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IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37°C

International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)[1), 2)]

Scientific Division, Committee on Reference Systems for Enzymes (C-RSE)[3)]

Part 8. Reference procedure for the measurement of catalytic concentration of α-amylase

[α-Amylase: 1,4-α-D-glucan 4-glucanohydrolase (AMY), EC 3.2.1.1]

Gerhard Schumann[1,*], Ryoji Aoki[2], Carlo A. Ferrero[3], Glenn Ehlers[4], Georges Férard[5], F.-Javier Gella[6], Poul Jørgen Jørgensen[7], Takahashi Kanno[8], Art Kessner[9], Rainer Klauke[1], Hans-Joachim Kytzia[10], Jean-Marc Lessinger[5], W. Gregory Miller[11], Rolf Nagel[10], Jean Pauwels[12], Heinz Schimmel[12], Lothar Siekmann[13], Gerhard Weidemann[14], Kiyoshi Yoshida[2] and Ferruccio Ceriotti[3]

[1] Klinische Chemie, Medizinische Hochschule Hannover, Hannover, Germany
[2] Research & Development, Asahi Chemical Industry Co., Tokyo, Japan
[3] Laboratorio Standardizzazione, Diagnostica e Ricerca San Raffaele s.p.a., Milano, Italy
[4] Ortho-Clinical Diagnostics, Rochester, NY, USA
[5] Laboratoire de Biochimie appliquée, Faculté de Pharmacie, Université Louis Pasteur, Illkirch, France
[6] BioSystems, S.A., Barcelona, Spain
[7] Department of Clinical Chemistry, Odense University Hospital, Odense, Denmark
[8] Department of Laboratory Medicine, Hamamatsu University, School of Medicine, Japan
[9] Beckman Coulter, Inc., Brea, CA, USA
[10] Roche Diagnostics GmbH, Mannheim, Germany
[11] Virginia Commonwealth University, Richmond, VA, USA
[12] European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Geel, Belgium
[13] Institut für Klinische Biochemie, Universität Bonn, Bonn, Germany
[14] Institut für Klinische Chemie und Laboratoriumsmedizin, Klinikum der Stadt, Nürnberg, Germany

Abstract

This paper is the eighth in a series dealing with reference procedures for the measurement of catalytic activity concentrations of enzymes at 37°C and the certification of reference preparations. Other parts deal with: Part 1. The concept of reference procedures for the measurement of catalytic activity concentrations of enzymes; Part 2. Reference procedure for the measurement of catalytic concentration of creatine kinase; Part 3. Reference procedure for the measurement of catalytic concentration of lactate dehydrogenase; Part 4. Reference procedure for the measurement of catalytic concentration of alanine aminotransferase; Part 5. Reference procedure for the measurement of catalytic concentration of aspartate aminotransferase; Part 6. Reference procedure for the measurement of catalytic concentration of γ-glutamyltransferase; Part 7. Certification of four reference materials for the determination of enzymatic activity of γ-glutamyltransferase, lactate dehydrogenase, alanine aminotransferase and creatine kinase at 37°C. The procedure described here is deduced from the previously described 30°C IFCC reference method. Differences are tabulated and commented on.
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[3)] Members: L. Siekmann[a] (DE), T. Kanno[a] (JP), F. Ceriotti[b] (IT), G. Férard[b] (FR), F. Canalias[c] (ES), P.J. Jørgensen[c] (DK), W.G. Miller[c] (USA), D. Kang[d] (JP), G. Schumann (DE); [a]member until 2004, [b]member until 2005, [c]member since 2005, [d]member since 2006.

*Corresponding author: Prof. Gerhard Schumann, Klinische Chemie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30623 Hannover, Germany
Phone: +49-511-5322523, Fax: +49-511-5325671, E-mail: schumann.gerhard@mh-hannover.de
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Abbreviations: AMY, α-amylase; EPS, 4,6-ethylidene(G1)-4-nitrophenyl(G7)-α-(1→4)-D-maltoheptaoside; G, α-(1→4)-D-glucopyranosyl-; 4-NP, 4-nitrophenoxide; G7-4-NP, 4-nitrophenyl-α-(1→4)-D-maltoheptaoside; IRMM, Institute for Reference Materials and Measurements.

Introduction

This paper is the eighth in a series dealing with reference procedures for the measurement of catalytic activity concentrations of enzymes at 37°C and the certification of reference prepartions (1–7).

Reaction principle

$$\text{EPS} + H_2O \xrightarrow{\alpha\text{-Amylase}} \text{4,6-Ethylidene-G x} + \text{4-Nitrophenyl-G (7–x)}$$

$$\text{4-Nitrophenyl-G (7–x)} + (7–x)\ H_2O \xrightarrow{\alpha\text{-Glucosidase}} (7–x)\ \text{Glucose} + \text{4-Nitrophenoxide}$$

Specimens

Calibration materials, control specimens and human sera.

