**IFCC METHODS FOR THE MEASUREMENT OF CATALYTIC CONCENTRATION OF ENZYMES
PART 9. IFCC METHOD FOR α -AMYLASE (1-4- α -D-GLUCAN 4-GLUCANOHYDROLASE, EC 3.2.1.1)**

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This paper forms part of a series of recommendations on measurements of catalytic concentrations of enzymes. Others deal with:

- Part 1. General considerations. Approved recommendation, 1979 (1).
- Part 2. Method for aspartate aminotransferase. Approved recommendation, 1985 (2).
- Part 3. Method for alanine aminotransferase. Approved recommendation, 1985 (3).
- Part 4. Method for γ -glutamyltransferase (4).
- Part 5. Method for alkaline phosphatase (5).
- Part 6. Reference materials for enzyme measurements (Stage 1, draft 1989-copy available from Committee Chairman).
- Part 7. Method for creatine kinase. Approved recommendation, 1991 (6).
- Part 8. Method for lactate dehydrogenase. Approved recommendation, 1994 (7).

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Appendix A : Description of pertinent factors in obtaining optimal conditions for measurement.

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1. Introduction

α -Amylase in human serum consists of at least two isoenzymes originating from either the pancreas or all salivary glands, but hundred-to thousand-fold lower concentrations of both types have been found in various other tissues (8), and a novel type was detected in lung carcinoid tumour (9). The human pancreatic and salivary isoenzymes are single polypeptide chains forming three structural domains composed of 496 amino acids with about 97 per cent homology (10,11) with one calcium and one chloride ion and almost identical catalytic and immunogenic properties which can only be distinguished by monoclonal antibodies (12).

More than two hundred α -amylase methods based on various polymeric substrates had been developed before the era of kinetic measurements which use maltotetraose, maltopentaose or, mostly, numerous nitrophenylated oligosaccharides together with α -glucosidase (α -D-glucoside glucohydrolase, EC 3.2.1.20). The latter tests have become increasingly popular despite their complicated stoichiometry. However, recently the components of an assay have become available, which uses a novel-type α -glucosidase completely hydrolyzing nitrophenylated substrates. Thus, the cleavage of one α -glucosidic linkage by α -amylase results in the release of one molecule of 4-nitrophenol. The following method describes the optimized conditions of this assay (13).

The principles applied in the selection of the conditions of measurement are those stated in a previous publication by this committee (1). Since elevated α -amylase activity in serum and urine is generally accepted as a reliable sign of pancreatic disease, the method was optimized for pancreatic amylase, but it is also suited, although less optimally, for the determination of salivary amylase.

2. Principle

α -Amylase hydrolyzes 4,6-ethylidene (G7)-4-

nitrophenyl (G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside to different 4,6-ethylidene- α -(1 $\rightarrow$ 4)-D-glucopyranosyl oligosaccharides and 4-nitrophenyl- α -(1 $\rightarrow$ 4)-D-glucopyranosyl oligosaccharides the latter ones being degraded to 4-nitrophenol and glucose by α -glucosidase:

4,6-Ethylidene (G7)-4-nitrophenyl (G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside* + H₂O $\xrightarrow{\alpha\text{-Amylase}}$

4,6-Ethylidene- α -(1 $\rightarrow$ 4)-D-glucopyranosyl-Gx +

4-Nitrophenyl- α -(1 $\rightarrow$ 4)-D-glucopyranosyl-G(7-x)

4-Nitrophenyl- α -(1 $\rightarrow$ 4)-D-Glucopyranosyl-G(7-x) + H₂O $\xrightarrow{\alpha\text{-Glucosidase}^{**}}$ (7-x) x Glucose + 4-Nitrophenol

The reaction is continuously monitored by measuring the increase of the yellow product 4-nitrophenoxide at 405 nm.

3. IFCC Conditions for Measurement

All reaction conditions have been chosen on the basis of uni- and multivariate experiments with purified human pancreatic amylase (14,15) and have been confirmed with sera from patients suffering from acute pancreatitis.

These IFCC conditions are optimized reaction conditions which are most favourable for both the kinetic reactions and the technical aspects of the measurement, i.e. they do not necessarily provide maximal activity (1).

* 4,6-Ethylidene-(G7)-4-nitrophenyl (G1)- α -D-maltoheptaoside, O-4,6-ethylidene- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-O- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-O- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-O- α -D-Glucopyranosyl-(1 $\rightarrow$ 4)-O- α -D-Glucopyranosyl-(1 $\rightarrow$ 4)-D-1-(4-nitrophenyl)-glucopyranoside.

G1 and G7 denote the glucosyl moieties (numbered from the reducing end) of the oligosaccharide.

** α -Glucosidase, α -D-glucoside glucohydrolase, EC 3.2.1.20.

Table 1. Optimized conditions for measurement.

Reaction temperature	30,0 $\pm$ 0,05°C
pH (30°C)	7,10 $\pm$ 0,05
N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid	50 mmol / l
Sodium chloride	70 mmol / l
Calcium chloride	1,0 mmol / l
4,6-Ethylidene (G7)-4-nitrophenyl(G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside	5,0 mmol / l
α Glucosidase (EC 3.2.1.20) from bacteria	80 μ kat / l
Volume fraction of sample	0,03226 (1:31)

Note: The concentrations apply to the complete reaction mixture.

4. Instrumentation and Equipment

A spectrometer or spectrum-line spectrometer (preferably a recording instrument suitable for accurate measurement at 405 nm) with constant temperature cuvette compartment is required. Specifications for the equipment (e.g. volumetric glassware, pH meter, performance of the spectrometer, and temperature control), sample and reagent handling should meet those of previous IFCC recommendations (1-7).

5. Reagents

1. Sodium chloride, NaCl, Mr 58,44
2. Calcium chloride, dihydrate, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Mr 147,02
3. N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid, $\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$, Mr 238,31
4. Sodium hydroxide, NaOH, Mr 40,00 (0,2 mol/l)
5. α -Glucosidase (EC 3.2.1.20) from *Bacillus stearothermophilus* (lyophilized). Specific activity higher than 400 μ kat / g (30°C).
6. 4,6-Ethylidene (G7)-4-nitrophenyl (G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside, $\text{C}_{50}\text{H}_{77}\text{NO}_{38}$, Mr 1300,2

6. Purity of Reagents

Specifications regarding the purity of reagents must conform to the criteria given in previous recommendations (1). 4,6-Ethylidene (G7)-4-nitrophenyl (G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside must be of high-

est available purity ($\geq 0,95$ by high performance liquid chromatography, mass fraction $\geq 0,90$, pH 6,20 in aqueous solution). α -Glucosidase must contain $\geq 400 \mu\text{kat} / \text{g}$ (24 kJ/g at 30°C) corresponding to $\geq 667 \mu\text{kat} / \text{g}$ (40 kJ/g at 37°C). Its relative contamination with α -amylase should not exceed 1×10^{-4} kat/kat. Calcium chloride, sodium chloride, sodium hydroxide, and N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid must be of Analytical Reagent grade. Commercial preparations (crystalline or off-white powder) generally meet these requirements. Further details are given in Appendix B.

7. Preparation of Solutions

To prevent the growth of microorganisms in solutions, use sterile containers. Solutions must be prepared in volumetric glassware of Class A specifications at the calibration temperature with doubly distilled deionized water (sterile; conductivity below 2 μS ; pH 6-7; silicates $< 0,1 \text{ mg} / \text{l}$). The pH of solutions must be adjusted at 30°C with a pH meter calibrated at 30°C with standardized reference buffers (1).

- I Stock solution of calcium chloride (calcium chloride, 258 mmol / l).

Dissolve 3,80 g of calcium chloride, dihydrate, in about 80 ml and make up to 100 ml with water.

- II Solution of effectors in buffer (sodium chloride, 72,3 mmol / l; calcium chloride, 1,03 mmol / l; N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 51,7 mmol / l; pH 7,12 at 30°C).

Dissolve 1,057 g of sodium chloride and 3,078 g of N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid in about 200 ml of water, add 1,0 ml of solution I, adjust to pH 7,12 at 30°C with approximately 24 ml of sodium hydroxide, 0,2 mol / l, and add water to exactly 250 ml.

