

SPECIFICATION

Process and reagent for the determination of α -amylase

The present invention is concerned with a process and a reagent for the determination of α -amylase.

The determination of α -amylase in serum is an important clinical parameter for the function of the pancreas. The commercially available reagents for the determination of α -amylase are preponderantly based upon a system in which starch is broken down by α -amylase and the fragments formed are determined in the visible range of light or in the UV range, depending upon whether coloured starch or native starch is used in the test as the substrate for the amylase. An important disadvantage of these processes and reagents is that starch, which is a macromolecule, cannot be sufficiently standardised and characterised so that the rate of reaction of individual batches can vary very greatly and, when carrying out measurements, a standard must always be included. For better results, a more uniform substrate would be necessary which provides dependable results in the case of fission.

A step forwards in the direction of a more uniform substrate occurred with the use of maltopentaose. This is split by α -amylase into maltotriose and maltose and maltotriose and maltose are converted by α -glucosidase into glucose which can then be determined by any desired method, for example by the known hexokinase method.

Besides maltopentaose, it has also already been proposed to use maltotetraose and maltohexaose as substrate (see U.S. Patent Specifications Nos. 3,879,263 and 4,000,042). However, in this case, the results obtained with the tetraose were markedly poorer than those obtained with the pentaose and with hexaose even worse results were obtained than with the tetraose. Thus, in the case of maltotetraose and -pentaose, it is still possible to obtain a stoichiometric reaction whereas in the case of the hexaose, just still tolerable deviations from the stoichiometric reaction were ascertained.

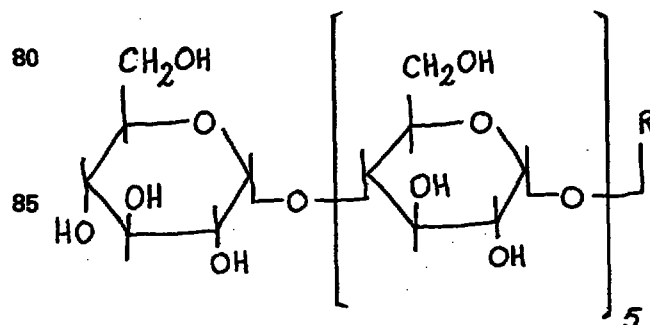
A disadvantage of maltopentaose, which was also found in the case of the tetraose, is, however, that a considerable reagent blank occurs, i.e. the measurement reaction already starts before the sample to be determined is added. Furthermore, this reagent blank is not constant in the case of comparatively high substrate concentrations but rather changes for more than 25 minutes before a constancy of this side reaction is achieved.

It has also been ascertained that the assumed different fission of maltopentaose by pancreas α -amylase and saliva α -amylase, which would have enabled a differentiation, does not actually exist (see J. BC., 1970, 245, 3917 — 3927; J. Biochem., 51, p. XVIII, 1952).

Therefore, it is an object of the present invention to provide a process and a reagent for the determination of α -amylase in which a substrate is used which has a higher degree of purity and uniformity than the known substrates, is readily obtainable and satisfies the requirements with regard to the blank

value without serum, the length of the lag phase and the achievable maximum activity. Furthermore, a simple measurement without expensive and complicated apparatus is to be possible and a suitability for rapid diagnostics, such as test strips, is to be provided.

Thus, according to the present invention, there is provided a process for the determination of α -amylase by the enzymatic fission of an α -amylase substrate and measurement of a fission product, wherein, as substrate, there is used a compound of the general formula:—



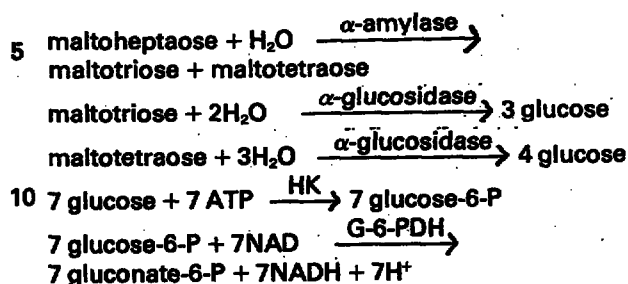
in which R is a glucoside, phenylglucoside, mononitrophenylglucoside, dinitrophenylglucoside, sorbitol or gluconic acid group.

We have, surprisingly, found that maltoheptaose possesses superior properties as a substrate for α -amylase, although in the case of the oligomaltoses from maltopentaose to maltohexaose, which have already been suggested for this purpose, a decrease in suitability has been ascertained since the maltohexaose gives results which are substantially poorer than those achieved with the pentaose. Therefore, it was to have been expected that with a further lengthening of the maltose oligosaccharide chain, no longer tolerable errors would occur. Surprisingly, however, better results are achieved than with the pentaose. Thus, for example, the reagent blank value in the case of 0.02 ml. of sample with maltopentaose as substrate amounts to 73% but with maltoheptaose amounts to only 13%, referred to the end-point of the determination.

Furthermore, we have found that instead of maltoheptaose itself, certain maltoheptaose derivatives can also be employed which, by the action of α -amylase, form a derivatised fission product which can be determined especially advantageously.

The process according to the present invention is especially suitable for the determination of the fission products by means of α -glucosidase or maltose phosphorylase.