Measurement conditions

Concentrations in the final reaction mixture and the measurement conditions are listed in Tables 1 and 2.

Note: Compliance with the prescribed tolerances for the parameters for temperature, pH, light path and wavelength is confirmed if the combined expanded uncertainty of the calibration is equal to or less than the tolerances **and** if the range of uncertainty of the calibration overlaps the prescribed tolerance intervals.

Table 2 Conditions for the measurement of α-amylase.

Temperature	37.0°C ± 0.1°C
Wave length	405 nm ± 1 nm
Band width	≤ 2 nm
Light path	10.00 mm ± 0.01 mm
Incubation time	60 s
Delay time	180 s
Measurement interval	180 s
Readings (measurement points)	≥ 6

Reagents

1. N-2-Hydroxyethylpiperazine-N′-ethanesulfonic acid [HEPES] ($C_8H_{18}N_2O_4S$), $M_r = 238.31$
2. 4,6-Ethylidene(G1)-4-nitrophenyl(G7)-α-(1→4)-D-maltoheptaoside [EPS], ($C_{50}H_{77}NO_{38}$), $M_r = 1300.1$
3. α-Glucosidase (EC 3.2.1.20)
4. Sodium chloride (NaCl), $M_r = 58.44$
5. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), $M_r = 147.02$
6. Sodium hydroxide solution (NaOH), $M_r = 40.00$, 0.2 mol·l^{-1}
7. Bovine serum albumin, fraction V, $M_r = 68000$
8. Aqueous sodium chloride solution (NaCl), $M_r = 58.44$, 0.154 mol·l^{-1}
9. Water (analytical grade), $M_r = 18.02$

Reagents of the highest purity must be used. The quality of the reagents has to be proved by certificates of analysis from the suppliers.

If a chemical is suspected of containing impurities affecting the catalytic activity of the analyte, further investigations must be performed, e.g., comparisons with products from different manufacturers and different lots. It is recommended to use reagents that have been tested and approved in comparisons.

EPS: Contamination of the substrate EPS by 4-nitrophenyl-α-D-maltoheptaoside (G7-4-NP) may cause a prolonged lag phase and may then interfere with the determination of the catalytic concentration of α-amylase. The mass fraction of G7-4-NP in EPS is limited to 0.001. If the mass fraction of G7-4-NP is unknown to the user or not clearly specified in a certificate, the mass concentration of G7-4-NP has to be determined (see Appendix 2).

α-Glucosidase: Only enzyme preparations of α-glucosidase that split all glucosidic bonds of the α-amy-

Table 1 Concentrations in the final complete reaction mixture for the measurement of α-amylase.

N-2-Hydroxyethylpiperazine-N′-ethanesulfonic acid	50 mmol·l^{-1}
pH (37°C)	7.00 ± 0.03
4,6-Ethylidene(G1)-4-nitrophenyl(G7)-α-(1→4)-D-maltoheptaoside	5 mmol·l^{-1}
Sodium chloride	70 mmol·l^{-1}
Calcium chloride	1 mmol·l^{-1}
α-Glucosidase (37°C)	135 μkat·l^{-1} (8100 U·l^{-1})*
Volume fraction of sample	0.0323 (1:31)

*Uninhibited catalytic concentration. *Note*: The indicated catalytic concentration of α-glucosidase shall be measured in the final complete reaction mixture if 9 g·l^{-1} (154 mmol·l^{-1}) sodium chloride solution is used as the sample (no inhibition by the sample matrix). *Note*: Besides the substances listed in Table 1, the final complete reaction mixture contains 0.1 g·l^{-1} albumin, which is originally a component of Solution 3. The presence of albumin in the Reaction Solution and consequently in the final complete reaction mixture stabilizes the α-glucosidase.

lase reaction products are suitable (e.g., from *Bacillus stearothermophilus*).

Bovine serum albumin: The bovine serum albumin used must be free from proteases as declared in the manufacturer's specifications.

Calibration of the pH meter

The molar absorption coefficient of the indicator strongly depends on the pH. Therefore the calibration of the pH-meter and the adjustments of the pH of solution 2 and the starting reagent solution must be very accurate. At least two standard buffer solutions have to be used for the calibration procedure. The standard buffer solutions must have certified values in the range from pH 6 to pH 8 and shall include the adjusting pH of the reagent solutions. The uncertainty of the certified pH has to be $\leq$0.01 pH.

Note: The use of standard buffer solutions containing potassium dihydrogen phosphate (anhydrous) and disodium hydrogen phosphate (anhydrous) is recommended. Appropriate certified standard reference buffer solutions traceable to national or international standard reference materials are commercially available.

Note: The molar absorption coefficient of the indicator (4-nitrophenoxide) strongly depends on the pH. A deviation in pH of 0.01 causes a change in the kinetics of approximately 1%. Therefore, the pH has to be adjusted very accurately.

