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(54) **An assay method for amylase activity**

(57) The present invention provides an assay method for amylase activity, comprising assaying a substrate decomposed by the enzyme amylase, wherein the substrate used is a glucose polymer having a modified reducing terminal glucose residue or is a cyclo glucose polymer (cyclodextrin).

A new maltose dehydrogenase is described.

GB 2 088 052 A

FIG. 1

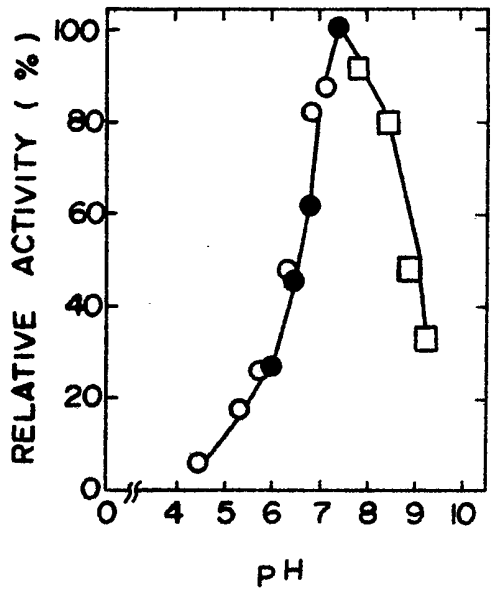



FIG. 2

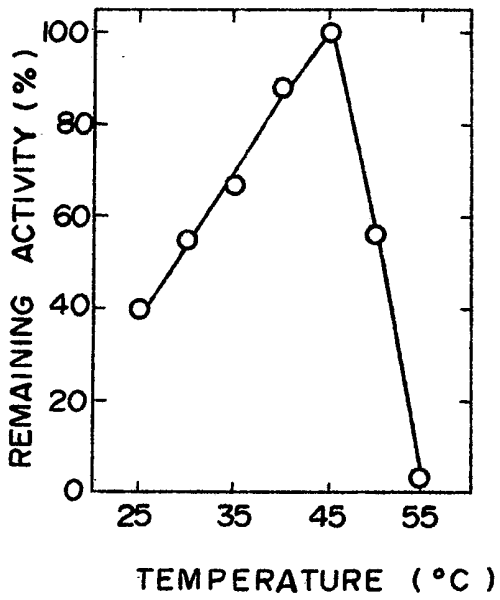



FIG. 3

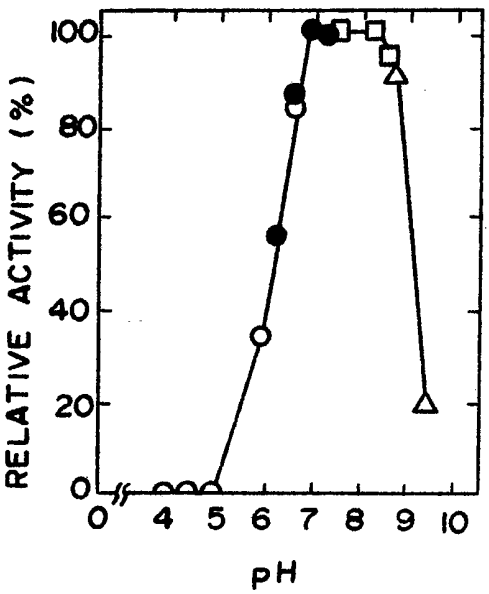



FIG. 4

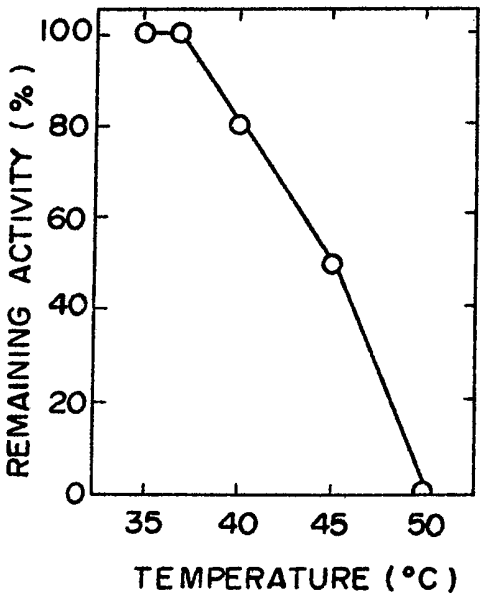


FIG. 5

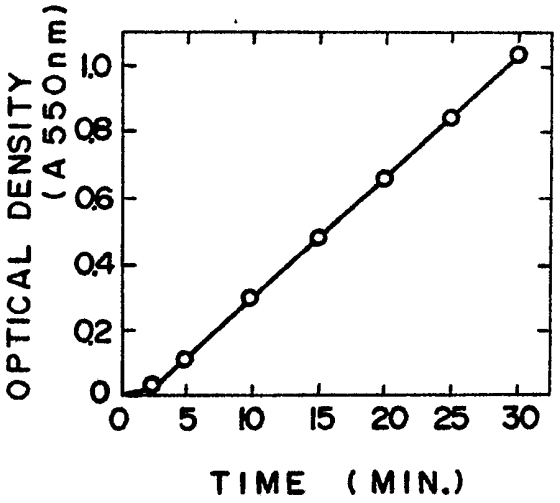



FIG. 6

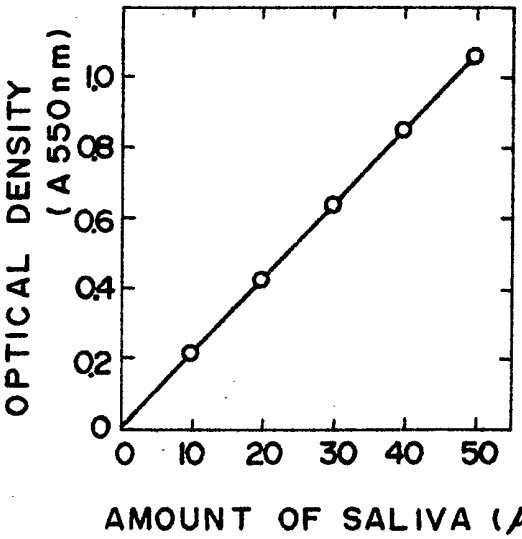



FIG. 7

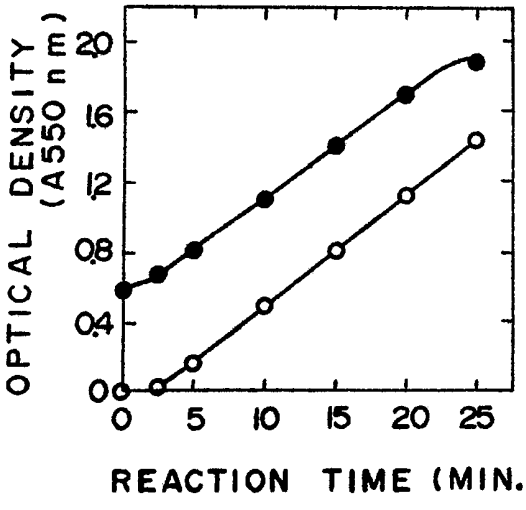
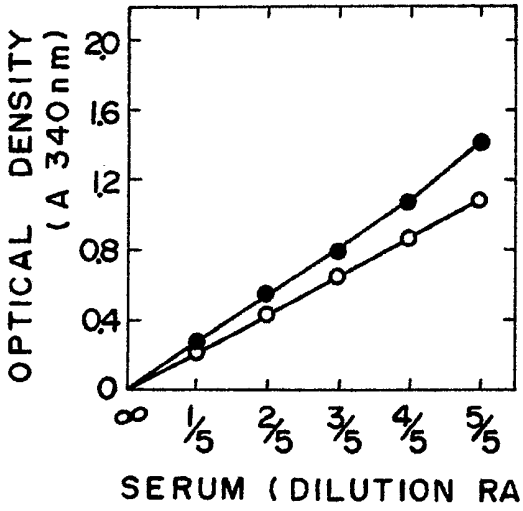



FIG. 8



SPECIFICATION

**Assay method for amylase**

The present invention is concerned with a novel assay method for amylase activity.

More particularly, the invention is concerned with an assay method for amylase activity in samples of, for example, serum, saliva and urine, which comprises assaying the decomposed substrate by the enzyme amylase, using a glucose polymer as a substrate.

Hitherto known assay methods of amylase activity are based upon the hydrolysis of a glucose polymer substrate, such as starch, by the action of amylase to give glucose, maltose or oligosaccharides. Examples of such assay methods include determining the decrease of viscosity of starch by the action of the amylase; iodometry; the reaction of glucose with glucose oxidase or glucose dehydrogenase and NAD (NADP), glucose being formed by an action of $\alpha$-glucosidase on maltose, which latter is produced by the action of amylase on starch; and the blue starch method or maltose phosphorylase method, wherein the soluble pigment produced by the action of amylase on insoluble pigment-bound starch is measured colorimetrically (see Japanese Patent Specification No. 55—27800).