In the case of the determination with α -glucosidase and a compound of general formula (I) in which R is a glucoside group, the fission products of the maltoheptaose, i.e. maltotetraose and maltotriose, are further split to give glucose, which can then be measured in known manner. For the measurement of the glucose formed in the presence of α -glucosidase, the hexokinase process is particularly preferred. The principle of this embodiment of the process according to the present invention can be illustrated by the following equations:



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In the above equations, HK means hexokinase, NAD means nicotinamide - adenine - dinucleotide, NADH means the reduced form thereof, G - 6 - PDH means glucose - 6 - phosphate dehydrogenase and ATP means adenosine triphosphate.

For this embodiment of the present invention, i.e. use of α -glucosidase there are also especially useful the maltoheptaose derivatives employed according to the present invention, i.e. compounds of general formula (I) in which R is other than a glucoside group. By the action of the two enzymes α -amylase and α -glucosidase, the substituent of R, i.e. a phenol group, a mononitrophenyl group or a dinitrophenyl group or the terminal sorbitol or gluconic acid residue, is split off and can easily be determined. The phenyl groups can be in the α - or β - position. In the case of the α -position, splitting off thereof takes place by the action of α -amylase and α -glucosidase alone and the substituted or unsubstituted phenols split off can then be easily determined by known colour reactions. However, the present invention can also be employed in the case of β -positioned substituents, in which case, in addition to α -glucosidase, β -glucosidase is also employed.

In the case of the dinitrophenyl groups, the two nitro groups can be present in any desired position, for example as 2,4-, 2,6- or 3,5-substituents.

The nitrophenols or dinitrophenols liberated by the splitting off of the nitro group-containing substituents are themselves coloured compounds which can easily be determined optically. If phenol itself is split off, then this can be determined by known methods, for example by reaction with a nucleophilic reagent, such as 3 - methyl - 6 - sulphonyl-benzthiazolone - hydrazone - (2) (HSK), in the presence of monophenol oxidase, a red coloured material being formed which can be measured.

In the case of splitting off sorbitol, this can be oxidised, for example, by sorbitol dehydrogenase to give fructose and, at the same time, NAD present is reduced to NADH. The formation of NADH can then easily be determined in known manner with a UV spectrophotometer. If this is not available, then, by reaction with a tetrazolium salt, for example 2 - (p - iodophenyl) - 3 - (p - nitrophenyl) - 5 - phenyl - tetrazolium chloride (INT), in the presence of diaphorase or some other electron carrier, a coloured formazan is formed which can be measured in the visible spectrum.

In an analogous manner, liberated gluconic acid can be determined by known methods, for example with gluconate kinase, 6 - phosphogluconate dehydrogenase and nicotinamide - adenine - dinucleotide phosphate (NADP), as well as optionally a tetrazolium salt and an electron carrier.

The process according to the present invention is generally carried out at a pH value of from 5 to 9. However, it is preferable to use a pH value of from 7 to 7.5 since, in this case, the best results are obtained in the shortest reaction times. If, according to the present invention, nitrophenyl compounds are employed, then the range of the most suitable pH values is somewhat narrower and is generally from 6 to 8.5.

The buffers used can be any of those which are effective in the main activity range of the enzymes employed. Preferred buffers include phosphate buffers, HEPES (N - (2 - hydroxyethyl) - piperazine - N - 2 - ethane - sulphonic acid) and glycylglycine. The preferred buffer concentrations are from 10 to 200 mMol/litre.

The α -glucosidase is generally employed in an amount of from 0.1 to 5000 U/ml. It is an especial advantage of the process according to the present invention that relatively large amounts of this enzyme can be used so that the α -amylase fission is the rate-determining step. It is, of course, also possible to employ even larger amounts of this enzyme but this provides no further advantages.

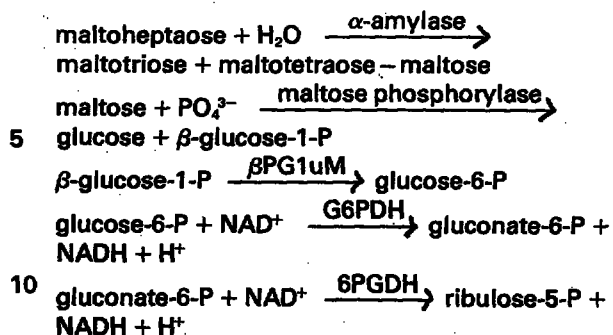
The compounds of general formula (I) employed according to the present invention are generally used in amounts of from 0.1 to 250 mMol/litre, amounts of from 0.5 to 100 mMol/litre being preferred.

Substrate saturation of the α -amylase with maltoheptaose is present at a concentration of 8 to 10 mMol. Therefore, it is preferable to use a minimum concentration of 8 mMol of maltoheptaose if it is desired to work under the conditions of substrate saturation, which is usually the case.

Furthermore, an activation agent for the α -amylase is preferably added. Such activation agents are known, the preferred ones including sodium chloride and potassium chloride.

