses. We performed preliminary experiments using *p*-nitrophenyl-maltoheptaoside with different amylase catalytic activities, different incubation times and dilution media such as NaCl and albumin. As a result of these studies, the substrate was incubated at 37 °C for 30 minutes using 5 µkat/l amylase catalytic concentration (the samples were diluted, if necessary, with 50 g/l albumin).

We found that between 4 and 7% of *p*-nitrophenyl-maltoheptaoside was hydrolysed by the samples with either normal isoamylase distribution or with pancreatic amylase dominance ($P > 0.65$). In the case of samples with increased salivary amylase ($S > 0.55$), between 4 and 12% of *p*-nitrophenyl-maltoheptaoside was hydrolysed. The samples with the broad salivary band were responsible for more hydrolysis. Whereas *p*-nitrophenyl-maltoside, -maltotrioside and -maltotetraoside were the main reaction products, *p*-nitrophenyl-glucoside was found in 3 of the samples with the abnormal salivary bands. We found that the ratio of *p*-nitrophenyl-maltotetraoside to -maltotrioside depended on the isoamylase pattern. A mean ratio of 1.6 was obtained for purified pancreatic amylase and for the samples where $P > 0.65$. A mean ratio of 2.0 was found for saliva, purified salivary amylase and samples with $S > 0.55$. However, in the case of 4 samples with a broad salivary amylase band, this ratio varied between 1.3 and 1.4. In the fifth sample belonging to this category, the ratio was 1.8. We also observed that more *p*-nitrophenyl-maltoside was produced in the samples with salivary amylase dominance using either *p*-nitrophenyl-maltoheptaoside, -maltotetraoside or -maltotrioside as subtrates.

Adding increasing catalytic activities of salivary amylase to a constant pancreatic component resulted in an increase of the *p*-nitrophenyl-maltotetraoside to -maltotrioside ratios (fig. 2), and in more production of *p*-nitrophenyl-maltoside, despite the fact that the final amylase catalytic activity concentration in each sample was kept constant at 5 μkat/l.

For the hydrolysis of *p*-nitrophenyl-maltoheptaoside, the main pathway appears to be the production of *p*-nitrophenyl-maltotrioside and -maltotetraoside. It appears feasible that more of *p*-nitrophenyl-maltotrioside is produced by pancreatic amylase, and more of *p*-nitrophenyl-maltotetraoside is produced by salivary amylase. Two alternative pathways may be responsible for the production of *p*-nitrophenyl-maltoside. First, a primary cleavage from *p*-nitrophenyl-maltoheptaoside. Second, a further cleavage of either *p*-nitrophenyl-maltotrioside or -maltotetraoside, and any *p*-nitrophenyl-maltopentaoside produced. When we used *p*-nitrophenyl-maltotrioside as the substrate, we found *p*-nitrophenylglucoside and -maltoside as the products. Since *p*-nitrophenyl-glucoside was found in only three of the samples, we concluded that *p*-nitrophenyl-maltotrioside is not likely to be subjected to further degradation in the presence of excess *p*-nitrophenyl-maltopheptaoside. However, it is evident from the HPLC studies that *p*-nitrophenyl-maltotetraoside is degraded further, at least by samples with salivary amylase dominance.