These known methods suffer from a number of disadvantages, for example, non-uniformity of the hydrolysis by the reagent used and the reaction conditions employed and the inhibitory action due to glucose and maltose present. The blue starch method is also complicated by the centrifugal separation procedure required, which makes automisation difficult and in the maltose phosphorylase method, four enzymatic steps are required, which is expensive and laborious.

In the course of investigations for accurate and automated amylase assay, we have now found that amylase activity can be assayed simply and with good accuracy by using as the substrate a glucose polymer, the reducing terminal glucose residue of which is esterified, etherified or oxidised to form a modified reducing terminal group which cannot act as a substrate for maltose dehydrogenase, i.e. a glucose polymer with a modified reducing terminal glucose residue is used as a substrate for the assay of amylase activity. We have also found that amylase can advantageously be assayed when, instead of a glucose polymer with a modified reducing terminal glucose residue, a cyclic glucose polymer is used as substrate. Further advantages arise due to the fact that the decomposed substrate produced by the enzymatic reaction is dehydrogenated by maltose dehydrogenase in the presence of NAD or NADP, the reduced NAD or reduced NADP formed being measured directly or indirectly by the use of a chromogen. Still further advantages are achieved when the samples for the amylase assay, such as serum, saliva or urine, have been previously treated with $\alpha$-glycosidase or a kinase, such as hexokinase, in the presence of magnesium ions and adenosine triphosphate (ATP).

Thus, the present invention provides an amylase assay method which is simple and accurate and can be automated.

Examples of substrates which may be used according to the present invention include glucose polymers with a modified reducing terminal glucose residue or a cycloglucose polymer, preferably with a degree of polymerisation of more than 5.