In the case of another preferred embodiment of the process according to the present invention, the determination of the fission products takes place by reaction with maltose phosphorylase, with the formation of glucose - 1 - phosphate, which is then determined in known manner. According to an especially preferred embodiment, the glucose - 1 - phosphate is determined by conversion into glucose - 6 - phosphate by means of β -phosphoglucomutase (β -PGluM) and oxidation of the glucose - 6 - phosphate formed with NAD in the presence of glucose - 6 - phosphate dehydrogenase, with the formation of gluconate - 6 - phosphate and NADH, the formation of the latter being easily monitored photometrically. The measurement signal can be intensified by further oxidation with NAD in the presence of 6 - phosphogluconate dehydrogenase with the formation of ribulose - 5 - phosphate and a further molecule of NADH.

The principle of this embodiment is illustrated by the following equations:—



An advantage of this embodiment of the present invention is that maltose phosphorylase is more specific than α -glucosidase and, therefore, endogenous glucose does not disturb.

With regard to the buffer, there again applies what was stated above with regard to the embodiment of the present invention using α -glucosidase process.

Apart from the two above-mentioned embodiments of the process according to the present invention, the determination of the fragments formed from the maltoheptaose, i.e. maltotetraose, maltotriose and the maltose formed therefrom, can also take place by other methods known for this purpose.

The present invention also provides a reagent for the determination of α -amylase which comprises an α -amylase substrate and a system for the determination of a fission product formed from the α -amylase substrate by the α -amylase, the substrate used being a compound of general formula (I).

A preferred system for the determination of the fission products is the α -glucosidase system which contains α -glucosidase, an alkali metal chloride and a buffer. If the substrate consists of maltoheptaose itself, then, as enzymes, there are also necessary hexokinase (HK) and glucose - 6 - phosphate dehydrogenase (G6PDH), as well as NAD, ATP and magnesium ions.

A reagent based upon the α -glucosidase system preferably comprises the following components:
 5×10^3 to 3×10^4 U/l. α -glucosidase,
 10^3 to 5×10^4 HK,
 10^3 to 5×10^4 G6PDH,
 0.5 to 8 mM/l. NAD,
 0.5 to 5 mM/l. ATP,
 1 to 3 mM/l. Mg^{2+} ,
 25 to 100 mM/l. NaCl or KCl,
 25 to 200 mM/l. buffer (pH 6.2 to 7.8) and
 5 to 100 mM/l. maltoheptaose,
 or a multiple or fraction thereof, in dry or dissolved form.

According to another preferred embodiment, the reagent comprises α -glucosidase, potassium chloride or sodium chloride, buffer and substrate. A reagent of this type comprises, in particular, 10^2 to 5×10^6 U/l. α -glucosidase, 1 to 100 mMol/l. sodium chloride or potassium chloride, 10 to 250 mMol/l. buffer (pH 5 to 9) and 0.1 to 250 mMol/l. of a maltoheptaose derivative of general formula (I), referred to the concentration in the test. The reagent can be present in dry and especially in lyophilised form or also in the form of a solution, as a mixture of all components or separately.

According to a further embodiment of the reagent

according to the present invention of the above-described type, α -glucosidase and/or phenol oxidase and HSK can additionally be present.

According to yet another embodiment of the reagent according to the present invention, in addition to α -glucosidase, sodium chloride or potassium chloride, buffer and substrate, it also contains sorbitol dehydrogenase and NAD or gluconate kinase, ATP, 6 - phosphogluconic acid dehydrogenase and NADP, and optionally also a tetrazolium salt and diaphorase or phenazine - methosulphate (PMS).

A preferred reagent of this type comprises 1×10^2 to 3×10^6 U/l. α -glucosidase, 2×10^3 to 5×10^4 U/l. sorbitol dehydrogenase, 1×10^3 to 5×10^4 U/l. hexokinase, 0.5 to 50 mMol/l. ATP, 10 to 500 U/l. diaphorase (*Chlostridium kluyveri*), 0.01 to 0.5 mMol/l. tetrazolium salt, 0.1 to 10 mMol/l. NAD, 0.2 to 5 mMol/l. magnesium chloride, 0.5 to 20 mMol/l. maltoheptaol 1 to 100 mMol/l. sodium chloride or potassium chloride and 10 to 250 mMol/l. buffer (pH 5.5 to 8.5).

If desired, a non-ionic surface-active agent can also be present, for example in an amount of from 5 to 50 mMol/l.

Another preferred embodiment of the reagent comprises 100 to 3×10^6 U/l. α -glucosidase, 10 to 10^4 U/l. 6 - phosphogluconate dehydrogenase, 20 to 2×10^4 U/l. gluconate kinase, 0.5 to 25 mMol/l. ATP, 0.05 to 10 mMol/l. NADP, 1.0 to 20 mMol/l. maltoheptagluconic acid, 0.5 to 5 mMol/l. magnesium chloride, 1 to 100 mMol/l. sodium chloride and 10 to 250 mMol/l. buffer (pH 5.5 to 8.5).

Yet another reagent according to the present invention comprises 0.1 to 250 mMol/l.

α -nitrophenyl - maltoheptaoside or dinitrophenyl - maltoheptaoside, 1×10^2 to 2.5×10^6 U/l. α -glucosidase, 1 to 100 mMol/l. sodium chloride or potassium chloride and 10 to 250 mMol/l. phosphate buffer (pH 7.0 to 8.0).

Yet another embodiment of the reagent according to the present invention comprises:

0.1 to 250 mMol/l. α -phenylmaltoheptaoside,
 1×10^2 to 1.5×10^6 U/l. α -glucosidase,
 10 to 10^3 U/l. monophenol oxidase,
 0.1 to 10 mMol/l. HSK,
 1 to 100 mMol/l. sodium chloride or potassium chloride,
 10 to 250 mMol/l. buffer.