- III Working solution of buffer-effector-enzyme mixture (sodium chloride, 72,3 mmol / l; calcium chloride, 1,03 mmol / l; α -glucosidase 100 μ kat / l (6 kU / l); N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 51,7 mmol / l; pH 7,12 at 30°C).

Dissolve 10 μ kat (600 U) of α -glucosidase from microorganisms, e.g. 25 mg of a preparation containing 400 μ kat / g, in 100 ml of solution II.

- IV Substrate solution (initiating reagent: 4,6-ethylidene (G7)-4-nitrophenyl (G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside, 31 mmol / l; sodium chloride, 72,3 mmol / l; calcium chloride, 1,03 mmol / l; N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 51,7 mmol / l; pH 7,06 at 30°C):

Dissolve 896 mg of 4,6-ethylidene (G7)-4-nitrophenyl(G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside (mass fraction 0,90) in 20 ml of solution II.

- V Diluent (sodium chloride, 154 mmol / l).

Dissolve 0,9 g of sodium chloride in 100 ml of water.

8. Stability of Solutions

Solutions I and V are indefinitely stable at 20°C. If not sterilized by boiling, solution II has a shelf life of at least four weeks at 20°C. Solution III and IV are at least stable for two weeks at 5°C. Bacterial or fungal contamination limit their stability. Do not add azide as a biocide.

9. Specimen Procurement, Stability and Storage

Serum and heparinized plasma are acceptable materials. Fluoride and ethylenediaminetetraacetate must not be used as anticoagulants, although the calcium concentration of solutions III and IV reactivates the enzyme. Collect blood by venipuncture with minimal stasis. Blood cells should be removed from serum or plasma within two hours of collection. Urine may be a specimen for special clinical requests.

α -Amylase is relatively stable. Single freezing and thawing of serum does not impair its activity which is fully retained during storage for 4 days at 20°C, 2 weeks at 5°C, 1 year at -28°C or 5 years at -75°C. Urine should not be frozen but should be analyzed within 12 hours at 20°C or 5 days at 5°C, and preservatives should not be added.

10. Measurement

10.1 Measurement conditions

Wavelength: 405 nm (± 1 nm)
 Bandwidth: ≤ 2 nm
 Light pathlength: $10,0 \pm 0,01$ nm
 Final volume of reaction mixture: 620 μ l
 Temperature $30,0 \pm 0,05^\circ\text{C}$ (thermostatted cuvette compartment)

10.2 Handling of solutions

Before pipetting, reagents and samples must be brought to the pipettes' calibration temperature. However, use of other temperatures results in a relative error of only 0,000025 for each $^\circ\text{C}$ difference from the calibration temperature. During the preincubation period the solution in the cuvettes must attain a temperature of $30,0^\circ\text{C}$ before initiating the reaction by addition of the substrate.

10.3 Subprocedures that constitute one measurement

The measurement consists of two subprocedures: the overall reaction comprising the catalysed plus the spontaneous substrate hydrolysis

Analytical system for measurement of the overall α -amylase rate of conversion.

Pipette into the cuvette		Concentration in the final, complete mixture	
Solution III	500 μ l	Calcium chloride	1,0 mmol / l
		Sodium chloride	70 mmol / l
		α -Glucosidase	80 μ kat / l
		N-2-hydroxyethylpiperazine-	
		N'-ethanesulfonic acid	50 mmol / l
Sample	20 μ l	Volume fraction	0.03226 (1:31)

Mix thoroughly without removing any of the mixture from the cuvette. Incubate at 30°C until the mixture has attained this temperature (at least 300 s).

Solution IV	100 μ l	4,6-ethylidene(G7)-4-nitro-	
		phenyl(G1)- α -(1 $\rightarrow$ 4)-D-	
		maltoheptaoside	5,0 mmol / l
		pH	7,10 (30°C)

Mix again and wait 180 s for the end of the lag phase. Monitor the increase in absorbance at 405 nm as a function of time at least in additional time of 300 s.

Note: The final pH value results from the slightly more acidic solution IV added to the mixture of solution III and sample.

and the reagent blank reaction, representing the latter only, which is very small, viz. about 0,0005 $\Delta A/\text{min}$. For practical purposes it may be disregarded. A sample blank measurement is not necessary.

10.4 Measurement interval

A lag phase of 180 s must be observed. The values for $\Delta A/\Delta t$ of the overall reaction are constant over a period of at least 300 s following the lag phase for samples with catalytic concentrations of α -amylase up to 11,5 μ kat / l (or 12,5 μ kat / l for sera, resp., see Calculation). If the increase of absorbance is greater than 0,235 per 60 s corresponding to 11,5 μ kat / l [689 U / l], the sample must be exactly diluted with solution V, and the resulting $\Delta A/\Delta t$ has to be adequately multiplied.

10.5 Reagent blank

The same procedure is followed for the measurement of the reagent blank except that the sample is replaced by solution V. A reagent blank absorbance change must be determined for each series of samples. The initial absorbance should not be above 0,120 and

the change of absorbance should be less than 0,0005 per 60 s at 405 nm. Otherwise the purity of the reagents must be reassessed.

10.6 Correction for blank reaction

The reagent blank has to be subtracted from the overall reaction as follows: ($\Delta A/\Delta t$) overall- ($\Delta A/\Delta t$) corrected = a.

CALCULATION

From the following equation the catalytic concentration, b, is given by

$$b = a \times \frac{V}{\epsilon \times l \times v}$$

The molar absorption coefficient, ϵ , of 4-nitrophenoxide (30°C, 405 nm) = $1056,8 \pm 12 \text{ m}^2/\text{mol}$. The light pathlength, l, is 0,01 m. Let the increase in absorbance ($\Delta A/\Delta t$) per second at 405 nm be a, s^{-1} . V denotes the total reaction volume $0,62 \times 10^3$ l and v the sample volume, $0,02 \times 10^3$ l. Under the conditions of the assay,

$$b = a \times \frac{0.62 \times 10^{-3}}{1056,8 \times 0,01 \times 0,02 \times 10^3} \times \frac{s^{-1} \times l}{m^2 \times mol^1 \times m \times l}$$

$$b = a \times \frac{31}{10,568} \times \frac{s^{-1}}{m^3 \times mol^1}$$

$$= a \times 2,933 \mu\text{kat} / m^3$$

$$= a \times 2933 \mu\text{kat} / l$$

* With human sera (total protein concentration between 65 and 77 g / l) as samples, $\epsilon = 975,0 \pm 18 m^2/mol$, corresponding to a calculation factor of 3179,5.

Note: These values were calculated for a measurement time of 1 s. Let the increase per 60 s at 405 nm be A, $(60 s)^{-1}$.

$$b = A \times 2933 U / l \text{ and } b = A \times 3179 U / l \text{ (for sera, resp.)}$$

12. Analytical Variability

Performance data with the described measurement procedure are given in table 2.

Table 2: Imprecision data from 15 manual α -amylase determinations with human sera.

Catalytic concentration $\mu\text{kat} / l \text{ U} / l$	Relative standard deviation	
	Intra-assay	Inter-assay
0,59 35,3	0,034	0,047
1,48 88,9	0,019	0,029
5,04 302	0,020	0,025

Reference Values

Preliminary reference ranges were derived by assessing α -amylase in 100 sera of apparently healthy people according to a *priori* selection (16) with following exclusion criteria: Postprandial glucose above 6,4 mmol / l, creatinine above 90 (females) or 110 (males) $\mu\text{mol} / l$, γ -glutamyltransferase above 0,45 $\mu\text{kat} / l$ (30°C; 4) and total protein below 58 g / l. Ten groups were established, each comprising 10

women or 10 men in each of the five decennia between 20 and 59 years of age. Since no sex- or age-related differences between these groups were observed by the Mann-Whitney U test, the 0,95-reference interval of 0,40 $\mu\text{kat} / l$ (24 U / l) to 1,10 $\mu\text{kat} / l$ (66 U / l) was generated from all data, whose natural logarithms showed gaussian distribution. Separate reference intervals for both sexes are presented in table 3.