Example of glucose polymers with a modified reducing terminal glucose residue include amylose, amylopectin, starch and starch hydrolysates, such as soluble starch (so-called dextrin), in which the reducing terminal glucose residue of the glucose polymer has been modified.

The modified reducing terminal glucose residue used according to the present invention is a residue not having a reducing activity. Examples thereof include reducing terminal groups etherified or esterified by conventional methods and gluconic acid residues of oxidised glucose residues and esterified derivatives thereof.

Examples of these modifications include the following:

A solution of a polysaccharide, such as soluble starch, is reacted with 4% methanolic hydrochloric acid at 70°C. for 4 hours, neutralised and a fraction corresponding to a molecular weight of more than 1000 is obtained by gel-filtration column chromatography, this being a methyl etherified soluble starch.

Soluble starch is added to acetic anhydride (3.5 ml.) in dry pyridine, reacted at 0°C. overnight and precipitated by adding acetone, a fraction corresponding to a molecular weight of more than 1000 being obtained by gel-filtration column chromatography, this being an acetylated soluble starch.

Soluble starch in Fehling's reagent is boiled for 5 minutes for 20 hours and then concentrated. Insolubles are removed after the addition of methanol and a fraction corresponding to a molecular weight of more than 1000 is obtained, this being a compound in which the reducing terminal glucose residue is oxidised to a gluconic acid residue, which may be optionally esterified.

These modifications of the glucose residues are not limited within the above and can be carried out by other conventional methods, for example the methyl etherification can be replaced by an ethyl or isopropyl etherification or an acetylation can be replaced by a propionylation, or the gluconic acid residue can be replaced by an anhydride such as of the gluconolactone type. However, the gluconolactone type is usually unstable so that the gluconic acid residue is preferable. The degree of polymerisation of the glucose may not be limited to a uniform degree of polymerisation, various degrees of polymerisation being possible. Examples of cyclic glucose polymers include dextrins containing more than 6 glucose units obtained from starch, such as $\alpha$-, $\beta$-, $\gamma$-, $\delta$ or $\varepsilon$- cyclodextrin. These substrates are,

as explained hereinabove, hydrolysed by amylase in a test sample at 37°C. in a buffer of pH 6 to 8 to give a decomposed substrate, such as glucose, maltose and other oligosaccharides. The amylase activity in a sample is assayed by measuring the substrates thus formed, this preferably being carried out with maltose dehydrogenase and NAD or NADP at 37°C. and at pH 6 to 8.

A preferred example is maltose dehydrogenase, produced by culturing *Bacillus megaterium* B—0779 FERM-P No. 5662 (see Japanese Patent Application of the 29th August, 1980 entitled "A process for manufacturing maltose dehydrogenase") (deposited on the 29th July, 1980).

The strain *Bacillus megaterium* B—0779 was isolated from a soil sample from a water-melon field in Ukihashi, Ohito-cho, Tagata-gun, Shizuoka-ken, Japan and has the following taxonomical properties:

A. Growth characterisations:

(a) Nutrient agar slant medium:

Good growth, grown on straight, opaque, dull, greyish white to grey. No soluble pigment formation.

(b) Nutrient agar plate medium:

Colonies; round, edges wrinkled and surface undulated.

(c) Peptone liquid medium:

Weak growth, uniformly turbid, later flocculent precipitation.

B. Morphological characterisation:

Single, double or short twisted chains. Straight large rods; round edges, 1.0—1.5×2.0—3.0$\mu$, seldom 1.0—1.5×1.5×7.0$\mu$. Capsules formed. No motility. Spores difficult to identify with centre or nearly edges in cells.

C. Biochemical and physiological properties:

| | | |
|---|---|---|
| Gram stain: | + | |
| Acid-fact stain: | — | |
| OF test: | O | (oxidative) |
| anaerobic growth: | — | |
| gelatine liquefaction: | + | |
| starch hydrolysis: | — | |
| casein hydrolysis: | (+) | |
| esculin hydrolysis: | — | |
| catalase: | + | |
| oxidase: | (+) | |
| lecithinase: | — | |
| urease: SSR medium: | — | |
| Christenssen medium: | — | |
| $H_2S$ formation: | — | |
| VP, MR-test: | — | |
| phosphatase formation: | — | |
| lysozyme resistance: | — | |
| indole formation: | — | |
| nitrate reduction: | + | |
| citrate utilisation (Simons medium): | + | |

Acid formation from carbohydrates (no gas formation):
Acid formation:
L(+) arabinose, cellobiose, fructose, glucose, glycerol, inulin, maltose, raffinose, sucrose, trehalose, xylose.
No acid formation:
adonitol, dulcitol, mesoerythritol, fucose, galactose, inositol, lactose, mannose, melezitose, melibiose, L(+)-rhamnose, salicine, L-sorbose, sorbitol, starch.
The G + C content of DNA: 40.1%.

The strain B—0779 has been identified as belongining to the genus *Bacillus* on the basis of the characteristics of being Gram positive, catalase and oxidase positive, sporulating aerobic large bacilli, no acetoin formation, not lysozyme resistant, no lecithinase production and acid formation from arabinose, mannitol and xylose (Bergey's Manual of Determinative Bacteriology, 7th Ed. (1957 and 8th Ed. (1974). Manual for the identification of Medicinal Bacteriology (1974) and Agriculture Handbook, p. 427, The genus Bacillus).