Another preferred system for the determination of the fission products is the maltose phosphorylase system which consists essentially of maltose phosphorylase, β -phosphoglucomutase BPGluM, glucose - 6 - phosphate dehydrogenase (G6PDH), glucose - 1,6 - diphosphate (Gl, 6DP), NAD, buffer, maltoheptaose and optionally 6 - phosphogluconate dehydrogenase (6PGDH).

An especially preferred reagent with this detection system comprises:

0.5×10^3 to 2×10^4 U/l. maltose phosphorylase,
 1×10^2 to 1×10^4 β -PGluM,
 2×10^2 to 3×10^4 U/l. glucose - 6 - phosphate dehydrogenase,
 0.5 to 10 mMol/l. NAD,
 0.001 to 1 mMol/l. glucose - 1,6 - diphosphate,
 0.5 to 100 mMol/l. buffer (pH 6.0 to 7.5),
 5 to 50 mMol/l. maltoheptaose and optionally

1x10² to 1x10⁴ U/l. 6-phosphogluconate dehydrogenase.

The reagent according to the present invention can be present in dry or dissolved form and it can also be impregnated or incorporated into a sheet-like carrier, for example a film, an absorbent paper or the like. In the latter case, it preferably consists of at least three layers or laminae, the first of which contains the substrate, the second of which serves as a barrier layer and the third of which contains the system for the determination of the fission products. If such a multi-layer reagent material, which can be used for a simple rapid test for α -amylase, is brought into contact with a liquid α -amylase-containing sample, then the α -amylase splits the substrate and the fragments diffuse through the intermediate layer into the third layer containing the determination system. As detection reaction, in this case there is preferably employed a colour-forming reaction in order to make visible the concentration of the α -amylase on the basis of the resultant coloration if the fission products themselves are not already coloured.

The maltoheptaose derivatives employed according to the present invention can be prepared in various ways and some processes for the preparation of the compounds of general formula (I), in which R is a phenylglucoside, mono- or dinitro phenyl-glucoside, sorbitol or gluconic acid group are described and claimed in our copending Patent Application No. 8033088. In the case of the phenylated derivatives, there can be used not only chemical but also enzymatic methods. The chemical syntheses are, in principle, based upon the reaction of peracetylated maltoheptaose with the appropriate phenol in the presence of a Friedel-Crafts catalyst. This method can be used not only for phenol itself but also for mononitrophenol and dinitrophenol. On the other hand, it is also possible first to prepare the phenyl derivative and subsequently to nitrate it, for example with the use of the process described in Bull. Chem. Soc. Japan, 34, 718/1961. This method is particularly suitable for the mononitro derivative. Under certain circumstances, a separation of the resultant *o* and *p*-nitrophenyl derivatives can also be carried out.

This reaction is preferably carried out by melting or boiling under reflux in non-polar solvents with zinc chloride, stannic chloride or titanium tetrachloride as Friedel-Crafts catalyst. After the introduction of the phenol or nitrophenol, the protective groups are split off in known manner, for example with sodium methylate, ammonia, potassium hydroxide or barium methoxide, in each case in methanolic solution, or with aqueous barium hydroxide solution or the like.

The enzymatic preparation of the phenyl derivatives can take place by transglucosidation of the phenyl glucoside or of the corresponding nitrated phenyl glucoside with α -cyclodextrin, amylase or soluble starch in the presence of a specific microbial transferase. For this purpose, it is preferred to use a transferase from *Bacillus macerans*. In this case, there can be used for this transglucosidation the known amylase from *Bacillus macerans* (E. C. 2.4.1.19.DSM 24; isolation see J. A. de Pinto, L. L.

Campbell, Biochemistry, 7, 114/1968; transfer reac-

tion see Methods in Carbohydrate Chemistry, Vol. II, 347 (1963)) which besides its hydrolytic and cyclising action, clearly also displays a glucosyl-transfering effectiveness.

Those compounds of general formula (I) in which R is a sorbitol residue can be obtained from maltoheptaose by reduction with sodium borohydride under mild conditions.

Finally, the compounds of general formula (I) in which R is a gluconic acid group can be prepared chemically or enzymatically by methods known for the preparation of gluconic acid from maltoheptaose, for example by oxidation with bromine (see Methods in Carbohydrate Chemistry, Vol. II, 13 (1963)).

As already mentioned, the present invention not only provides a rapid and specific process for the determination of α -amylase but also completely or substantially removes the lag phase, which is of particular importance when the process is used in automatic analysers. Furthermore, the process according to the present invention can be carried out in many embodimental forms without the use of complicated apparatus for the evaluation and is, therefore, especially useful for rapid diagnostics and for optical determination in the visible range of light. However, at the same time, the various embodiments of the process according to the present invention can also be determined with UV measurement devices. Further advantages are the strict proportionality and the absence of disturbance by chemically related components of the blood.

The substrates employed according to the present invention are readily obtainable in a high state of purity. A simple process for the preparation of maltoheptaose is described in German Patent Specification No. P 27 41 191.2. The process can be used for the determination of α -amylase in biological fluids, such as serum, heparin plasma, urine and the like, as well as in other liquid and solid materials.