Table 3: Preliminary 0,95-reference intervals for α -amylase in 100 healthy adults.

Population	Number of samples	Proposed method (30°C)	
		$\mu\text{kat} / \text{l}$	U / l
Women	50	0,41 - 1,08	24,4 - 64,8
Men	50	0,42 - 1,10	25,2 - 66,0

REFERENCES

1. Bowers, Jr., G.N., Bergmeyer, H.U., Hørdér, M. and Moss, D.W. (1979/1980) Approved recommendations on IFCC methods for the measurements of catalytic concentrations of enzymes. Part 1. General considerations concerning the determination of the catalytic concentration of an enzyme in the blood serum or plasma of man. Clin. Chim. Acta. (1979) 98, 163F-174F; J. Clin. Chem. Clin. Biochem (1980) 18, 89-95.
2. Bergmeyer, H.U., Hørdér, M. and Rej, R. (1985) Approved recommendation on IFCC methods for the measurements of catalytic concentrations of enzymes. Part 2, IFCC method for aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1). J. Clin. Chem. Clin. Biochem. (1986) 24, 497-510
3. Bergmeyer, H.U., Hørdér, M. and Rej, R. (1985) Approved recommendation on IFCC methods for the measurement of catalytic concentrations of enzymes. Part 3. IFCC method for alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2). J. Clin. Chem. Clin. Biochem. (1986) 24, 481-495.
4. Shaw, L.M., Strømme, J.H., London, J.L. and Theodorsen, L. (1983) IFCC methods for the measurement of catalytic concentrations of enzymes. Part 4. IFCC method for γ -glutamyltransferase [γ -glutamyl]-peptide: amino acid γ -glutamyltransferase, EC 2.3.2.2]. J. Clin. Chem. Clin. Biochem. 21, 633-646.
5. Tietz, N.W., Rinker, A.D. and Shaw, L.M. (1983) IFCC methods for the measurement of catalytic concentrations of enzymes, Part 5. IFCC method for alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1). Clin. Chim. Acta 135, 339F-367F; J. Clin. Chem. Clin Biochem. 21, 731-748.
6. Hørdér, M., Elser, R.C., Gerhardt, W., Mathieu, M. and Sampson, E.J. (1991) Approved recommendation on IFCC methods for the measurement of catalytic concentrations of enzymes. Part 7. IFCC method for creatine kinase (ATP: creatine N-phosphotransferase, EC 2.7.3.2). J. Clin. Chem. Clin. Biochem. 29, 435-456. JIFCC (1989) 1, 130-139; JIFCC (1990) 2, 26-35; JIFCC (1990) 2, 80-83.
7. Bais, R. and Philcox, M. (1994) Approved recommendation on IFCC methods for the measurement of catalytic concentrations of enzymes. Part 8. IFCC method for lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27). Eur. J. Clin. Chem. Clin. Biochem. 32, 639-655.
8. Whitten, R.O., Chandler, W.L., Thomas, M.G.E., Clayson, K.J. and Fine, J.S. (1988) Survey of α -amylase activity and isoamylases in autopsy tissue. Clin. Chem. 34, 1552-1555.
9. Tamita, N., Horii, A., Doi, S., Yokouchi, H., Shiosaki, K., Higashiyama, M., Matsuura, N., Ogawa, M., Mori, T. and Matsubara, K. (1989) A novel type of human α -amylase produced in lung carcinoid tumor. Gene 76, 11-18.
10. Nishide, T., Emi, M., Nakamura, Y. and Matsubara, K. (1986) Corrected sequences of cDNAs for human salivary and pancreatic α -amylases. Gene 50, 371-372.
11. Brayer, G.D., Luo, Y.G. and Withers, S.G. (1995) The structure of human pancreatic α -amylase at 1.8 Å resolution and comparison with related enzymes. Prot. Sci. 4, 1730-1742.
12. Gerber, M., Naujoks, K., Lenz, H., Gerhardt, W. and Wulff, K. (1985) Specific immunoassay of α -amylase isoenzymes in human serum. Clin. Chem. 31, 1331-1334.
13. Lorentz, K., Gütschow, B. and Renner, F. (1996) Improved α -amylase determination with a protected 1,4- α -D-4-nitrophenylmaltoheptaoside with use of a novel α -glucosidase. In preparation.

14. Lorentz, K. (1982) Properties of human α -amylases from urine, pancreas, and saliva. *Enzyme* 28, 233-241.
15. Gella, F.J., Gubern, G. and Canalias, F. (1991) The feasibility of the preparation of a purified human pancreatic α -amylase as a reference material standard. *Commiss. Eur. Communities: BCR Information, Report EUR 13629 EN*.
16. Petit Clerc, C. and Solberg, H.E. (1987) Approved recommendation (1987) on the theory of reference values. Part 2. Selection of individuals for the production of reference values. *J. Clin. Chem. Clin. Biochem.* 25, 639-644.

APPENDIX A

Description of Pertinent Factors in Obtaining Optimal Conditions for Measurement

1. Introduction

The optimization of measurement conditions for α -amylase in this coupled test was not influenced by the indicator system, so that all analytical variables of the primary reaction could be investigated without limitation (except some buffers noted below). We applied univariate experiments, since there were no interactions between buffer species and strength, pH, substrate, and effectors. The results, based on the release of 4-nitrophenol/4-nitrophenoxide, were confirmed by response surface methodology, and the choice of conditions considers enzyme kinetics as well as the technical aspects of performance that are consistent with robustness and transferability of the method.

2. Buffer and pH

On principle, both α -amylases display broad optima at neutral pH with less than 2% difference in activity between pH 6,9 and pH 7,2 slightly depending on buffer type and temperature. The maximum for human pancreatic amylase lies close to pH 7,10, whereas that of the salivary component is between pH 6,9 and 7,0 under the conditions of the assay (figure 1). N-(2-Acetamido) iminodiacetic acid complexes the intrinsic calcium atom of α -amylase, while the amino type buffers bis (2-hydroxyethyl) amino-tris(hydroxy-methyl) methane and 1,3-bis [tris(hydroxymethyl)-methylaminopropane inhibit the indicator enzyme α -glucosidase (table 1).

Both isoamylases show higher activities in zwitterionic buffers than in phosphate, so that 3-(N-morpholino)propanesulfonic acid (pKa 7,09), N,N'-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (pKa 7,09) and N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (pKa 7,41, at 30°C each) were considered for measure-

ment. Although the pK-values of the former two closely correspond to the optimal pH of the pancreatic isoenzyme, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid was chosen by two reasons: Most of the sera from patients with acute pancreatitis produce higher conversion rates with this buffer, and an adaptation of the proposed method for routine use (at 37°C) shifts the maximum to pH 7,15 and lowers the pKa to 7,31. Trimethylpyridine is not throughout available in the required quality and was, therefore, declined.

α -Amylase activity does not decrease with increasing concentration of N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid up to 200 mmol / l in contrast to phosphate, and the dissociation of the indicator is only insignificantly suppressed by higher buffer strength (see Appendix B).

Fig. 1. Dependence of human salivary (above, open circles) and pancreatic α -amylase activity (below, closed circles) on pH in N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (solid lines) and phosphate (dashed lines), 50 mmol / l, each.

Conditions at 30°C: 4,6-Ethylidene-(G7)-4-nitrophenyl(G1)- α -D-maltoheptoside, 5 mmol / l; sodium chloride, 70 mmol / l; calcium chloride, 1,0 mmol / l; α -glucosidase, 80 μ kat / l.

Table 1. Fractional catalytic concentration of human α -amylases in various buffers, 50 mmol / l (if not otherwise stated), under optimized conditions as given in figure 1 at pH 7,10 (pancreatic) and pH 6,95 (salivary isoenzyme) and 30°C.