The strain is also referred to as *Bacillus megaterium* on the basis of a detailed comparison as shown in Table 1 and is designated as being *Bacillus megaterium* B—0779.

| | Strain B–0779 | *Bacillus megaterium* (Agriculture Handbook 427 the genus *Bacillus* |
|---|---|---|
| Gram stain | + | + |
| Capsule | + | d |
| size of cells | 1.0 – 1.5 × 2.0 – 3.0 | 1.2 – 1.5 × 2.0 – 5.0 |
| anaerobic growth | – | – |
| lecithinase | – | – |
| urease | – | – |
| lysozyme resistance | – | – |
| acetoin formation | – | – |
| phosphatase formation | – | |
| arabinose (acid) | + | d |
| mannitol (acid) | + | + |
| xylose (acid) | + | d |

Maltose dehydrogenase used according to the present invention can be obtained by culturing the maltose dehydrogenase-producing micro-organism of the present invention in a concentional medium for antibiotic or enzyme production. Cultivation can be carried out in a solid or liquid medium, submerged aeration culture being preferable for industrial production. Nutrient sources for the medium are those conventional for micro-organism cultivation. Nutrient sources include assimilable nitrogen sources, such as corn steep liquor, peptone, casein, soya bean powder, yeast extract and meat extracts. Carbon sources include assimilable carbon sources, such as molasses, glucose, glycerol, sucrose and dextrin. Inorganic salts, such as sodium chloride, potassium chloride, magnesium sulphate, potassium dihydrogen phosphate and potassium hydrogen phosphate may also be added. The culture temperature can be varied, depending upon the growth of the micro-organisms and the maltose dehydrogenase production and is preferably 25 to 30°C. The culture time depends upon the conditions used and is usually 30 to 72 hours. Culturing is terminated at the stage of maximum production of the enzyme. The maltose dehydrogenase is present in the cultured cells.

The enzyme can be isolated from the wet cultured cells by treatment with lysozyme in Tris-HCl buffer, by exposure to ultrasonic waves or by French press treatment to give a crude maltose dehydrogenase solution. The crude enzyme solution may be treated by known enzyme isolation and purification procedures to obtain the purified enzyme. Organic solvent precipitation, for example with acetone, methanol or ethanol, and salting out with ammonium sulphate, sodium chloride or aluminium sulphate can also be applied. Further purification can be achieved by adsorption chromatography, using an ion exchanger, such as diethylaminoethylcellulose, diethylaminoethyldextran gel or

triethylaminoethyldextran gel, or a gel filtration agent, such as dextran gel or polyacrylamide gel, and lyophilisation to give a purified maltose dehydrogenase powder.

The assay method and the biochemical properties of the thus obtained maltose dehydrogenase are as follows:

(1) Assay method:
A reaction mixture (1.00 ml.) consisting of the following:

| | | |
|---|---|---|
| 0.2 M Tris-HCl buffer (pH 7.5) | 0.4 | ml. |
| 1% bovine serum albumin | 0.1 | ml. |
| 0.25% nitrotetrazolium blue (NTB) | 0.1 | ml |
| 1% "Triton" X—100 | 0.1 | ml. |
| 10 mM NADP | 0.1 | ml. |
| 0.05% phenazine methosulphate (PMS) | 0.02 | ml. |
| 1M maltose | 0.1 | ml. |
| distilled water | 0.08 | ml. |
| Total | 1.00 | ml. |

("Triton" is a Registered Trade Mark) is preincubated at 37°C for 3 minutes. 0.05 ml. of enzyme solution is added to the reaction mixture and incubated at 37°C for 10 minutes. The reaction is stopped by adding 2.0 ml. 0.1 N hydrochloric acid and the amount of $NTBH_2$ is colorimetrically measured at 550 nm (optical density: As). 0.05 ml. Water is added as a control and measured at 550 nm (Ab).

The enzymatic reaction of the above is shown as follows:

$$\text{maltose} + NAD(P)^+ \longrightarrow GGL^* + NAD(P)H + H^+$$

$$NAD(P)H + NTB + H^+ \xrightarrow{PMS} NAD(P)^+ + NTPH_2$$

*GGL: O-$\alpha$-D-glucopyranosyl (1→4)-$\delta$-gluconolactone

One unit is defined as being the amount which forms 1 $\mu$ mole of $NTBH_2$ in 1 minute, the enzyme activity being calculated from the following equation:—

$$\text{Activity (unit/ml)} = \frac{(As—Ab) \times 3.05}{12.4 \times 10 \times 0.05}$$

$$= (As—Ab) \times 0.49$$

(2) Substrate specificity:

Maltose in the above assay method is replaced by the substrates in the following Table 2 (each 0.1 M, 0.1 ml.) and assayed according to the assay method:

TABLE 2

| substrate | Relative activity (%) |
|---|---|
| maltose | 100 |
| xylose | 0.9 |
| ribose | 2.5 |
| glucose | 15 |
| galactose | 3.9 |
| mannose | 2.8 |
| fructose | 2.9 |
| sucrose | 0 |
| lactose | 98 |
| cellobiose | 44 |
| raffinose | 0 |
| maltotriose | 53 |
| maltopentaose | 11 |
| inositol | 0 |
| sorbitol | 0 |
| mannitol | 0 |
| glycerol | 0 |
| 0.5% soluble starch | 0.7 |

As shown in Table 2, the maltose dehydrogenase used according to the present invention has the substrate specificity for maltose and lactose.