Charts for the adjustment and control of pH

Procedure for the adjustment of pH at temperatures diverging from 37°C

Both the thermometer and the pH electrode are suspended in the mixed solution simultaneously. The stirred solution is then titrated to the pH listed in Tables 3 and 4 for the actual temperature measured. The speed of agitation should be the same during calibration and control of the pH meter and adjustment of the pH of the reagent solutions. The pH electrode should be positioned in the center of the stirred solution.

The fact that the temperature can change during the titration must be taken into account. For this reason, the temperature in the proximity of the target value should be controlled again and the target pH has to be corrected if necessary. The same applies for the adjustment of the temperature compensation of the pH meter.

Table 3 Dependence of the pH of Solution 2 on temperature.

Temperature, °C	pH	Temperature, °C	pH	Temperature, °C	pH
15.00	7.254	23.50	7.154	32.00	7.056
15.25	7.251	23.75	7.151	32.25	7.053
15.50	7.248	24.00	7.148	32.50	7.051
15.75	7.245	24.25	7.145	32.75	7.048
16.00	7.242	24.50	7.142	33.00	7.045
16.25	7.239	24.75	7.139	33.25	7.042
16.50	7.236	25.00	7.136	33.50	7.039
16.75	7.233	25.25	7.133	33.75	7.036
17.00	7.230	25.50	7.130	34.00	7.034
17.25	7.227	25.75	7.128	34.25	7.031
17.50	7.224	26.00	7.125	34.50	7.028
17.75	7.221	26.25	7.122	34.75	7.025
18.00	7.218	26.50	7.119	35.00	7.022
18.25	7.215	26.75	7.116	35.25	7.020
18.50	7.212	27.00	7.113	35.50	7.017
18.75	7.209	27.25	7.110	35.75	7.014
19.00	7.206	27.50	7.107	36.00	7.011
19.25	7.203	27.75	7.105	36.25	7.008
19.50	7.200	28.00	7.102	36.50	7.006
19.75	7.197	28.25	7.099	36.75	7.003
20.00	7.194	28.50	7.096	37.00	7.000
20.25	7.192	28.75	7.093	37.25	6.997
20.50	7.189	29.00	7.090	37.50	6.994
20.75	7.186	29.25	7.087	37.75	6.992
21.00	7.183	29.50	7.085	38.00	6.989
21.25	7.180	29.75	7.082	38.25	6.986
21.50	7.177	30.00	7.079	38.50	6.983
21.75	7.174	30.25	7.076	38.75	6.981
22.00	7.171	30.50	7.073	39.00	6.978
22.25	7.168	30.75	7.070	39.25	6.975
22.50	7.165	31.00	7.068	39.50	6.972
22.75	7.162	31.25	7.065	39.75	6.969
23.00	7.159	31.50	7.062	40.00	6.967
23.25	7.157	31.75	7.059		

Table 4 Dependence of the pH of the starting reagent solution on temperature.

Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)	pH
15.00	7.270	23.50	7.165	32.00	7.058
15.25	7.267	23.75	7.162	32.25	7.055
15.50	7.264	24.00	7.158	32.50	7.052
15.75	7.261	24.25	7.155	32.75	7.049
16.00	7.258	24.50	7.152	33.00	7.046
16.25	7.255	24.75	7.149	33.25	7.043
16.50	7.252	25.00	7.146	33.50	7.040
16.75	7.249	25.25	7.143	33.75	7.037
17.00	7.246	25.50	7.139	34.00	7.034
17.25	7.243	25.75	7.136	34.25	7.032
17.50	7.240	26.00	7.133	34.50	7.029
17.75	7.237	26.25	7.130	34.75	7.026
18.00	7.234	26.50	7.127	35.00	7.023
18.25	7.231	26.75	7.124	35.25	7.020
18.50	7.227	27.00	7.120	35.50	7.017
18.75	7.224	27.25	7.117	35.75	7.014
19.00	7.221	27.50	7.114	36.00	7.011
19.25	7.218	27.75	7.111	36.25	7.008
19.50	7.215	28.00	7.108	36.50	7.006
19.75	7.212	28.25	7.105	36.75	7.003
20.00	7.209	28.50	7.102	37.00	7.000
20.25	7.206	28.75	7.099	37.25	6.997
20.50	7.203	29.00	7.095	37.50	6.994
20.75	7.199	29.25	7.092	37.75	6.992
21.00	7.196	29.50	7.089	38.00	6.989
21.25	7.193	29.75	7.086	38.25	6.986
21.50	7.190	30.00	7.083	38.50	6.984
21.75	7.187	30.25	7.080	38.75	6.981
22.00	7.184	30.50	7.077	39.00	6.978
22.25	7.181	30.75	7.074	39.25	6.976
22.50	7.177	31.00	7.071	39.50	6.973
22.75	7.174	31.25	7.068	39.75	6.970
23.00	7.171	31.50	7.064	40.00	6.968
23.25	7.168	31.75	7.061		

Preparation of solutions

The mass given for compounds for the preparation of solutions refers to 100% content. If the content of the reagent chemical employed is less (e.g., yz %), the amount equivalent to the given mass is calculated using a factor:

$$F_{content} = \frac{100}{yz}$$

Impurities in the water for the preparation of the reagent solutions for α-amylase measurements may cause inhibition of the catalytic activity. Guidelines describing the preparation and testing of reagent water are published elsewhere (9). However, standards prescribing the quality of water specifically for enzyme measurements are not yet defined.