The following Examples are given for the purpose of illustrating the present invention:—

Example 1.

Maltogen method (α -glucosidase system).

A reagent mixture containing α -glucosidase, G6PDH, HK, NAD, ATP, maltoheptaose, magnesium ions, sodium chloride and phosphate buffer is dissolved in 2.0 ml. distilled water. The storage stability of the reagent at ambient temperature is about 1 hour and at temperatures below 8°C. is 6 hours.

The solution obtained contains the following concentrations of the reagents:

phosphate buffer	50 mMol/l.; pH 7.0
sodium chloride	50 mMol/l.
magnesium ions	2 mMol/l.
maltoheptaose	10 mMol/l.
ATP	1.2 mMol/l.
NAD	2 mMol/l.
HK	≥ 2 U/l.
G6PDH	≥ 2 U/l.
α -glucosidase	≥ 10 U/l.

The solution is mixed at 25°C. with 0.02 ml. serum sample, filled in a cuvette of 1 cm. path length and

then the absorbance determined at 365 nm, 340 nm or 334 nm in a photometer. After 10 minutes, the extinction is read off and then, at intervals of one minute, reading off is repeated five times.

- 5 From the absorbance differences per minute ($\Delta A/\text{min.}$) there is determined the average value, the reagent blank value is subtracted and the corrected value used for the calculation. The calculation is made as follows:

$$\begin{aligned} 10 \quad U/1. (25^\circ\text{C}) &= 4244 \times \Delta A / \text{min.} & \begin{matrix} [365 \text{ nm}] \\ [340 \text{ nm}] \\ [334 \text{ nm}] \end{matrix} \\ &= 2290 \times \Delta A / \text{min.} \\ &= 2335 \times \Delta A / \text{min.} \end{aligned}$$

- 15 The Figure of the accompanying drawings shows the results obtained with a series of dilutions of human serum with physiological saline solution using this method.

If the reagent is divided up into two batches, one

- 20 of which contains the maltoheptaose and the other the mixture of all the other reagents, then the storage stability of the solutions produced therewith can be increased. For the mixture of the reagents at temperatures of up to 8°C. , the stability is up to 30 hours and at ambient temperature is about 8 hours. The corresponding storage stability of the maltoheptaose solution is 6 weeks and about 1 month, respectively.

Example 2.

- 30 *Determination with the maltose phosphorylase system.*

Two reagents are prepared consisting of the amylase substrate and the maltose phosphorylase system. One reagent contains the maltoheptaose substrate and the other contains soluble starch according to the prior art. After dissolving in water, the reagent concentration is as follows:

	according to the invention	comparison
phosphate buffer	20 mM; pH 6.5	20 mM; pH 6.5
NAD	2 mM	2 mM
maltoheptaose	10 mM	—
soluble starch	—	5 mg./ml.
glucose-1, 6-diphosphate	traces	traces
maltose phosphorylase (micro-organism)	3 U/1.	3 U/1.
β -phosphoglucosidase (micro-organism)	1 U/1.	1 U/1.
G6PDH (<i>Leuconostoc mesenteroides</i>)	9 U/1.	9 U/1.
6PGDH (<i>Leuconostoc mesenteroides</i>)	1 U/1.	1 U/1.

- The solution obtained is incubated at 30°C. , mixed with the sample solution and the absorbance difference measured in a photometer at Hg 334 nm. After a pre-incubation period of 10 minutes, the extinction difference is measured over a period of 10 minutes. For 2.0 ml. of reagent and 0.10 ml. of sample, there is then given the following calculation formula:

$$45 \quad \Delta E/\text{min.} \times \frac{2.1 \times 1000}{6.18 \times 0.1 \times 2.0} = \Delta E/\text{min.} \times 1699 \text{ U/1.}$$

- 50 In the case of the use of five different human sera, the following values are found with the above reagents:

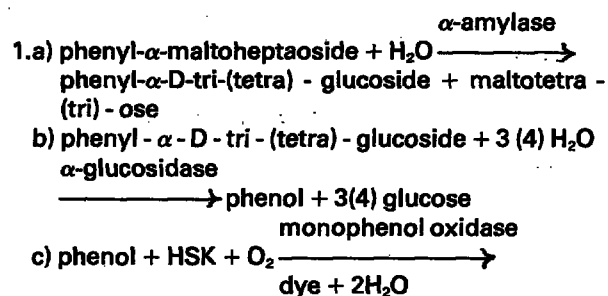
serum	present invention	starch
1	37.4 U/1.	15.3 U/1.
2	61.2 U/1.	27.3 U/1.
3	95.1 U/1.	39.1 U/1.
4	114 U/1.	44.2 U/1.
5	374 U/1.	161 U/1.

Example 3

- 55 *Detection of α -amylase with phenyl - α - maltoheptaoside as substrate.*

- Phenyl - α - maltoheptaoside is split with α -amylase into phenyl - α - maltotriglucoside or -tetraglucoside which is converted by α -glucosidase into phenol and glucose. The liberated phenol is oxidatively coupled by monophenol oxidase with the nucleophilic reagent 3 - methyl - 6 - sulphonyl - benzthiazolone - hydrazone - (2) (HSK) to give a red coloured material, the rate of formation of which is proportional to the amylase activity in the sample and can be monitored photometrically.