(3) Coenzymes:

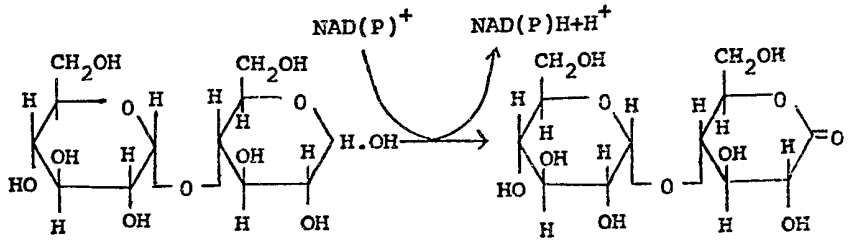
NADP in the above assay method is replaced by NAD or is omitted and the activity is measured according to the assay methods, the results obtained being shown in Table 3. The maltose dehydrogenase used according to the present invention requires NADP or NAD as a coenzyme.

TABLE 3

| Coenzyme | Relative activity (%) |
|---|---|
| NADP | 100 |
| NAD | 45 |
| no addition | 0 |

(4) Enzyme action:

The enzyme catalyses the following reaction:—



in which GGL and reduced NAD(P) are formed from maltose and the coenzyme $NAD(P)^+$.

(5) Optimum pH:

| | | |
|---|---|---|
| 1M maltose | 0.1 | ml. |
| 10 mM NADP | 0.1 | ml. |
| 0.2 M buffer at various pH | 0.3 | ml. |
| distilled water | 2.5 | ml. |
| total | 3.0 | ml. |

The above reaction mixture is preincubated at 37°C. for 3 minutes. Enzyme solution (1 U/ml., 0.03, ml.) is added thereto and incubated at 37°C. for 10 minutes. The increased amount of reduced NADP is measured at 340 nm to determine the optimum pH.

The results are shown in Fig. 1 of the accompanying drawings, wherein o-o: 0.2 M dimethylglutarate-NaOH buffer (pH 4.5—7.2), ●-●: 0.2 M phosphate buffer (pH 6.1—7.4) and □—□: 0.2 M Tris-HCl buffer (pH 7.9—9.3).

The optimum pH of the maltose dehydrogenase used acocrding to the present invention is approximately pH 7.5.

(6) Optimum temperature:

The reaction mixture (1.00 ml.) described in the above assay method is heated at various temperatures of from 25 to 55°C. for 3 minutes. Enzyme solution (0.05 U/ml., 0.05 ml.) is added thereto and incubated at a set up temperature for 10 minutes. 0.1 N HCl (2.0 ml.) is added to stop the enzyme reaction and then the increased amount of $NTBH_2$ is measured by optical density at 550 nm. The results obtained are shown in Fig. 2 of the accompanying drawings. The optimum temperature of the maltose dehydrogenase used according to the present invention is approximately 45°C.

(7) pH stability:

A mixture of enzyme solution (10 U/ml., 0.1 ml.), buffer solution (0.1 ml., at various pH) and distilled water (0.8 ml.) is preincubated at 37°C. for 60 minutes. The reaction mixture is immediately cooled in ice-cold water and the enzyme activity is assayed according to the above assay method. The results obtained are shown in Fig. 3 of the accompanying drawings. In this Figure, o-o: 0.2 M dimethylglutarate-NaOH buffer (pH 4—6.6), ●-●; 0.2 M phosphate buffer (pH 6.3—7.4), □—□: 0.2 M Tris-HCl buffer (pH 7.6—8.6) and △—△: 0.2 M glycine-NaOH buffer (pH 8.7—9.5). The pH stability of the maltose dehydrogenase used according to the present invention is pH 7 to 8.5 at 37°C. for 60 minutes treatment.

(8) Heat stability:

A mixture of enzyme solution (10 U/ml., 0.1 ml.), 0.2 M Tris-HCl buffer (pH 7.5, 0.1 ml.) and distilled water (0.8 ml.) is preincubated at various temperatures of from 37 to 60°C. for 10 minutes. After treatment, the reaction mixture is cooled in ice-water, whereafter the enzyme activity of the mixture is assayed according to the above assay method. The results obtained are shown in Fig. 4 of the accompanying drawings. The maltose dehydrogenase used according to the present invention has the heat stability below 37°C. at pH 7.5 for 10 minutes treatment.

(9) Molecular weight:

Approximately 93000 (Gel-filtration method using "Sephacryl" S—200) ("Sephacryl" is a Registered Trade Mark).