In the case of suspected influence of the water on determination of the catalytic activity concentration, systematic investigations have to be performed.

The expanded (k=2) combined uncertainty (normally distributed) of each weighing procedure (including the uncertainty of the purity of the substance) shall be ≤1.5% and the mass on the display of the balance shall not differ from the target mass by more than ±0.5%.

Table 5 Analytical system for the measurement of α-amylase.

2.000 ml	Reaction solution Equilibrate to 37.0°C
0.080 ml	Sample Mix thoroughly and incubate for 60 s. At the end of the incubation time, the temperature of the solution in the cuvette shall have reached 37.0°C
0.400 ml	Starting reagent solution Mix thoroughly, wait 180 s and monitor time and absorbance for an additional 180 s

Solution 1

6.14 g (417.5 mmol·l^{-1}) calcium chloride, dihydrate.

- Dissolve in approximately 80 ml of water.
- Transfer to a 100-ml volumetric flask.
- Equilibrate the volumetric flask and water to 20°C.
- Fill with water (20°C) up to the calibration mark of the volumetric flask.

Stability at 20°C: 3 months.

Solution 2

3.10 g (52.10 mmol·l^{-1}) N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid.

1.26 g (86.13 mmol·l^{-1}) sodium chloride.

- Dissolve in approximately 200 ml of water.
- Add 0.75 ml of solution 1.
- Adjust to pH (37°C) 7.00 with 0.2 mol·l^{-1} sodium hydroxide solution.
- Transfer to a 250-ml volumetric flask.
- Equilibrate the volumetric flask and water to 20°C.
- Fill with water (20°C) up to the calibration mark of the volumetric flask.

Stability at 2°C–8°C: 5 weeks.

Diluent for reagent enzymes

1.20 g bovine albumin.
0.900 g (154 mmol l^{-1}) NaCl.

- Dissolve in approximately 80 ml of water.
- Transfer to a 100-ml volumetric flask.
- Equilibrate the volumetric flask and water to 20°C.
- Fill with water (20°C) up to the calibration mark of the volumetric flask.

Stability at 2°C–8°C: at least 3 months.

Solution 3

16.9 mkat·l^{-1} (1014 kU·l^{-1}) α-glucosidase at 37°C.

- Determine the catalytic α-glucosidase concentration according to Appendix 1.
- Reconstitute the lyophilized α-glucosidase with a volume of the Diluent for Reagent Enzymes to obtain a catalytic concentration of the reconstituted material of 16.9 mkat·l^{-1} (1014 kU·l^{-1}) at 37°C.
- Freeze the enzyme solution in portions of 0.25 ml at –25°C.

Stability at −25°C: at least 6 months.

Reaction solution

- Mix 25 ml of solution 2 with 0.25 ml of solution 3.

Stability at 2°C–8°C: 2 weeks.

Starting reagent solution

1.01 g (31.00 mmol·l^{-1}) 4,6-ethylidene(G1)-4-nitrophenyl(G7)-α-(1→4)-D-maltoheptaoside.
0.310 g (52.10 mmol·l^{-1}) N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid.

- Dissolve in approximately 20 ml of water.
- Adjust to pH (37°C) 7.00 with 0.2 mol·l^{-1} sodium hydroxide solution.
- Transfer to a 25-ml volumetric flask.
- Equilibrate the volumetric flask and water to 20°C.
- Fill with water (20°C) up to the calibration mark of the volumetric flask.

Stability at 2°C–8°C: 2 weeks.

Measurement procedure

Equilibrate only an adequate volume (≈0.6 ml per measurement) of the starting reagent solution at 37°C shortly before the measurement procedure. The remaining volume of the starting reagent solution should be stored at 2°C–8°C.

Pipette the volumes listed in Table 5 one after another into the cuvette.

A high-performance spectrometer and high-accurate volumetric devices shall be used. The inaccuracy of the kinetic photometric measurements and the inaccuracy of the volume fraction of sample shall be determined by test procedures with known standard uncertainties.