Type of buffer	Pancreas	Saliva
Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane	0,84	0,69
N-(2-Acetamido)iminodiacetic acid	0,15	0,23
Piperazine-N-N'-bis(2-ethanesulfonic acid)	0,96	0,89
1,3-Bis[tris(hydroxymethyl)-methylamino]propane	0,83	0,70
N-(2-Acetamido)-2-aminoethanesulfonic acid	0,96	0,96
3-(N-Morpholino)propanesulfonic acid	1,00	0,97
N,N'-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid	0,99	0,95
Phosphate, 50 mmol / l	0,97	0,88
100 mmol / l	0,93	0,85
200 mmol / l	0,75	0,70
Diethylmalonic acid	0,91	0,79
2,4,6-Trimethylpyridine	1,02	0,94
N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid	0,94	0,87
N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid, 50 mmol / l	1,00	1,00
100 mmol / l	0,97	0,99
200 mmol / l	0,98	0,94

3. Substrate, Chloride and Calcium Concentration

The dependence of reaction velocity on substrate concentration was followed up to 4,6-ethylidene(G7)-4-nitrophenyl(G1)- α -D-maltoheptaoside, 10 mmol / l, where maximum velocity (V_{max}) is attained. Substrate inhibition does not take place, and high concentrations do not noticeably increase the reagent blank. For practical (mainly economic) reasons 5,0 mmol / l, which yield $\geq 0,96 V_{max}$ for both isoamylases, were selected.

α -amylases undergo allosteric activation by halides and other monovalent anions chloride being the most effective one (1). Hence, the activity profile of human pancreatic and salivary amylase by varying the chloride concentration was recorded by increasing the sodium chloride concentration up to 150 mmol / l. Maximal velocity with the pancreatic isoenzyme occurs at 80 mmol / l, whereas human salivary α -amylase requires about 120 mmol / l. At 70 mmol / l, the concentration chosen, $\geq 0,99 V_{max}$ and $0,95 V_{max}$, respectively, are obtained.

Both substrate and chloride dependence strictly obey the Michaelis-Menten equation $v = V_{max} [S] / (K_m + [S])$, so that the K_m values could be determined using zero order kinetics. From repeated measure-

ments using linear reaction intervals between 0,025 and 2,5 mmol / l of substrate and from 0,5 to 100 mmol / l of chloride (figures 2 and 3) the Michaelis constants (K_m) were estimated with both Hanes-Woolf and Eadie-Hofstee plots in accordance varying slightly with buffer type and pH value (table 2).

Fig. 2. Effect of substrate concentration on human salivary (---○---) and pancreatic α -amylase activity (---○---). Insert: Eadie-Hofstee plot.

Conditions at 30°C: N-2-Hydroxyethylpiperazine-N β -ethanesulfonic acid, 50 mmol / l, pH 7,10; sodium chloride, 70 mmol / l; calcium chloride, 1 mmol / l; α -glucosidase, 80 μ kat / l.

Fig. 3. Effect of chloride concentration on human salivary (---○---) and pancreatic α -amylase activity (—●—). Insert: Eadie-Hofstee plot.

Conditions at 30°C: N-2-Hydroxyethylpiperazine -N'-ethanesulfonic acid, 50 mmol / l, pH 7,10; substrate 5 mmol / l; calcium chloride, 1 mmol / l; α -glucosidase, 80 μ kat / l.

Table 2. Kinetic constants of 4,6-ethylidene-(G7)-4-nitrophenyl(G1)- α -D-maltoheptaoside and chloride for human salivary and pancreatic α -amylase (mean, standard deviation, n=8).

Conditions at 30°C: Calcium chloride, 1,0 mmol / l; α -glucosidase, 80 μ kat / l; sodium chloride, 70 mmol / l (substrate dependence); substrate, 5,0 mmol / l (chloride dependence); buffer, 50 mmol / l.

Variable	Pancreatic amylase	Salivary amylase
N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid pH 7,10		
Substrate, Km (mmol / l)	0,091 $\pm$ 0,002	0,132 $\pm$ 0,008
Chloride, Km (mmol / l)	5,0 $\pm$ 0,4	7,0 $\pm$ 0,6
Phosphate pH 7,0		
Substrate, Km (mmol / l)	0,085 $\pm$ 0,008	0,139 $\pm$ 0,015
Chloride, Km (mmol / l)	4,6 $\pm$ 0,5	6,6 $\pm$ 0,6

Like chloride, which is located near the active site in domain A of the three structural domains, the calcium ion in a pocket of domain B (2) forms an essential component of α -amylase stabilizing the enzyme molecule and retaining its activity (3). No effect was observed by varying the calcium chloride concentration between 0 and 10 mmol / l without changing the other conditions of the optimized assay. However, to protect the enzyme against complexing agents in the sample (citrate, traces of ethylenediaminetetraacetate) calcium chloride, 1,0 mmol / l, was included in the assay.

4. The Catalyzed Reaction

Both human pancreatic and salivary α -amylase degrade 4,6-ethylidene-(G7)-4-nitrophenyl(G1)- α -D-maltoheptaoside to patterns consisting of only three substituted oligosaccharides, viz. 4-nitrophenyl- α -D-maltoside, -maltotrioside, and -maltotetraoside. The frequency distribution of bond cleavage does not shift with time, provided that the substrate consumption does not exceed 0,05 of the initial concentration. The data fairly well agree with those of Hägele et al. (4; table 3).

Table 3. Product patterns of 4,6-ethylidene-(G7)-4-nitrophenyl(G1)- α -D-maltoheptoside hydrolysis by human α -amylases (mean, standard deviation, n=5).

Conditions at 30°C: Substrate, 5,0 mmol / l (with conversion < 250 μ mol/l); sodium chloride, 70 mmol / l; calcium chloride, 1,0 mmol / l, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 50 mmol / l, pH 7,10.

Product	Fractional amount	Hägele et al.(4)
Pancreatic amylase		
4-Nitrophenyl- α -D-maltoside	0,40 $\pm$ 0,03	0,39 $\pm$ 0,02
4-Nitrophenyl- α -D-maltotriose	0,40 $\pm$ 0,04	0,40 $\pm$ 0,02
4-Nitrophenyl- α -D-maltotetraose	0,20 $\pm$ 0,02	0,21 $\pm$ 0,02
Salivary amylase		
4-Nitrophenyl- α -D-maltoside	0,50 $\pm$ 0,05	0,43 $\pm$ 0,02
4-Nitrophenyl- α -D-maltotriose	0,29 $\pm$ 0,04	0,33 $\pm$ 0,01
4-Nitrophenyl- α -D-maltotetraose	0,21 $\pm$ 0,03	0,24 $\pm$ 0,01

Fig. 4. Active site model of α -amylase according to Robyt and French (5) with the catalytic group (thick line) between the subsites 2 and 3. The subsites per glucose residue (-O-) are numbered from the reducing-end side.

Possible binding modes of 4,6-ethylidene(G7)-4-nitrophenyl(G1)- α -D-maltoheptoside producing 4-nitrophenyl- α -D-maltoside, B; 4-nitrophenyl- α -D-maltotriose, C; and 4-nitrophenyl- α -D-maltotetraose, D.

Accepting the five-subsite model (5) for the active site of α -amylase with the catalytic site between subsites 2 and 3 (figure 4A), where substrates are exclusively cleft between two glucose residues required for productive binding at these subsites. This endo-type activity of α -amylase towards substrates consisting of five or more unmodified glucose residues yields maximal reaction rates about pH 7,0 yielding maltose and maltotriose as terminal products.

The release of three reaction products from 4,6-ethylidene(G7)-4-nitrophenyl(G1)- α -D-maltoheptoside conforms well to this model, because all subsites are specifically occupied by glucose residues in the distinctly favoured cleavage to 4-nitrophenyl- α -D-maltoside (B) and 4-nitrophenyl- α -D-maltotriose (C). Subsite 3 absolutely requires unmodified glucose, whereas subsites 2 and 4 allow minor modifications viz. hydroxyethylation, at C-3 and C-6 or C-2 and C-6 of the respective residues (6). The specificity of subsite 5 is poor, thus allowing a weak productive binding of the bulky ethylidene glucose with the subsequent production of the small 4-nitrophenyl- α -D-maltotetraose fraction (D).