Test principle:



Measurement conditions: 25°C., measurement wavelength 492 nm, 1 cm. cuvette; the following Table shows the composition of the reagent:

TABLE

reagent	concentration in the test
phosphate buffer, pH 7.4	0.5 mol/l.
sodium chloride	0.05 mole/l.
phenyl- α -D-maltoheptaoside	10 mMole/l.
HSK	1 mMole/l.
monophenol oxidase	1 U/ml.
α -glucosidase	10 U/ml.

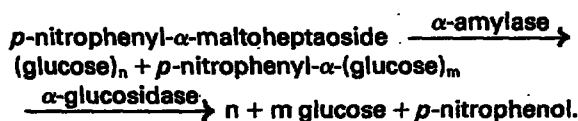
test volume: 2.0 ml.

Instead of phosphate buffer, glycine, glycyglycine, Hepes, tris, tra and other buffers have also proved to be useful.

Example 4.

Determination of α -amylase with *p*-nitrophenyl-maltoheptaoside.

Test principle:



Test system:

reaction batch	ml.	concentration in test
Na-/K-PO ₄ buffer 0.02 mol/l. + Triton X100 2 ml./100 ml. + NaCl 10 mMol/l., pH 6.9	1.92	12.8 mMol/l. PO ₄ ³⁺
MgCl ₂ 0.3 mol/l.	0.01	6.4 mMol/l.
maltoheptite 100 mMol/l.	0.10	1.0 mMol/l.
NAD, c = 40 mg./ml.	0.05	3.3 mMol/l.
INT, c = 0.6 mg./ml.	0.05	1.0 mMol/l.
diaphorase (<i>Clostr. kluyveri</i>) 5 mg./ml. ⁺	0.20	0.09 mMol/l.
ATP, c = 50 mg./ml.	0.05	167 U/l.
hexokinase 280 U/ml. ⁺	0.10	3.29 mMol/l.
SDH 180 U/ml. ⁺	0.05	4666 U/l.
α -glucosidase 1120 U/ml. ⁺	0.30	18000 U/l.
sample (serum)	0.20	74670 U/l.
	0.02	

⁺ in the test buffer

(Triton is a registered Trade Mark).

After splitting the maltoheptaoside, the fission products are broken down into glucose and *p*-nitrophenol. The *p*-nitrophenolate ion has a yellow colour in alkaline solution and can, therefore, be directly measured optically.

Test system:

reaction mixture: 50 mM/1. phosphate buffer, pH 7.4

50 mM/1. sodium chloride

5 to 50 U/ml. α -glucosidase

5 to 10 mM/1. *p*-nitrophenyl- α -maltoheptaoside.

Test batch:

1 ml. reaction mixture + 50 μ l. sample (serum)

temperature: 30°C.

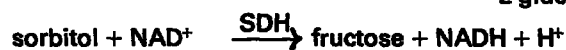
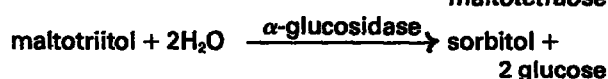
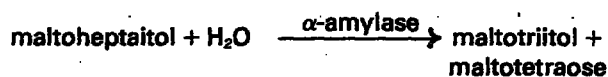
wavelength: 405 nm

Serum start after achieving the measurement temperature (10 minutes pre-incubation).

Example 5.

α -Amylase determination with maltoheptaoside

Test principle:

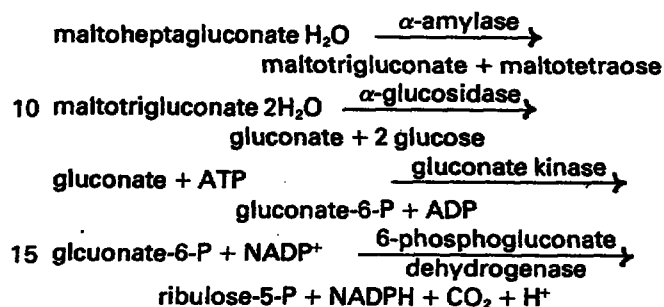


Start by adding sample: measurement is performed at 492 nm; temperature: 25°C.

Example 6.

α -Amylase determination with maltoheptagluconate

5 Test principle:



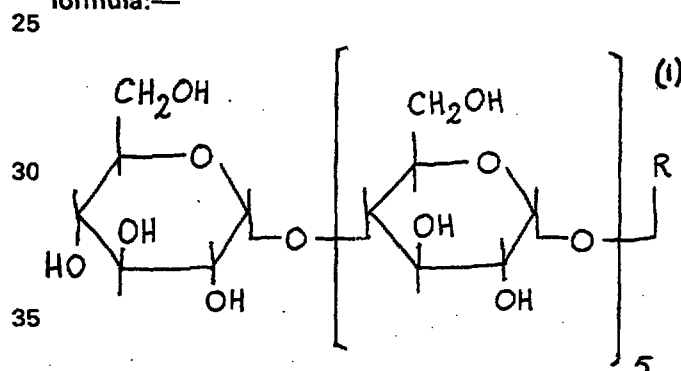
Test system:

reaction batch	ml.	concentration in test
Na-/K-PO ₄ buffer 0.02 mol/l.	2.19	14.6 mMol/l. phosphate
+ NaCl 10 mMol/l., pH 6.9		7.3 mMol/l. NaCl
MgCl ₂ 1 mol/l.	0.01	3.3 mMol/l.
maltoheptagluconic acid 150 mMol/l.	0.10	5 mMol/l.
NADP, c = 10 mg./ml.	0.10	0.38 mMol/l.
ATP, c = 50 mg./ml.	0.10	2.7 mMol/l.
gluconate kinase 40 U/ml.	0.03	1066 U/l.
6-phosphogluconate dehydrogenase 24 U/ml.	0.10	800 U/l.
α -glucosidase 1120 U/ml.	0.30	11.2 x 10 ⁴ U/l.
sample (serum)	0.02	

Start by adding sample; measurement is performed at 340 nm or 365 nm; temperature: 25°C.; test volume: 3.00 ml.