Reagent blank rate

To determine the reagent blank rate, the sample is replaced by 9 g·l^{-1} (154 mmol·l^{-1}) sodium chloride solution. The measurement procedure is then carried out as described above. The initial absorbance should not exceed 0.35 and the change in absorbance of the reagent blank rate should be less than 3.3×10^{-5} s^{-1} (0.002 min^{-1}). Otherwise, sources of contamination by salivary amylase should be identified and excluded or the purity of the reagents must be reassessed.

Sample blank rate

For determination of the sample blank rate, the starting reagent solution is replaced by 9 g·l^{-1} (154 mmol·l^{-1}) sodium chloride solution. The measurement procedure is then carried out as described above.

Note: The sample blank is determined and documented, but not taken into account for calculation of the catalytic concentration of α-amylase in control sera and calibrators. If the value of the sample blank exceeds 1% of total α-amylase, a warning should be given that the material is not appropriate for calibration.

Note: The reagent blank for the sample blank is determined by replacing the starting reagent solution **and** the sample by 9 g·l^{-1} sodium chloride solution.

Note: Omission of the starting reagent means omission of the substance that forms the indicator. Therefore, interference by the sample matrix on the indicator reaction is not identified.

Upper limit of the measurement range

If the change in absorbance exceeds 0.0039 s^{-1} (0.235 min^{-1}) in the measurement interval, an analytical portion of the sample must be diluted with 9 g·l^{-1} (154 mmol·l^{-1}) aqueous sodium chloride solution and the measurement procedure must be repeated with the diluted sample. The value obtained must then be multiplied by the corresponding dilution factor.

Sources of error

The catalytic concentration of α-glucosidase in the final complete reaction mixture is differently inhibited by the specific matrix of the specimen investigated (see the respective comments in Appendix 3). Therefore, determination of the matrix-dependent inhibition

of α-glucosidase is necessary for each material investigated (see Appendix 1).

Calcium complexing agents reduce the available concentration of this essential component and can lead to reduced measurement values.

Contamination by salivary amylase (e.g., by finger contact with the solutions or the inner sides of the solution containers) leads to falsely increased catalytic concentrations of α-amylase or to increased reagent blank rates.

Calculation

The temporal change in absorbance (s^{-1}) is calculated using regression analysis (least squares method). After subtraction of the reagent blank rate (s^{-1}), the corrected change in absorbance (s^{-1}) is multiplied by the factor

F = 3063
[measurement at 405 nm, ε_{405}(4-NP) = 1012 $m^2 \cdot mol^{-1}$]

Note: Use of the molar absorption coefficient ε_{405}(4-NP) = 1012 $m^2 \cdot mol^{-1}$ is recommended by IFCC and IRMM (10).

The catalytic concentration of α-amylase is calculated in $\mu kat \cdot l^{-1}$.

$\frac{\Delta A}{\Delta t}_{Amylase}$ Change in absorbance (s^{-1}) after correction for the reagent blank rate (s^{-1})

$b_{Amylase}$ Catalytic concentration of α-amylase ($\mu kat \cdot l^{-1}$)

$$b_{Amylase} = 3063 \frac{\Delta A}{\Delta t}_{Amylase}$$

The catalytic concentration in $\mu kat \cdot l^{-1}$ can be converted to $U \cdot l^{-1}$ by multiplication by the factor f = 60.

Preliminary upper and lower reference limits

Reference values

Preliminary reference intervals were obtained in two different reference laboratories by measuring α-amylase activity in serum samples from two reference cohorts (146 males and 89 females). The reference cohorts comprised serum samples from employees of an annual wellbeing check (first cohort) and from blood donors (second cohort). Samples were excluded from the reference cohort if the concentration at least one of the following quantities in serum exceeded the corresponding upper reference limit: creatinine, glucose, AST, ALT, GGT (and/or ALP) and lipase (cohort 2 only). No significant differences (t-test, $p < 0.05$) could be observed between the two reference cohorts or between samples from males and females.

The preliminary reference limits for men and women (≥ 17 years) are:

Lower reference limit* (and 90% confidence interval for the 2.5th percentile)
0.52 $\mu kat \cdot l^{-1}$ (0.42 $\mu kat \cdot l^{-1}$–0.60 $\mu kat \cdot l^{-1}$)
31 $U \cdot l^{-1}$ (25 $U \cdot l^{-1}$–36 $U \cdot l^{-1}$)

Upper reference limit* (and 90% confidence interval for the 97.5th percentile)
1.78 $\mu kat \cdot l^{-1}$ (1.71 $\mu kat \cdot l^{-1}$–2.00 $\mu kat \cdot l^{-1}$)
107 $U \cdot l^{-1}$ (103 $U \cdot l^{-1}$–120 $U \cdot l^{-1}$)

*The lower and upper reference limits are the 2.5th and 97.5th percentiles of the reference cohorts. Values in parentheses are the 90% confidence intervals for the 2.5th and 97.5th percentiles.