(10) Isoelectric point:

Approximately pH 5.1 (electrophoresis using ampholite carrier)

(11) Km value:

$Km = 3.4 \times 10^{-10}$ M (for maltose)

(12) Effect of metallic ions, PCMB and EDTA:

| | | |
|---|---|---|
| 0.2 M Tris-HCl buffer (pH 7.5) | 0.4 | ml. |
| 0.5% NTB | 0.05 | ml. |

| | | |
|---|---|---|
| 2% Triton X—100 | 0.05 | ml. |
| 1 M maltose | 0.1 | ml. |
| 10 mM NADP | 0.1 | ml. |
| 0.05% PMS | 0.02 | ml. |
| metallic ions, PCMB or EDTA solution | 0.1 | ml. |
| distilled water | 0.18 | ml. |
| total | 1.00 | ml. |

The above reaction mixture is preincubated at 37°C. for 3 minutes. The enzyme solution (0.05 U/ml., 0.5 ml.) is added thereto and incubated at 37°C. for 10 minutes, the enzymatic reaction then being stopped by adding 0.1 N HCl (2.0 ml.). The optical density at 550 nm is measured for observing the effect of each metallic ion, PCMB and EDTA. The results obtained are shown in Table 4:

TABLE 4

| metallic ion, EDTA or PCMB | relative activity (%) | metallic ion, EDTA or PCMB | relative activity (%) |
|---|---|---|---|
| (no addition) | 100 | 0.01 M $CoCl_2$ | 0 |
| 0.1 M KCl | 91 | 0.01 M $ZnCl_2$ | 0 |
| 0.1 M NaCl | 104 | 0.001 M $CuCl_2$ | 0 |
| 0.1 M $NH_4Cl$ | 35 | 0.01 M $NaN_3$ | 100 |
| 0.1 M LiCl | 89 | 0.0004 M PCMB | 126 |
| 0.01 M $CaCl_2$ | 77 | 0.01 M EDTA | 134 |
| 0.01 M $MgCl_2$ | 16 | 0.001 M EDTA | 114 |
| 0.01 M $BaCl_2$ | 106 | 0.0001 M EDTA | 110 |
| 0.01 M $MnCl_2$ | 0 | | |

(13) Effect of surface active agents:

| | | |
|---|---|---|
| 1 M maltose | 0.1 | ml. |
| 10 mM NADP | 0.1 | ml. |
| 0.2 M Tris-HCl buffer (pH 7.5) | 0.4 | ml. |
| surface active agent | 0.1 | ml. |
| distilled water | 2.3 | ml. |
| total | 3.0 | ml. |

The above reaction mixture is preincubated at 37°C. for 3 minutes. The enzyme solution (1 U/ml., 0.05 ml.) is added thereto and incubated at 37°C. for 10 minutes. The increased optical density at 340 nm caused by the reduced NADP is a measure of the effect of the surface active agent. The results obtained are shown in Table 5.

TABLE 5

| surface active agent (%) | relative activity (%) |
|---|---|
| control | 100 |
| "Triton" X–100 (0.1) | 98 |
| " (0.5) | 105 |
| " (1 ) | 107 |
| "Adekatol" SO–145 (0.1) | 105 |
| " (0.5) | 102 |
| " (1 ) | 126 |
| sodium dodecyl sulphate (0.1) | 6 |
| " (0.5) | 0.5 |
| " (1 ) | 1 |
| sodium laurylbenzene sulphate (0.1) | 0.5 |
| " (0.5) | 1 |
| " (1 ) | 1 |
| "Tween"–60 (0.1) | 92 |
| " (0.5) | 99 |
| "Tween"–80 (0.1) | 98 |
| " (0.5) | 100 |
| "Cation" DT (0.1) | 100 |
| " (0.5) | 100 |
| cetyltrimethylammonium chloride (0.1) | 7 |
| " (0.5) | 4 |
| sodium deoxylcholate (0.1) | 116 |
| " (0.5) | 127 |

("Triton", "Adektatol", "Tween" and "Cation" are Registered Trade Marks).

The above biochemical properties indicate that the enzyme used according to the present invention has weak substrate specificities for lactose, cellobiose and maltotriose and strong specific activity for maltose. These substrate specificities, other enzymatic actions and other properties indicate that the enzyme is preferred to the known maltose dehydrogenase (NAD(P)-dependent maltose dehydrogenase; see *Agr. Biol. Chem.*, 44(1), 41—47/1980).

The maltose dehydrogenase used according to the present invention is not restricted to the above-described enzyme. For example, maltose dehydrogenase obtained from culturing the strain *Corynebacterium* sp. No. 93—1 (see *Agr. Biol. Chem.*, 44(1), 41—47/1980) can also be used.

As explained hereinbefore, the decomposed substrate, such as maltose, glucose or oligosaccharide, such as maltotriose and maltopentose, can be dehydrogenated with the formation of reduced NAD or NADP.

The reduced NAD or reduced NADP thus formed can be directly measured by absorption at 340 nm for the amylase activity assay or reduced NAD or reduced NADP can be reacted with a hydrogen transport colour reagent, hydrogen being transferred to the other transport system, which is indirectly measured for determining the amylase activity.

Examples of hydrogen transport colour reagents for the indirect assay system include a

tetrazolium salt and diaphorase or a tetrazolium salt and phenazinemethosulphate.

The resultant reaction product is measured colorimetrically by absorption at 550 nm.

Preferred examples for the amylase assay system are reaction mixtures comprising 0.4 parts 0.2 M Tris-HCl buffer, 0.1 parts 1% bovine serum albumin, 0.1 parts 0.25% NTB, 0.1 parts 1% Triton X—100, 0.1 parts 10 mM NAD(P), 0.02 parts 0.05% PMS, 0.1 parts 10% substrate solution, 0.05 parts 200 U/ml. maltose dehydrogenase and 0.03 parts distilled water; or a mixture comprising 0.3 parts 0.2 M Tris-HCl buffer, 0.1 parts 10 mM NAD(P), 0.1 parts 10% substrate solution, 0.05 parts 200 U/ml. maltose dehydrogenase and 0.45 parts distilled water.