5. Indicator Reaction

Addition of α -glucosidase is necessary to release the chromophore from the three nitrophenylated reaction products mentioned above. Table 4 shows the relationship between the catalytic concentration of the indicator enzyme and the time lag before achieving linear conversion rates after initiating the reaction.

Table 4. Dependence of lag time on catalytic concentration of α -glucosidase under the proposed conditions of the α -amylase assay.

Catalytic concentration $\mu\text{kat} / \text{l}$ (U / l) at 30°C	Lag phase time seconds	Fractional conversion rates after 180 s
80 (4800)	110	1,00
60 (3600)	150	1,00
40 (2400)	180	1,00
20 (1200)	240	0,85
10 (600)	>300	0,60

Table 5. Fractional release of 4-nitrophenol/4-nitrophenoxide with time from different chromogenic oligosaccharides, 250 $\mu\text{mol} / \text{l}$, by α -glucosidase, 80 $\mu\text{kat} / \text{l}$ (4800 U / l), under the proposed conditions of the α -amylase assay.

Substrate	Chromophore production with increasing reaction intervals after start		
	60 s	120 s	180 s
4-Nitrophenyl- α -D-maltoside	0,85	0,95	1,0
4-Nitrophenyl- α -D-maltotrioside	0,90	>0,98	1,0
4-Nitrophenyl- α -D-maltotetraoside	0,85	>0,95	1,0

Table 6. Activation energies and temperature coefficients of 4,6-ethylidene-(G7)-4-nitrophenyl(G1)- α -D-maltoheptaoside hydrolysis by human amylases in different temperature intervals (n=5).

Isoenzyme	Interval °C	Activation energy kJ/mol (cal/mol)	Temperature coefficient Q10
Pancreatic	20 - 25	3,44 $\pm$ 0,08 (14380)	2,29 $\pm$ 0,07
	25 - 40	1,79 $\pm$ 0,06 (7490)	1,63 $\pm$ 0,06
Salivary	20 - 25	2,87 $\pm$ 0,08 (12000)	1,78 $\pm$ 0,07
	25 - 40	1,75 $\pm$ 0,06 (7330)	1,61 $\pm$ 0,05

Other experiments showed that 80 $\mu\text{kat} / \text{l}$ of α -glucosidase completely hydrolyzed all reaction products which might have been generated by a sample of α -amylase containing 43 $\mu\text{kat} / \text{l}$ (corresponding to 1,39 $\mu\text{kat} / \text{l}$ in the reaction mixture) during the lag phase (table 5).

6. Temperature Dependence

Plotting the logarithm of the respective reaction velocity against the reciprocal absolute temperature gives a straight line between 25 and 37°C with a discontinuity of this slope below the transition temperature of 25°C (Fig. 5) yielding different activation

energies and temperature coefficients above and below 25°C (table 6).

7. Technical Aspects

7.1 Volume fraction of the sample, preincubation time and initiation of reaction.

The volume fraction of the sample is not critical between 0,004 and 0,047. α -Amylase solutions with catalytic concentrations between 0,50 (30) and 2 $\mu\text{kat} / \text{l}$ (120 U / l) yield concordant results within a relative standard deviation of $\pm 0,028$. This is also true for sera in the same range, if proper corrections for the different protein concentrations are made.

Fig. 5. Arrhenius plot: Logarithm of reaction velocity versus reciprocal absolute temperature for salivary (—○—) and pancreatic amylase (—●—). The dashed lines show the calculated slope from measurements above 25°C.

tween a sample start and the proposed substrate start for samples containing between 0,50 (30) and 6,0 $\mu\text{kat} / \text{l}$ (360 U / l) of α -amylase.

7.2 Measurement interval, time of linear response and dynamic range

The lag phase has to be obeyed. Linear conversion rates are always observed for catalytic concentration up to 12,2 $\mu\text{kat} / \text{l}$ (735 U / l), i.e. 11 times the upper reference limit, if the reaction is monitored no longer than 300 s (table 7).

The data of table 7 allow measurements up 0,235A/60 s, since the assay of those samples diluted with nine volumes of sodium chloride, 154 mmol / l, leads to fractional activities of 0,97 - 1,03 after multiplication. Therefore, the dynamic range is

Table 7. Fractional conversion rates of different catalytic concentrations with time. The initial velocity (180 s after start of the reaction) is 1,00 (mean $\pm$ relative standard deviation, n=8). Pancreatic enzyme, HPA; salivary enzyme, HSA.

Sample	Conversion rate $\Delta A_{405\text{nm}/60\text{s}}$	Catalytic concentration $\mu\text{kat} / \text{l}$ U / l	Time after start s	Fractional rate
HSA	0,250	12,2 735	300	1,00 $\pm$ 0,025
	0,350	17,1 1028	300	0,95 $\pm$ 0,030
HPA	0,208	10,2 610	600	0,99 $\pm$ 0,028
Serum	0,235	12,5 747	300	1,00 $\pm$ 0,029

set according to the results of figure 5, and sera containing more than 12,5 $\mu\text{kat} / \text{l}$ (750 U / l) should be adequately diluted.

Fig. 6. Dynamic range of α -amylase. Dilution of a highly active serum sample with nine, four, one and 0,25 volumes of sodium chloride, 154 mmol/l.

Preincubation of the sample with solution III for up to 15 minutes does not affect the activity. Since sera and enzyme solutions are equally stable, a protective effect of protein is less conceivable than stabilization by calcium. There is no difference be

7.3 Reagent stability, reagent blank and sensitivity of the assay

As stated above, solutions III and IV are prone to microbial and fungal growth, but their stability may be enhanced, e.g. by methylisothiazolone, 0,1 g / l. Solution IV proved stable only in the proposed formulation or with N,N'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid but not with phosphate as buffer substance. 4,6-Ethylidene (G7)-4-nitrophenyl (G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside may be stored for at least two months at 5°C in aqueous stock solutions above 50 mmol / l.

Spontaneous substrate hydrolysis is extremely low, so that the average reagent blank is 0,25 U / l. Criteria for the sensitivity of the assay as proposed by Gautschi et al. (7) are shown in table 8.

Table 8. Data for the analytical efficiency of the proposed method calculated from 15 determinations of different solutions, given in U / l. SD, standard deviation; RSD, relative standard deviation.

Solution replacing sample	Critical Limit (Lc) (mean plus 3 SD)	Limit of detection (Ld) calculated from Lc	Limit of quantification (Lq, at a tolerated CV of 10 p.c.)
Distilled water	0,282	0,84	3,3
Sodium chloride, 154 mmol / l	0,300	0,95	4,5
Human serum albumin, 50 g / l	0,327	1,28	5,1

7.4 Interferences and sample blank

The detrimental effect of calcium complexing agents has been already stressed. Although the calcium concentration in the reaction mixture restores the activity of the enzyme, the use of anticoagulants other than heparin (up to 200 U/l or sodium heparinate, 1 g/l) is not recommended. 100 ± 3 p.c. α -amylase recovery (at 1,2 μ kat / l) is noted with following concentrations of endogenous serum components: Glucose, 55 mmol / l, triacylglycerols, 25 mmol / l, bilirubin, 500 μ mol/l, ascorbic acid 1 mmol / l, and haemoglobin, 2,5 g / l.

8. Evaluation of the Selected Conditions by Multivariate Analysis

The method is optimized for human pancreatic amylase but is also well suited for the measurement of salivary amylase as can be deduced by the results of computerized Response Surface Methodology.

Optimal concentrations of substrate, chloride and pH were not interdependent, interactions between these variables were weak.

Maximal activity for pancreatic amylase is observed in rather broad plateaus around substrate, 5,5 mmol / l. chloride, 70 mmol / l, and pH 7,25. Apart from pH the apparent maxima lie close to the proposed conditions. The same is true for salivary amylase (figures 7 and 8).

Fig. 7. Optimization of chloride, pH and 4,6-ethylidene(G7)-4-nitrophenyl(G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside for pancreatic amylase.

a - at chloride, 50 mmol / l b - at pH 7,10
c - at substrate, 3,7 mmol / l

Fig. 8. Optimization of chloride, pH and 4,6-ethylidene(G7)-4-nitrophenyl(G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside for salivary amylase.

a - at chloride, 50 mmol / l b - at pH 7,10
c - at substrate, 3,7 mmol / l

Fig. 7. Response Surface Optimization of pH, 4,6-ethylidene(G7)-4-nitrophenyl(G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside and chloride for pancreatic amylase at 30°C.