CLAIMS

- 20 1. Process for the determination of α -amylase by the enzymatic splitting of an α -amylase substrate and measurement of a fission product, wherein, as substrate, there is used a compound of the general formula:—



in which R is a glucoside, phenylglucoside, mononitrophenylglycoside, dinitrophenylglycoside, sorbitol or gluconic acid group.

- 40 2. Process according to claim 1, wherein the fission products are converted by α -glucosidase into

glucose which is then determined in known manner.

3. Process according to claim 2, wherein the con-
45 version is carried out at a pH value of from 5 to 9.

4. Process according to any of the preceding claims, wherein 0.1 to 250 mMol/l. of the compound of general formula (I) are used.

5. Process according to claim 4, wherein 0.5 to
50 100 mMol/l. of the compound of general formula (I) are used.

6. Process according to any of the preceding claims, wherein, in the case of a β -position substituent on the sugar residue of the group R, β -
55 glucosidase is additionally employed.

7. Process according to claim 1, wherein the fission products are reacted with maltose phosphorylase with the formation of glucose-1-phosphate which is then determined in known manner.

- 60 8. Process according to claim 7, wherein, for the determination of the glucose-1-phosphate formed, this is converted with phosphoglucomutase into glucose-6-phosphate and with the latter NAD is reduced to NADH in the presence of glucose-6-phosphate dehydrogenase, the NADH being meas-
65 ured.

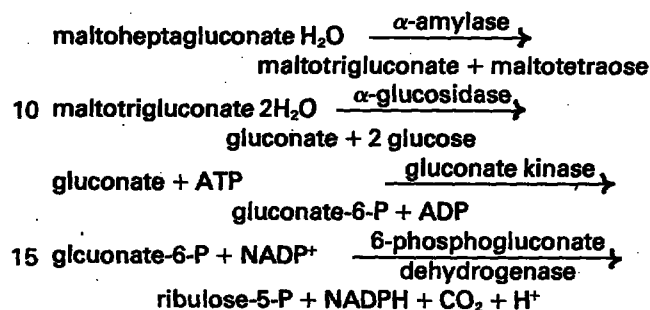


Start by adding sample: measurement is performed at 492 nm; temperature: 25°C.

Example 6.

α-Amylase determination with maltoheptagluconate

5 Test principle:



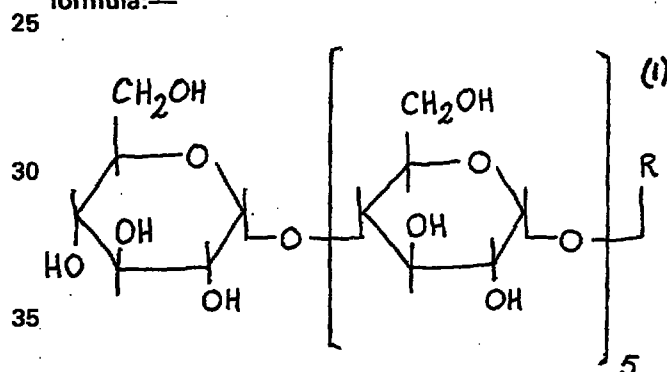
Test system:

reaction batch	ml.	concentration in test
Na-/K-PO ₄ buffer 0.02 mol/l.	2.19	14.6 mMol/l.
+ NaCl 10 mMol/l., pH 6.9		phosphate
MgCl ₂ 1 mol/l.	0.01	7.3 mMol/l. NaCl
maltoheptagluconic acid		3.3 mMol/l.
150 mMol/l.	0.10	5 mMol/l.
NADP, c = 10 mg./ml.	0.10	0.38 mMol/l.
ATP, c = 50 mg./ml.	0.10	2.7 mMol/l.
gluconate kinase 40 U/ml.	0.03	1066 U/l.
6-phosphogluconate		
dehydrogenase 24 U/ml.	0.10	800 U/l.
α-glucosidase 1120 U/ml.	0.30	11.2 x 10 ⁴ U/l.
sample (serum)	0.02	

Start by adding sample; measurement is performed at 340 nm or 365 nm; temperature: 25°C.; test volume: 3.00 ml.

CLAIMS

- 20 1. Process for the determination of α - amylase by the enzymatic splitting of an α - amylase substrate and measurement of a fission product, wherein, as substrate, there is used a compound of the general formula:—



in which R is a glucoside, phenylglucoside, mono-nitrophenylglycoside, dinitrophenylglycoside, sorbitol or gluconic acid group.

- 40 2. Process according to claim 1, wherein the fission products are converted by α - glucosidase into

glucose which is then determined in known manner.