Generally one part of these reaction mixtures is mixed with 0.01—0.5 parts of sample and incubated at 37°C. for an appropriate time, such as 5 minutes. After termination of the reaction, the mixture is measured by optical density determination at a conventional wavelength.

Samples to be assayed include serum, urine and saliva, which is optionally diluted before assay. Sometimes the samples also contain glucose or maltose. Maltose can be removed by converting it into glucose with $\alpha$-glucosidase and the glucose is phosphorylated by a kinase, such as hexokinase or glucokinase, in the presence of ATP and $Mg^{++}$ (preferably $MgCl_2$) to give glucose-6-phosphate. Maltose can also be phosphorylated by maltose phosphorylase. By means of these treatments, previously present glucose and maltose are converted into the compounds which do not affect the soluble oxygen in the sample and the amylase activity assay.

As hereinbefore explained, the present invention provides an assay method for amylase activity, such as $\alpha$- or $\beta$-amylase activity, in a sample which comprises decomposing a substrate, such as a glucose polymer having a modified reducing terminal glucose residue, or a cyclic glucose polymer by the action of the amylase activity in the sample, preferably reacting the decomposed substrate with maltose dehydrogenase and NAD or NADP and quantitatively measuring, directly or indirectly, the reduced NAD or reduced NADP formed. More preferably, the present invention can be performed with an additional step in which previously present glucose or maltose is converted into glucose 6-phosphate by a kinase in the presence of $\alpha$-glucosidase, $Mg^{++}$ and ATP.

The amylase activity assay method of the present invention is a simple and accurate method which may be performed by a kit comprising the above reagents. It can be used for an automatic assay.

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

EXAMPLE 1.

| | | |
|---|---|---|
| 0.2 M Tris-HCl buffer (pH 7.4) | 0.4 | ml. |
| 1% bovine serum albumin | 0.1 | ml. |
| 0.25% NTB | 0.1 | ml |
| 1% Triton X—100 | 0.1 | ml. |
| 10 mM NADP | 0.1 | ml. |
| 0.05% PMS | 0.02 | ml. |
| 10% oxidised soluble starch* | 0.1 | ml. |
| 200 U/ml. maltose dehydrogenase** | 0.05 | ml. |
| distilled water | 0.03 | ml. |
| total | 1.0 | ml. |

* glucose polymer with modified reducing terminal glucose residue (gluconic acid residue) obtained in Example 9 (oxidised starch hydrolysate).

** produced according to Example 12.

The above reaction mixture (1.0 ml.) was preincubated at 37°C. for 3 minutes. Saliva (20 $\mu$l., 300 fold dilution) was added thereto and incubated at 37°C. for 0, 2.5, 5, 10, 20 and 30 minutes, respectively. After the reaction, 0.1 N HCl (2.0 ml.) was added and the mixture was measured at 550 nm by colorimetry.

The results are shown in Fig. 5 of the accompanying drawings, a good linearity being obtained except for an initial time-lag of 2.5 minutes.

EXAMPLE 2

The mixture (1.0 ml.) described in Example 1 was preincubated at 37°C. for 3 minutes. 1000 fold diluted saliva samples (0, 10, 20, 30, 40 and 50 $\mu$l., respectively) were added thereto and incubated at 37°C. for 10 minutes. After incubation, 0.1 N HCl (2.0 ml.) was added, followed by colorimetric assay at

550 nm. The results obtained are shown in Fig. 6 of the accompanying drawings, in which a good linearity was obtained.

EXAMPLE 3

Reaction mixture I:

| | | |
|---|---|---|
| 0.2 M Tris-HCl buffer (pH 7.5) | 0.2 | ml. |
| 1% bovine serum albumin | 0.1 | ml. |
| 0.25% NTB | 0.1 | ml. |
| 1%Triton X—100 | 0.1 | ml. |
| 10 nM NADP | 0.1 | ml |
| $\alpha$-glucosidase (200 U/ml.) | 0.1 | ml |
| hexokinase (100 U/ml.) | 0.1 | ml. |
| 100 mM $MgCl_2$ | 0.05 | ml. |
| 0.05% PMS | 0.02 | ml. |
| total | 0.87 | ml. |

Reaction mixture II:

| | | |
|---|---|---|
| 10% oxidised soluble starch* | 0.1 | ml. |
| 200 U/ml. maltose dehydrogenase | 0.05 | ml. |
| 200 mM EDTA | 0.05 | ml. |
| total | 0.2 | ml. |

*glucose polymer with modified reducing terminal glucose residue (gluconic acid residue) obtained in Example 9 (oxidised soluble starch).

Reaction mixture I (0.8 ml.) was preincubated at 37°C. Serum (50 $\mu$l.) was added thereto and incubated at 37°C. for 5 minutes to remove glucose and maltose present. Reaction mixture II (0.2 ml.) was added thereto and incubated at 37°C. for 0, 2.5, 5, 10, 15, 20 and 25 minutes, respectively. After the reaction, 0.1 N HCl (2.0 ml.) was added, followed by colorimetric measurement at 550 nm.

Reaction mixture I' without hexokinase was used as control.

The results obtained are shown in Fig. 7 of the accompanying drawings, wherein oo: reaction mixture I and II, ●-●: reaction mixture I' and II. As shown in Fig. 7, the assay method using reaction mixture I and II gave good results.