Values used for these experiments were pH (6.3, 6.6, 7.1, 7.6, 7.9), substrate (0.5, 1.8, 3.7, 5.6, 6.9 mmol/l) and chloride (5, 23, 50, 77, 95 mmol/l). The ciphers within the figures denote the fractional activity of the maximum (1,0).

a Variation of pH and substrate at chloride, 50 mmol / l

b Variation of chloride and substrate at pH 7,10

c Variation of pH and chloride at substrate, 3,7 mmol / l

Fig. 8. Response Surface Optimization of pH, 4,6-ethylidene(G7)-4-nitrophenyl(G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside and chloride for salivary amylase at 30°C.

Values used for these experiments were pH (6,3, 6,6, 7,1, 7,6, 7,9), substrate (0,5, 1,8, 3,7, 5,6, 6,9 mmol/l) and chloride (5, 23, 50, 77, 95 mmol/l). The ciphers within the figures denote the fractional activity of the maximum (1,0).

a Variation of pH and substrate at chloride, 50 mmol / l

b Variation of chloride and substrate at pH 7,10

c Variation of pH and chloride at substrate, 3,7 mmol / l

REFERENCES

1. Levitzki, A. and Steer, M.L. (1974) The allosteric activation of mammalian α -amylase by chloride. *Eur. J. Biochem.* 41, 171-180.
2. Brayer, G.D., Luo, Y.G. and Withers, S.G. (1995) The structure of human pancreatic α -amylase at 1.8 Å resolution and comparison with related enzymes. *Prot. Sci.* 4, 1730-1742.
3. Vallee, B.L., Stein, E.A., Sumerwell, W.N. and Fischer, E.H. (1959) Metal content of α -amylases of various origins. *J. Biol. Chem.* 234, 2901-2905.
4. Hägele, E.O., Kratzer, M., Schaich, E. and Rauscher, E. (1989) Mechanism of action of human pancreatic and salivary α -amylase on 4,6-ethylidene- α -4-nitrophenylmaltoheptaoside substrate. *Clin. Chem.* 35, 188-189.
5. Robyt, J.F. and French, D. (1970) The action pattern of porcine pancreatic α -amylase in relationship to the substrate binding site of the enzyme. *J. Biol. Chem.* 245, 3917-3927.
6. Chan, Y.-Ch., Braun, P.J., French, D. and Robyt, J.F. (1984) Porcine pancreatic α -amylase hydrolysis of hydroxyethylated amylose and specificity of subsite binding. *Biochemistry* 23, 5795-5800.
7. Gautschi, K., Keller, B., Keller, H., Pei, P. and Vonderschmitt, D.J. (1993) A new look at the limits of detection (LD), quantification (LQ) and power of definition (PD). *Eur. J. Clin. Chem. Clin. Biochem.* 31, 433-440.

APPENDIX B

Reagent Specifications

Properties of the Indicator and the Indicator Enzyme

1. Introduction

The thermodynamic ionization constant of 4-nitrophenol was found to be $7,10 \times 10^{-8}$ at 25°C (1) under conditions similar to those of the assay, i.e. almost equal amounts of 4-nitrophenol and 4-nitrophenoxide are released by the proposed method, and small variations in pH, ionic strength, tempera-

ture and protein concentration will considerably influence the absorbance of the final reaction product to be measured. Hence, these conditions have to be carefully examined and exactly observed.

Although the study of analytical variables has to concentrate on the measurement of α -amylase, relevant properties of α -glucosidase, e.g. pH profile and influence of effectors must fit the reaction conditions of the proposed method. The indicator enzyme must not attack 4,6-ethylidene (G7)-4-nitrophenyl(G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside, which can be ascertained by the reagent blank. Moreover,

α -glucosidase must completely release the chromophore without time lag from all 4-nitrophenyl oligosaccharides generated by α -amylase, and this has to be proved by assessing its required catalytic concentration and by high performance chromatography of the product patterns.

2. Spectral Properties of 4-Nitrophenol

Commercially available material must meet the specifications given by Bowers et al. (2) or it has to be purified best by sublimation according to their instructions. The calculation of molar absorption coefficients was based on measuring 4-nitrophenol solutions, 100 $\mu\text{mol/l}$, with a 0,5 nm bandwidth spectrometer. The spectrometric data presented in table 1 and figure 1 should be met within a range of ± 2 p.c. with instruments used for this method.

Perusal of table 1 shows that the ionization of

Table 1. Molar absorption coefficients of 4-nitrophenol derived from 8 measurements (mean $\pm$ standard deviation) in different solutions. The pH values were adjusted with sodium hydroxide.

The protein content denotes its concentration in the sample, n.d. = not determined. Total protein and human serum albumin were quantified by biuret method.

Molar absorption coefficient, ϵ (m^2/mol), at 405 nm					
In sodium hydroxide, 10 mmol / l		25°C	30°C	37°C	
		1815,1 $\pm$ 9, 5	1829,2 $\pm$ 7, 6	1848,0 $\pm$ 8, 2	
		In buffer, 50 mmol/l; sodium chloride, N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid		70 mmol / l Phosphate	
pH	Additive	30°C	37°C	30°C	37°C
6,90		n.d.	n.d.	832,5 $\pm$ 13	918,1 $\pm$ 15
6,95		n.d.	n.d.	886,1 $\pm$ 16	961,2 $\pm$ 11
7,00		955 $\pm$ 9	1051,6 $\pm$ 12	937,6 $\pm$ 12	1017,5 $\pm$ 16
7,05		1007,2 $\pm$ 8	1101,9 $\pm$ 7	991,2 $\pm$ 14	1068,3 $\pm$ 13
7,10		1056,8 $\pm$ 12	1151,8 $\pm$ 12	1042,0 $\pm$ 12	1116,2 $\pm$ 15
	Albumin,				
	15g / l	1012,2 $\pm$ 13		1007,6 $\pm$ 14	
	46g / l	968,0 $\pm$ 15		963,9 $\pm$ 15	
	92g / l	914,1 $\pm$ 14		906,8 $\pm$ 12	
	Serum: protein				
	67g / l	975,0 $\pm$ 16		970,2 $\pm$ 13	
	81g / l	970,2 $\pm$ 18		966,1 $\pm$ 16	
7,15		1112,0 $\pm$ 15	1200,5 $\pm$ 14	1092,5 $\pm$ 8	1166,4 $\pm$ 12
	Albumin,				
	15g / l	1166,9 $\pm$ 16	1127,9 $\pm$ 15		
	46g / l	1106,9 $\pm$ 13	1082,4 $\pm$ 13		
	92g / l	1063,6 $\pm$ 15	1042,6 $\pm$ 16		
	Serum: protein,				
	67g / l	1120,0 $\pm$ 15			1109,5 $\pm$ 16
	81g / l	1108,2 $\pm$ 18			1093,1 $\pm$ 17
7,20	1137,7 $\pm$ 10		1245,3 $\pm$ 11	1137,8 $\pm$ 6	1225,1 $\pm$ 7
7,25	1205,6 $\pm$ 13		1283,6 $\pm$ 16	1194,6 $\pm$ 15	1263,2 $\pm$ 10

Fig. 1. Molar absorption coefficient of 4-nitrophenol (ordinate) in N-2-hydroxyethyl-piperazine-N'-2-sulfonic acid (—●—, above and phosphate (—○—, below), 50 mmol / l each, with sodium chloride, 70 mmol / l; calcium chloride, 1,0 mmol / l; at 30°C versus different pH values (abscissa).

Increasing the buffer strength to 100 mmol / l or doubling the chloride concentration lowers the molar absorption coefficient by approximately 1% in solutions devoid of proteins.