Appendix 1: Determination of the catalytic concentration of α-glucosidase under the reaction conditions of the procedure for α-amylase

Additional reagents

4-Nitrophenyl-α-D-glucopyranoside ($C_{12}H_{15}NO_8$)
M_r = 301.26

Solution A

1.24 g (52.08 $mmol \cdot l^{-1}$) N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid.
0.419 g (71.63 $mmol \cdot l^{-1}$) NaCl.

- Dissolve in approximately 80 ml of water.
- Add 0.25 ml of solution 1 for the procedure for α-amylase (calcium chloride stock solution).
- Adjust pH (37°C) to 7.00 with 0.2 $mol \cdot l^{-1}$ sodium hydroxide solution.
- Transfer to a 100-ml volumetric flask.
- Equilibrate the volumetric flask and water to 20°C.
- Fill with water (20°C) to the calibration mark of the volumetric flask.

Stability at 2°C–8°C: 5 weeks.

Solution B

0.900 g (154.0 $mmol \cdot l^{-1}$) sodium chloride.

- Dissolve in approximately 80 ml of water.
- Transfer to a 100-ml volumetric flask.
- Equilibrate the volumetric flask and water to 20°C.
- Fill with water (20°C) to the calibration mark of the volumetric flask.

Stability at 20°C: 3 months.

Reaction solution for α-glucosidase

0.0392 g (5.208 $mmol \cdot l^{-1}$) 4-nitrophenyl-α-D-glucopyranoside

- Transfer to a 25-ml volumetric flask and dissolve in approximately 20 ml of solution A.
- Equilibrate the volumetric flask and solution A to 20°C.

Table 6 Concentrations in the final complete reaction mixture for the measurement of α-glucosidase.

N-2-Hydroxyethylpiperazine-N′-ethanesulfonic acid	50 mmol·l^{-1}
pH (37°C)	7.00 ± 0.03
4-Nitrophenyl-α-D-glucopyranoside	5 mmol·l^{-1}
Sodium chloride	70 mol·l^{-1}
Calcium chloride	1 mmol·l^{-1}
Volume fraction of sample	0.0320 (1:31.25)

Table 7 Conditions for the measurement of α-glucosidase.

Temperature	37.0°C ± 0.1°C
Wave length	405 nm ± 1 m
Band width	≤ 2 nm
Light path	10.00 mm ± 0.01 mm
Incubation time	60 s
Delay time	180 s
Measurement interval	180 s
Readings (measurement points)	≥ 6

Table 8 Analytical system for the measurement of α-glucosidase.

2.400 ml	Reaction solution for α-glucosidase Equilibrate to 37°C
0.080 ml	Solution B Mix thoroughly and wait 60 s
0.020 ml	Solution of α-glucosidase diluted with the Diluent for Reagent Enzymes Mix thoroughly, wait 180 s and monitor time and absorbance for an additional 180 s

Note: Solution B here replaces the sample in the α-amylase measurement system.

- Fill with solution A (20°C) up to the calibration mark of the volumetric flask.

  Stability at 2°C–8°C: 5 days.

Measurement conditions

Concentrations in the reaction mixture and measurement conditions are listed in Tables 6 and 7.

Preparation of α-glucosidase

1. Weight approximately 10 mg of the lyophilized enzyme (record the weighed mass; *m*) and reconstitute with 1000 µl of Diluent for Reagent Enzymes for the procedure for α-amylase.
2. Dilute 50 µl of the reconstituted α-glucosidase with 1000 µl of the Diluent for Reagent Enzymes. The dilution of the first step is 1:21.
3. Dilute 50 µl of the solution from step 1 with 1000 µl of the Diluent for Reagent Enzymes. The dilution of the second step is 1:21. The total dilution after step 1 and step 2 is 1:441. The dilution factor is thus 441.

Note: The indicated dilutions in step 1 and step 2 are appropriate for lyophilized α-glucosidase containing 0.40–1.65 µkat·mg^{-1} (25–100 U·mg^{-1}). The preparation of lyophilized α-glucosidase may necessitate other dilutions. The dilution factor must be changed corresponding to the dilution.

Measurement procedure

The catalytic concentration of α-glucosidase is determined under reaction conditions very close to those of the procedure for α-amylase.

The reaction is started with the diluted solution of α-glucosidase.

Pipette the volumes indicated in Table 8 sequentially into the cuvette.

Reagent blank rate

To determine the reagent blank rate, the diluted solution of α-glucosidase is replaced by the Diluent for Reagent Enzymes. The measurement procedure is then carried out as described above.

Calculation

The temporal change in absorbance (s^{-1}) is calculated using regression analysis (least squares method). The reagent blank rate is subtracted. The corrected change of absorbance is used for the calculation of the catalytic mass concentration:

Calculation of the catalytic mass concentration of α-glucosidase lyophilizate powder:

$$b_{lyo} = \frac{F_{dilution} 12350 \frac{\Delta A}{\Delta t}}{1000\ m}$$

Note: This formula is only valid if the volume for the reconstitution is 1000 µl (see step 1 in the section Preparation of α-glucosidase).