3. Process according to claim 2, wherein the conversion is carried out at a pH value of from 5 to 9.

- 45 4. Process according to any of the preceding claims, wherein 0.1 to 250 mMol/l. of the compound of general formula (I) are used.

5. Process according to claim 4, wherein 0.5 to 100 mMol/l. of the compound of general formula (I) are used.

6. Process according to any of the preceding claims, wherein, in the case of a β - position substituent on the sugar residue of the group R, β - glucosidase is additionally employed.

7. Process according to claim 1, wherein the fission products are reacted with maltose phosphorylase with the formation of glucose - 1 - phosphate which is then determined in known manner.

8. Process according to claim 7, wherein, for the determination of the glucose - 1 - phosphate formed, this is converted with phosphoglucomutase into glucose - 6 - phosphate and with the latter NAD is reduced to NADH in the presence of glucose - 6 - phosphate dehydrogenase, the NADH being measured.



9. Process according to any of the preceding claims, wherein, when R is a phenylglucoside group, phenol split off is determined with 3 - methyl - 6 - sulphonyl - benzthiazolone - hydrazone - (2) in the presence of monophenol oxidase.
10. Process according to any of claims 1 to 8, wherein, when R is a sorbitol group, sorbitol split off is determined with sorbitol dehydrogenase and NAD.
11. Process according to any of claims 1 to 8, wherein, when R is a gluconic acid group, gluconic acid split off is determined with gluconate kinase, 6 - phosphogluconate dehydrogenase and NAD(P).
12. Process according to claim 10 or 11, wherein, in the case of the reduction of NAD(P), NAD(P)H formed is reacted with a tetrazolium salt in the presence of an electron carrier to give a formazan and the latter is determined.
13. Process according to claim 12, wherein diaphorase or phenazine - methosulphate is used as electron carrier.
14. Process according to claim 1 for the determination of α - amylase, substantially as hereinbefore described and exemplified.
15. Reagent for the determination of α - amylase, comprising an α - amylase substrate and a system for the determination of a fission product formed from the amylase substrate by α - amylase, wherein the substrate is a compound of general formula (I) given in claim 1.
16. Reagent according to claim 15, consisting essentially of α - glucosidase, hexokinase, glucose - 6 - phosphate dehydrogenase, NAD, ATP, magnesium ions, NaCl, phosphate buffer and maltoheptaose as substrate.
17. Reagent according to claim 16, comprising: 5×10^3 to 3×10^4 U/l. α - glucosidase, 10^3 to 5×10^4 U/l. hexokinase, 10^3 to 5×10^4 U/l. glucose - 6 - phosphate dehydrogenase, 0.5 to 8 mMol/l. NAD, 0.5 to 5 mMol/l. ATP, 1 to 3 mMol/l. magnesium ions, 25 to 100 mMol/l. sodium chloride or potassium chloride, 25 to 100 mMol/l. buffer (pH 6.2 to 7.8) and 5 to 100 mMol/l. maltoheptaose or a multiple or fraction thereof, in dry or dissolved form.
18. Reagent according to claim 23, consisting essentially of maltose phosphorylase, β - phosphoglucomutase, glucose - 6 - phosphate dehydrogenase, glucose - 1,6 - diphosphate, NAD, buffer, substrate and optionally 6 - phosphogluconate dehydrogenase, the substrate being maltoheptaose.
19. Reagent according to claim 18, comprising: 0.5×10^3 to 2×10^4 U/l. maltose phosphorylase, 1×10^2 to 1×10^4 U/l. β - phosphoglucomutase, 2×10^2 to 3×10^4 U/l. glucose - 6 - phosphate dehydrogenase, 0.5 to 10 mMol/l. NAD, 0.001 to 1 mMol/l. glucose - 1,6 - diphosphate, 0.5 to 100 mMol/l. buffer (pH 6.0 to 7.5) 5 to 50 mMol/l. maltoheptaose and optionally 1×10^2 to 1×10^4 U/l. 6 - phosphogluconate dehydrogenase.
20. Reagent according to claim 15, comprising α - glucosidase, sodium chloride or potassium chloride, buffer and a compound of general formula (I) given in claim 1.
21. Reagent according to claim 15 or 20, comprising 10^2 to 5×10^3 U/l. α - glucosidase, 1 to 100 mMol/l. sodium chloride or potassium chloride, 10 to 250 mMol/l. buffer (pH 5 to 9) and 0.1 to 250 mMol/l. of a compound of general formula (I) given in claim 1, referred to the concentration in the test.
22. Reagent according to any of claims 15, 20 or 21, which additionally contains β - glucosidase or/and phenol oxidase and 3 - methyl - 6 - sulphonyl - benzthiazolone - hydrazone - (2).
23. Reagent according to any of claims 15 or 20 to 21, which additionally contains either sorbitol dehydrogenase or gluconate kinase and 6 - phosphogluconate dehydrogenase and NADP.
24. Reagent according to claim 23, which contains in addition a tetrazolium salt and diaphorase or PMS.
25. Reagent according to any of claims 15 to 24, wherein a surface-active agent is also present.
26. Reagent according to any of claims 15 to 25, whenever impregnated or incorporated into a sheet-like carrier material.
27. Reagent according to claim 15 for the determination of α - amylase, substantially as hereinbefore described and exemplified.