3. Properties of α -Glucosidase

In specifying optimized conditions the measurement of α -amylase is not compromised by the indicator reaction as asserted in Appendix A. This claim is seemingly at variance with the known preference of α -glucosidases (α -D-glucoside glucohydrolases, EC 3.2.1.20) for terminal α 1,4-glucosidic bonds of small substrates and decreasing hydrolysis rates with increasing size of the oligosaccharide (3), while all products from α -amylase attack, viz. 4-nitrophenyl- α -

D-maltoside-maltotriose and -maltotetraose, ought to be completely degraded to free the chromophore. Thus, conclusions from the following results must be drawn in the light of the primary reaction.

3.1 Measurement of the catalytic concentration of the indicator enzyme

The measurement conditions, if not otherwise stated, correspond to those applied to the assay of α -amylase in this IFCC method (table 1), as instrumentation, equipment and reagents, but 4,6-ethylidene(G7)-4-nitrophenyl(G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside is replaced by 4-nitrophenyl- α -D-glucopyranoside.

Table 2. Conditions for measurement of α -glucosidase (EC 3.2.1.20)

Reaction temperature	30,0 $\pm$ 0,05°C
pH (30°C)	7,10 $\pm$ 0,05
N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid	50 mmol / l
Sodium chloride	70 mmol / l
Calcium chloride	1,0 mmol / l
4-Nitrophenyl- α -D-glucopyranoside	5,0 mmol / l
Volume fraction of sample	0,03846 (1:26)

3.1.1 Preparation of solutions

- A Buffer (sodium chloride, 70 mmol / l; calcium chloride 1,0 mmol / l; N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 50 mmol / l; pH 7,1 at 30°C).

Dissolve 1,02 g of sodium chloride and 2,98 g of N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid in about 220 ml of water, add 0,07 ml of solution I, adjust to pH 7,1 at 30°C with approximately 24 ml of sodium hydroxide, 0,2 mol / l, and add water to exactly 250 ml.

- B Substrate solution (4-nitrophenyl- α -D-glucopyranoside, 5,2 mmol / l; sodium chloride, 70 mmol / l; calcium chloride 1,0 mmol / l; N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 50 mmol / l; pH 7,1 at 30°C).

Dissolve 157 mg of 4-nitrophenyl- α -D-glucopyranoside in solution A and fill up to exactly 100 ml. The solution is stable for 5 days at 5°C.

- C Enzyme stock solution (α -glucosidase, 1

mkat / l (60 kU/L); sodium chloride, 70 mmol / l; calcium chloride 1,0 mmol / l; N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 50 mmol / l; pH 7,1 at 30°C; human serum albumin, 20 g / l).

Dissolve 10 mg of α -glucosidase from microorganism (e.g. *Bacillus stearothermophilus*) containing 400 μ kat / g (24 kU/g, 30°C) and 80 mg of human serum albumin with N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 50 mmol / l; pH 7,12 at 30°C up to 4,0 ml. The solution may be stored at 5°C for four weeks or at -28°C for at least 1 year.

A working solution with about 5 μ kat / l (300 U / l) should be prepared within 5 hours before use by dilution with solution A.

3.1.2 Measurement procedure

The measurement consists of the overall reaction minus the reagent blank reaction as follows : $(\Delta A / \Delta t)_{\text{overall}} - (\Delta A / \Delta t)_{\text{blank}} = (\Delta A / \Delta t)_{\text{corrected}} =$
a. Solution A replaces the enzyme in the blank reaction, whose increase of absorbance is less than 0,001 $\Delta A / \text{min}$ and may be neglected for practical purposes.

Analytical system for measurement of the overall α -glucosidase rate of conversion.

Pipette into the cuvette		Concentration in the final, complete mixture	
Solution B	500 μ l	4-nitrophenyl- α -D-glucopyranoside	5,0 mmol / l
		Calcium chloride	1,0 mmol / l
		Sodium chloride	70 mmol / l
		N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid	50 mmol / l
		pH	
Incubate at 30°C until the mixture has attained this temperature (at least 300 s).			
Enzyme	20 μ l	Volume fraction	0.03846(1:26)
Mix thoroughly, wait 30 s and monitor the increase in absorbance at 405 nm for an additional time of 300s.

Fig. 2. Dependence of bacterial α -glucosidase on pH in phosphate, 50 mmol / l. Conditions at 30°C: Substrate 5,0 mmol / l (—○—, 4-nitrophenyl- α -D-glucopyranoside; —■—, 4-nitrophenyl- α -D-maltotrioxide).

3.1.3 Calculation

The catalytic concentration, b , is given by

$$b = a \times 0,52/0,02 \times 1/(1056,8 \times 0,01)$$

$$= a \times 2,46 \text{ kat/m}^3 \text{ (or } a \times 2460 \text{ } \mu\text{kat / l)}$$

The factors represent the volume ratio (assay/sample), ϵ 405 for 4-nitrophenol in N-2-hydroxyethylpiperazine-N' ethanesulfonic acid, 50 mmol / l, pH 7,10 at 30°C, and the light pathlength, respectively. The influence of the minimal protein concentration in the reaction mixture on the molar absorption coefficient of 4-nitrophenol can be disregarded.

3.2 Buffer and pH

The catalytic concentration of α -glucosidase can be reliably determined by the hydrolysis of 4-nitrophenyl- α -D-glucopyranoside, but it must be kept in mind that monitoring the cleavage of the maltoside, maltotrioxide and maltotetraoxide derivatives would

better reflect its pertinence as an indicator enzyme for α -amylase. Thus, additional experiments were carried out with 4-nitrophenyl- α -D-maltotrioxide, whose decrease was determined by chromatography (see 4).

To compare the bacterial enzyme with α -glucosidase from yeast a variation of activity with pH was done in phosphate (figure 2) showing an identical activity profile for both the glucoside and the maltotrioxide, a similar dependence on pH as the yeast enzyme (3,4) and a fairly good agreement with the requirements of the primary reaction. In a further approach, therefore, the influence of buffers with pK values near pH 7,1 was studied on enzyme activity under optimized conditions as well for α -glucosidase as for α -amylase measurement (table 3).

Table 3 confirms the inhibition of α -glucosidases by amino type buffers and displays only slight differences except N-(2-acetamido)-2-aminoethanesulfonic acid between the action on both substrates. From this point of view phosphate must be regarded as the optimal buffer.

Table 3. Fractional catalytic concentration, related to phosphate, of bacterial α -glucosidase in various buffers, 50 mmol / l; substrate, 5,0 mmol / l (A, 4-nitrophenyl- α -D-glucopyranoside, B, 4-nitrophenyl- α -D-maltotrioxide), at pH 6,5 (adjusted with acetic acid or sodium hydroxide) and 30°C.

Type of buffer	(A)	(B)
Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane	0,74	0,65
N-(2-Acetamido)iminodiacetic acid	0,98	0,91
Piperazine-N,N'-bis(2-ethanesulfonic acid)	0,93	0,87
1,3-Bis[tris(hydroxymethyl)-methylamino]propane	0,73	0,85
N-(2-Acetamido)-2-aminoethanesulfonic acid	0,88	0,56
3-(N-Morpholino)propanesulfonic acid	0,71	0,62
N,N'-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid	0,66	0,52
Phosphate	1,00	1,00
Diethylmalonic acid	0,90	0,73
2,4,6-Trimethylpyridine	0,44	0,70
N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid	0,55	0,57
N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid	0,82	0,76
N-Tris(hydroxymethyl)-aminomethane	0,05	0,01

Table 4. Fractional catalytic concentration, related to phosphate with sodium chloride, 70 mmol / l, of bacterial α -glucosidase in some buffers, 50 mmol / l; under the conditions of the α -amylase assay at 30°C Conditions : (A) 4-nitrophenyl- α -D-glucopyranoside, 5,0 mmol / l; sodium chloride, 70 mmol / l; calcium chloride, 1 mmol / l; pH 7,10, (B) idem, without chloride.

Type of buffer	(A)	(B)
3-(N-Morpholino)propanesulfonic acid	0,71	0,38
Phosphate	1,00	1,03
2,4,6-Trimethylpyridine	1,50	0,44
Diethylmalonic acid	0,90	0,90
N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid	0,86	0,85

This also proves right under the conditions of the α -amylase assay. Unexpectedly, chloride strongly activated the enzyme in 3-(N-morpholino)propanesulfonic acid and, especially, in 2,4,6-trimethylpyridine buffer, where activity was higher than in phosphate (table 4).