12350 Calculation factor [measurement at 405 nm, ε_{405}(4-NP) = 1012 m^2·mol^{-1}]

$\frac{\Delta A}{\Delta t}$ Change in absorbance after correction of the reagent blank rate (s^{-1}).

$F_{dilution}$ Dilution factor (441 for the example above)

m Weighed mass (mg) of the reconstituted α-glucosidase.

b_{lyo} Catalytic mass concentration [µkat·mg^{-1}] of α-glucosidase in the lyophilized material.

The catalytic mass concentration in µkat·mg^{-1} can be converted to U·mg^{-1} by multiplication by the factor f = 60.

The same procedure is also used to determine the relative inhibition of α-glucosidase caused by the matrix of some α-amylase samples. Two measure-

Table 9 Comparison of the IFCC methods for measurement temperatures of 30°C and 37°C.

37°C Reference procedure	30°C Reference method	Comment
Specimen investigated		
Calibration materials, control specimens and human sera	Human serum	The reference procedure is used primarily for the measurement of control samples and calibration materials
pH of the final complete reaction mixture		
The optimum pH is 7.00	The optimum pH is 7.10	The optimum pH depends on the measurement temperature
pH of solution 2		
There is no difference from the pH of the reaction mixture	The difference from the pH of the reaction mixture is 0.02 units	Addition of EPS to the final reaction mixtures lowers the pH in the procedure for 30°C
Tolerance for the pH adjustment		
pH $\pm$0.03	Not specified	
Tolerance for the temperature adjustment		
Uncertainty $\leq$0.1°C (k=2)	Bias less than $\pm$0.05°C, imprecision less than $\pm$0.1°C	High quality spectrophotometer with devices for temperature adjustment and control should provide temperature uncertainty (k=2) of $\leq$0.1°C
Incubation time		
60 s	At least 300 s	There is no need for a long incubation time of 300 s if the buffer solution and the sample are pre-warmed to 37°C. Contact between the sample and buffer should not be unnecessarily long
Measurement time		
180 s	At least 300 s	Higher signals at 37°C allow a shorter measurement time without increasing the imprecision
Catalytic α-glucosidase concentration		
8100 U·l^{-1}	4800 U·l^{-1}	Same amount of α-glucosidase for 30°C and 37°C. The higher temperature induces an increase in enzyme activity
Preparation of the starting reagent solution		
The starting reagent solution is buffered with HEPES. The pH has to be adjusted to 7.00. The pH of solution 2 is also adjusted to 7.00	The starting reagent solution is prepared with solution 2 (pH 7.12) as the solvent. The pH of the final reaction mixture is 7.10 owing to the acidity of EPS	The acidity of EPS seems to differ from lot to lot. Therefore, the pH of solution 2 and of the starting reagent solution has to be adjusted separately
Determination of the inhibition of α-glucosidase by the sample matrix		
Determination of the relative decrease in the catalytic concentration of α-glucosidase caused by the sample matrix is described and maximum allowable inhibition is defined	Did not deal with the problem of inhibition of α-glucosidase by the sample matrix	Relative decreases of 0–60% of the catalytic concentrations of α-glucosidase were observed (investigation by Schumann and Klauke of 17 control materials and calibrators, and 11 human sera)
Control of the mass fraction of G7-4-NP in EPS		
Contamination of EPS by G7-4-NP is limited to 0.1%. A procedure for checking compliance with this specification is included in the reference procedure	Did not deal with the problem of G7-4-NP contamination of the substrate	G7-4-NP causes a prolonged lag phase that can be longer than the delay time. Determination of the catalytic concentration of α-amylase is influenced in these cases. Therefore, contamination is limited to 0.1% in the EPS specification
Unit of catalytic enzymatic concentration		
μkat·l^{-1} and U·l^{-1}	μkat·l^{-1}	U·l^{-1} is the common unit in clinical chemistry, but μkat·l^{-1} is based on the SI system
Diluent for the reagent enzyme and preparation of the reaction solution		
Bovine serum albumin and sodium chloride in water are used for the preparation of stock α-glucosidase solution, which is stored at –25°C until use. The reaction solution is prepared by pipetting 0.25 ml of the α-glucosidase solution into 25 ml of solution 2	Lyophilized α-glucosidase is used for preparation of the reaction solution. The mass equivalent to 600 U necessary for 100 ml of reaction solution is weighed on a micro balance	The procedure for 37°C seems to be simpler. The preparation of volumes smaller than 100 ml of reaction solution is possible without loss of precision of the enzyme content. The α-glucosidase solution is stable for a long time (6 months at –25°C)

(Table 9 continued)