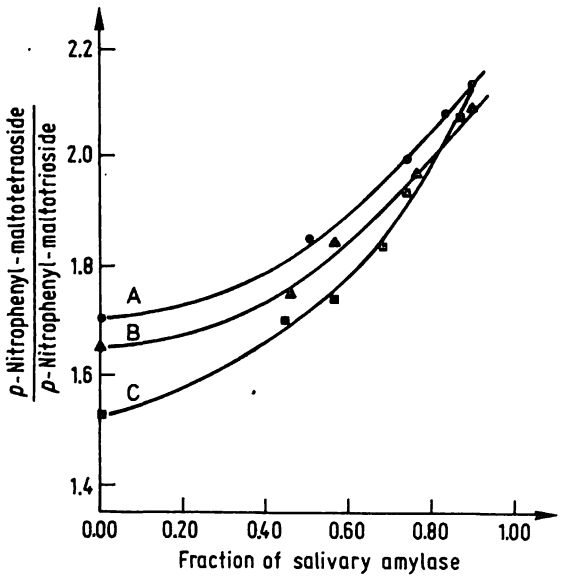

Fig. 2. Effect of salivary amylase on the *p*-nitrophenyl-maltotetraoside/-maltotrioside ratio. Three samples (A, B and C), containing between 0.50 and 0.85 pancreatic amylase, were diluted with saliva such that the salivary amylase catalytic activity increased whereas the pancreatic catalytic activity was constant. The samples were then diluted to final catalytic concentrations of 5 μkat/l and incubated with *p*-nitrophenyl-maltoheptaoside as described in "Materials and Methods". The products were separated by HPLC and the *p*-nitrophenyl-maltotetraoside/-maltotrioside ratios were calculated.

The calculation of the stoichiometric factor is based on the amylase reaction products that can be hydrolysed to free *p*-nitrophenol by α-glucosidase (5). These include *p*-nitrophenyl-glucoside, -maltoside and -maltotrioside. We calculated the stoichiometric factors from the amount of *p*-nitrophenol detected by HPLC (when α-glucosidase was included in the incubations). The mean stoichiometric factor for samples showing a normal isoamylase pattern was 2.99 (SD 0.16), and 3.25 (SD 0.31) for samples with $P > 0.65$. The mean factor for samples with $S > 0.55$ was 2.72 (SD 0.52). The most interesting deviations which result in discrepancies between the amylase catalytic activities are shown in table 2. It is evident that the stoichiometric factors in these samples vary from 2.0 to 4.0. In those cases where the stoichiometric factor was close to 3.0, the amylase catalytic activities by either the Phadebas method or the *p*-nitrophenyl-maltoheptaoside colorimetric method were comparable. When the factor was > 3.0, the catalytic activities with the Phadebas method were higher. This was seen in samples with pancreatic amylase dominance and particularly with samples displaying a double pancreatic amylase band. With a stoichiometric factor < 3.0, the catalytic activities

Tab. 2. Stoichiometric factors and method comparisons.

Sample	Fraction of P	Coefficient *p*-nitrophenol	Stoichiometric factor[a])	Amylase-Catalytic concentration (μkat/l) Phadebas	PNP-G7 (BM)[b])
1	1.00	0.34	2.94	7.2	7.1
2	0.87	0.34	2.94	14.5	13.8
3[c])	0.80	0.25	4.00	24	17
4[c])	0.80	0.26	3.85	61	51
5[c])	0.75	0.28	3.57	43	35
6	0.75	0.29	3.45	16	14
7	0.67	0.32	3.13	11.1	10.5
8	0.65	0.33	3.03	13.8	14
9[d])	0.46	0.48	2.08	14.2	21
10[d])	0.25	0.49	2.04	10	16.8
11[d])	0.20	0.50	2.00	8.9	15.3
12[d])	0.15	0.46	2.16	12.9	18.2
13	0.27	0.38	2.63	9.8	10.8
14	0.00	0.36	2.78	7.0	7.5

[a]) The stoichiometric factor is the reciprocal of the amount of *p*-nitrophenol detected.
[b]) *p*-Nitrophenyl-maltoheptaoside colorimetric method, Boehringer Mannheim.
[c]) Samples with double pancreatic (P) band.
[d]) Samples with abnormal, broad salivary (S) band.

with the Phadebas method were lower. This was seen in samples containing the abnormal, broad salivary band. In all the cases where the stoichiometric factor deviated significantly from 3.0, the HPLC examination of the products revealed that all the *p*-nitrophenyl-maltoside or -maltotrioside was not hydrolysed by α-glucosidase. The fact that more *p*-nitrophenol was detected with the samples containing the broad salivary band can be explained by our observation that more of substrate was hydrolysed by these samples.

In conclusion, we can state that for a great majority of the samples, the reaction mechanism and stoichiometry agree well with the results of *Hägele* et al (5). However, it is important to bear in mind that in some cases of pancreatitis, the amylase catalytic activity with the *p*-nitrophenyl-maltoheptaoside colorimetric method may be underestimated. Conversely, the enzyme catalytic activity in some of the samples containing predominantly salivary amylase may be overestimated.

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