3.3 Kinetic constants and product pattern

Contrasting with the yeast enzyme bacterial α -

glucosidase is claimed to cleave higher maltooligosaccharides faster than 4-nitrophenyl- α -D-glucopyranoside. Hence, kinetic constants, relative hydrolysis rates and product patterns were determined under the conditions of the α -amylase assay. The enzyme demonstrates ideal Michaelis-Menten kinetics with highest affinity to 4-nitrophenyl- α -D-maltotrioxide and maximal hydrolysis with 4-nitrophenyl- α -D-maltoside (table 5). For maximal velocity substrate concentrations above 20 mmol / l are needed.

Table 5. K_m values (mean $\pm$ standard deviation, $n = 5$) and relative hydrolysis rates at substrate, 4 mmol / l) of 4-nitrophenyl- α -D-maltooligosaccharides for bacterial- α -glucosidase at 30°C. Substrate consumption measurement by HPLC, estimation of K_m by Woolf-Hanes plot. Conditions: Calcium chloride, 1,0 mmol / l; sodium chloride, 70 mmol / l; N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, 50 mmol / l; substrate, 0,25, 0,5, 1,0, 2,0 and 4,0 mmol / l; pH 7,10.

Substrate	K_m (mmol / l)	Relative hydrolysis rate
4-Nitrophenyl- α -D-glucopyranoside	$2,33 \pm 0,06$	1,0
4-Nitrophenyl- α -D-maltoside	$1,20 \pm 0,03$	$2,42 \pm 0,08$
4-Nitrophenyl- α -D-maltotriose	$0,76 \pm 0,02$	$1,25 \pm 0,10$
4-Nitrophenyl- α -D-maltotetraose	$1,37 \pm 0,05$	$1,32 \pm 0,12$
4-Nitrophenyl- α -D-maltopentaose	$1,62 \pm 0,06$	$0,85 \pm 0,05$

K_m -values determined with 4-nitrophenyl- α -D-glucopyranoside by photometry are somewhat lower: Under the conditions of table 5 (i.e according to table 2) $2,16 \pm 0,06$ mmol / l are observed and $1,25 \pm 0,10$ mmol / l in phosphate at pH 6,5 (according to figure 2). The supplier reports 0,67 mmol / l in phosphate, 100 mmol / l, pH 7,0 at 37°C.

Digests of 4-nitrophenylated oligosaccharides

show the enzyme attacking preferably the ultimate bond at the non-reducing end thus acting like a glucoamylase. Obviously, only two products are formed, viz. the oligosaccharide minus and plus 1 glucose residue, but only 25% of the released glucose are transferred to the original substrate. If the degradation is restricted to 5-10% substrate consumption, degradation does not proceed to smaller products (table 6).

Table 6. Product pattern of 4-nitrophenyl- α -D-oligosaccharides hydrolyzed by α -glucosidase from *Bacillus stearothermophilus* under the conditions of the α -amylase assay, but with different substrates, 4,0 mmol / l. Incubation for 10 minutes at 30°C and quantification of products by high performance liquid chromatography (see 4.1 and 4.2).

Products	Substrates 4-Nitrophenyl- α -D-oligosaccharides				
	-glucoside	-maltoside	-maltotriose	-tetraose	-pentaose
4-Nitrophenol	3,05	-	-	-	-
4-Nitrophenyl- α -D-glucoside	94,5	7,36	-	-	-
4-Nitrophenyl- α -D-maltoside	2,43	90,5	4,67	0,44	-
4-Nitrophenyl- α -D-maltotriose	-	2,10	94,3	4,1	-
4-Nitrophenyl- α -D-maltotetraose	-	-	1,02	92,5	3,56
4-Nitrophenyl- α -D-maltopentaose	-	-	-	2,16	96,4

3.4 Temperature dependence and stability

The catalytic concentration of α -glucosidase under the conditions of the α -amylase assay at 30°C (as given in table 2) roughly corresponds to 60% of that in phosphate at pH 7,0 at 37°C. Arrhenius plots between 25 and 37°C yield straight lines with almost parallel slopes for the reaction mixture of the α -amylase assay (Q10 $2,05 \pm 0,06$, activation energy $3,15 \pm 0,1$ kJ/mol) and for phosphate, 50 mmol / l, pH 6,5 (Q10 $2,12 \pm 0,08$, activation energy $3,32 \pm 0,16$ kJ/mol).

The enzyme proved stable at 5°C for three weeks either in solution III (working solution) or in analogous mixtures, replacing N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid by 3-(N-morpholino) propanesulfonic acid or by 2, 4,6-trimethylpyridine, but it decayed after 12 days at 20°C storage in these solutions.

4. High Performance Liquid Chromatography of Reaction Products

4.1 Working parameters

Precolumn : Lichrocart (4x4 mm) with Lichrospher 5 μ m 100 NH₂
 Column : Lichrocart (125 x 4 mm) with Lichrospher 5 μ m 100 NH₂
 Sample volume : 10 μ l

Temperature:	20°C
Detection:	315 nm
Flow rate:	2 ml/60 s
Mobile phase:	Binary gradient system of A - acetonitrile/acetic acid, 20 mmol / l, 1/1 (v/v) and B - acetonitrile
Programme:	Step Time (s) A% B%
	1 0 10 90
	2 30 10 90
	3 210 50 50
	4 510 50 50
	5 660 10 90

4.2 Performance and results of chromatographic procedures

The system is capable to separate 4-nitrophenol and its glucosides to check the purity of the chromophore (figure 3) and to follow the action of α -glucosidase and α -amylase on 4-nitrophenyl- α -D-oligosaccharides as well. As an example, the hydrolysis of the substrate is stopped after various times by adding 0,05 volumes of concentrated acetic acid to the assay mixture followed by centrifugation for approximately 300 s with 12000 x g. The chromatography of the supernatant yields a typical order of peaks as demonstrated in figure 3.

Table 7. Retention times of 4-nitrophenol and some of its glucosides according to the working parameters.

Peak (minutes)	Retention time	Substance
1	$0,95 \pm 0,05$	4-Nitrophenol
2	$1,93 \pm 0,20$	4-Nitrophenol- α -D-glucopyranoside
3	$3,88 \pm 0,08$	4-Nitrophenol- α -D-maltoside
4	$4,85 \pm 0,07$	4-Nitrophenol- α -D-maltotrioside
5	$5,52 \pm 0,07$	4-Nitrophenol- α -D-maltotetraoside
6	$6,15 \pm 0,08$	4-Nitrophenol- α -D-maltopentaoside

Fig. 3. Separation of 4-nitrophenol and 4-nitrophenyl- α -D-oligosaccharides by high performance chromatography. Chromatographic conditions: see working parameters. The ciphers above the peaks correspond to those of table 7.

Fig. 4. Pattern of human pancreatic α -amylase reaction products from 4,6-ethylidene(G7)-4-nitrophenyl(G1)- α -(1 $\rightarrow$ 4)-D-maltoheptaoside. Chromatographic conditions: see working parameters. Peaks as in table 7.

REFERENCES

1. Robinson, R.A. and Biggs, A.I. (1955) The thermodynamic ionization constant of p-nitrophenol from spectrophotometric measurements. *Trans. Faraday Soc.* 51, 901-903.
2. Bowers, Jr., G.N., McComb, R.B., Christensen, R.G. and Schaffer, R. (1980) High-purity 4-nitrophenol; purification, characterization, and specifications for use as a spectrophotometric reference material. *Clin. Chem.* 26, 724-729.
3. Halvorson, H. (1966) α -Glucosidase from yeast. *Methods Enzymol.* 8, 559-562.
4. Needleman, R.B., Federof, H.J., Eccleshall, T.R., Buchferer, B. and Marmur, J. (1978) Purification and characterization of an α -glucosidase from *Saccharomyces carlsbergensis*. *Biochemistry* 17, 4657-4661.