37°C Reference procedure	30°C Reference method	Comment
Sample blank rate		
Not taken into account	Subtraction	Usually, sample blank rates are not subtracted in routine procedures. Therefore, assigned values for calibrators and control materials are only useful for routine methods if they include the sample blank rate
Temperature of the starting reagent solution before use		
The starting reagent solution should reach a temperature of 37°C before use	No information about the temperature	The use of starting reagent solution at ambient temperature decreases the temperature in the cuvette
Data collection		
Number of readings ≥ 6	No information about the number of measurement points	At least 6 readings should ensure sufficient precision of the measurement results
Determination of the slope (time vs. absorbance)		
Regression analysis using the least squares method	No information	A well-defined statistical method is necessary to ensure the reproducibility of the slope calculation
Reference range		
Women and men 0.52–1.78 $\mu kat \cdot l^{-1}$ (31–107 $U \cdot l^{-1}$)	Women 0.41–1.08 $\mu kat \cdot l^{-1}$ (24.4–64.8 $U \cdot l^{-1}$) Men 0.42–1.10 $\mu kat \cdot l^{-1}$ (25.2–66.0 $U \cdot l^{-1}$)	Reference values for women and men were investigated separately for the measurement temperature of 37°C
Molar absorption coefficient nitrophenoxide in the reaction mixture		
The molar absorption coefficient was determined as ε_{405}(4-NP) = 1012 $m^2 \cdot mol^{-1}$.	ε_{405}(4-NP) = 1056.8 $m^2 \cdot mol^{-1}$ in the reaction solution at 30°C.	The difference can be explained partly by the different pH of the reaction mixtures at 30°C and 37°C.
ε is independent of the protein content of the sample	ε_{405}(4-NP) = 975 $m^2 \cdot mol^{-1}$ if samples with total protein concentrations between 65 and 77 $g \cdot l^{-1}$ are added to the reaction mixture	The reason for the influence of protein at 30°C could not be explained. The use of ε_{405}(4-NP) = 1012 $m^2 \cdot mol^{-1}$ is recommended by IRMM/IFCC

ments are performed: one using Solution B and the other substituting Solution B by the α-amylase sample under investigation. In both measurements, the reaction is started with a solution of α-glucosidase diluted with the Diluent for Reagent Enzymes to a catalytic concentration between 8 $\mu kat \cdot l^{-1}$ and 33 $\mu kat \cdot l^{-1}$ (500 $\mu \cdot l$ and 2000 $U \cdot l^{-1}$).

Calculation of the matrix-dependent inhibition of α-glucosidase

The relative (%) inhibition of the catalytic concentration of α-glucosidase caused by the matrix of the sample in the final complete reaction mixture is calculated from the conversion rates obtained with and without the investigated sample.

$$Inh = 100\left(1 - \frac{\frac{\Delta A}{\Delta t}_{sample}}{\frac{\Delta A}{\Delta t}_{Sol\ B}}\right)$$

$\frac{\Delta A}{\Delta t}_{Sample}$ Change in absorbance in the presence of the sample investigated instead of Solution B (s^{-1})

$\frac{\Delta A}{\Delta t}_{Sol\ B}$ Change in absorbance using Solution B (s^{-1})

Inh Relative (%) inhibition of the catalytic concentration of α-glucosidase caused by the sample matrix in the final complete reaction mixture

Note: If the relative inhibition of the catalytic concentration of α-glucosidase by the test sample exceeds 45%, the lag phase may be not complete during the delay time. Such material should not be recommended as a calibrator or control material.

Appendix 2: Control of the mass fraction of G7-4-NP in EPS

Solution 3 of the reference procedure for α-amylase is diluted 1+4 with the Diluent for Reagent Enzymes. This solution is mixed with the starting reagent solution at 1+100. Another starting reagent solution is mixed at the same ratio with the Diluent for Reagent Enzymes. Both mixtures are incubated at ambient temperature and protected from light for 1 h.

The absorbance at 405 nm of the starting reagent solution containing α-glucosidase is measured at 37°C. Starting reagent solution without α-glucosidase is used as the reference.

The contamination of EPS by G7-4-NP is $\leq$0.1% if the measured absorbance is $\leq$0.32.

Note: The absorbance value of 0.32 is based on EPS with a content of 100%. Correction has to be performed if the lot of EPS used has a content $<$100%.

Appendix 3: Changes in the IFCC reference procedure for measurements at 37°C compared with the reference method for measurements at 30°C as described in the original IFCC document

The primary reference procedure is derived from the IFCC reference method (8), which provides optimized conditions for the measurement of catalytic activity concentrations of α-amylase. The measurement temperature of 37°C instead of 30°C requires only minimal changes of certain measurement parameters to retain the optimum measurement conditions. The modifications are listed and commented on in Table 9. Furthermore, if a more accurate specification in comparison to the 30°C reference method has become necessary to improve the high level of standardization of the measurements, it is also described here.

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