EXAMPLE 4

Reaction mixture I:

| | | |
|---|---|---|
| 0.2 M Tris-HCl buffer (pH 7.5) | 1.2 | ml. |
| 10 mM NADP | 0.3 | ml. |
| $\alpha$-glucosidase (200 U/ml.) | 0.1 | ml. |
| hexokinase (10 U/ml.) | 0.1 | ml. |
| 100 mM $MgCl_2$ | 0.15 | ml. |
| distilled water | 0.55 | ml. |
| total | 2.4 | ml. |

| Reaction mixture II: | | |
|---|---|---|
| 10% oxidised soluble starch* | 0.3 | ml. |
| 200 U/ml. maltose dehydrogenase | 0.05 | ml. |
| 200 mM EDTA | 0.15 | ml. |
| total | 0.5 | ml. |

* glucose polymer with modified reducing terminal glucose residue (gluconic acid residue) obtained in Example 9 (oxidised soluble starch).

Reaction mixture I (2.4 ml.) was preincubated at 37°C. Serum (50 $\mu$l., 1/5—5/5 fold dilution) was added thereto and incubated at 37°C. for 5 minutes. Reaction mixture II (0.5 ml.) was added thereto and incubated at 37°C. for exactly 20 minutes and then measured at 340 nm.

Reaction mixture I', which did not contain hexokinase, was used as control.

The results obtained are shown in Fig. 8 of the accompanying drawings, wherein o-o: reaction mixture I and II, ●-●: reaction mixture I' and II. Good results were obtained by assaying with reaction mixture I and II.

EXAMPLE 5

The substrate in reaction mixture II of Example 3 was replaced by a 10% solution (0.3 ml.) of the glucose polymer with modified reducing terminal glucose residue (methyl etherified glucose residue) obtained in Example 10 to give reaction mixture II, the procedure being the same as in Example 3.

The results obtained show a good linearity of the reaction time and an assay was obtained which is the same as shown in Fig. 7 of the accompanying drawings.

The optical absorption after 10 minutes was 0.52.

EXAMPLE 6

The substrate in reaction mixture II in Example 4 was replaced by a 10% solution (10.3 ml.) of the glucose polymer with modified reducing terminal glucose residue (methyl etherified glucose residue) obtained in Example 10 to give reaction mixture II, the procedure otherwise being the same as in Example 4, using serum of 5/5 fold dilution. The optical absorption is 1.06, showing a good result.

EXAMPLE 7

The substrate in reaction mixture II in Example 3 was replaced by a 10% solution (0.3 ml.) of the glucose polymer with modified reducing terminal glucose residue (acetylated glucose residue) obtained in Example 11 to give reaction mixture II, the procedure otherwise being the same as in Example 3. Good linearity was obtained in each reaction time, which was the same as in Fig. 7 of the accompanying drawings.

EXAMPLE 8

The substrate in reaction mixture II in Example 3 was replaced by a 5% solution (0.3 ml.) of $\gamma$-cyclodextrin to give reaction mixture II, the remaining operation being the same as in Example 3. The optical absorption after 30 minutes reaction was 0.12.

EXAMPLE 9

Soluble starch (50 g.) was dissolved in water (200 ml.), added to Fehring's reagent (1 litre containing 35 g. $CuSO_4 \cdot 5H_2O$, 173 g. sodium potassium tartrate and 65 g. sodium hydroxide) and boiled for 20 minutes. The reaction mixture was concentrated *in vacuo* to 300 ml. Methanol (100 ml.) was added thereto and the precipitate removed. The supernatant solution was concentrated to 100 ml. and applied to a column (7 x 100 cm.) of "Sephadex" G—100 ("Sephadex" is a Registered Trade Mark). Eluate corresponding to a molecular weight above 1000 was collected and lyophilised to give a glucose polymer (33.6 g.) with modified reducing terminal glucose residue (gluconic acid residue) (reducing terminal glucose residue in soluble starch was oxidised to a gluconic acid residue).

EXAMPLE 10

Soluble starch (20 g.) suspended in 4% methanolic hydrochloric acid (400 ml.) was reacted at 70°C. for 6 hours. The reaction mixture was neutralised with 5 N sodium hydroxide solution, desalted and concentrated *in vacuo*. The concentrate was applied to a column (5 x 100 cm.) of "Sephadex" G—25. Eluate corresponding to a molecular weight above 1000 was collected, concentrated *in vacuo* and lyophilised to give glucose polymer (8.3 g.) with modified reducing terminal glucose residue of methylated reducing terminal residue in soluble starch.

EXAMPLE 11.

Soluble starch (20 g.) in dry pyridine (200 ml.) and acetic anhydride (3.5 ml.) was reacted at 0°C.

overnight. Acetone (1 litre) was added thereto. The precipitate was washed with acetone (200 ml.), dissolved in water and applied to a column (5 x 100 cm.) of "Sephadex" G—25. Eluate corresponding to a molecular weight above 1000 was collected and lyophilised to give glucose polymer (10.3 g.) with modified reducing terminal glucose residue of acetylated reducing terminal residue in soluble starch.

EXAMPLE 12

A medium (100 ml.) comprising 1% dextrin, 1% yeast extract powder, 0.1% $K_2HPO_4$, 0.05% KCl and 0.05% $MgSO_4 \cdot 7H_2O$ (sterilised at 120°C. for 20 min., pH 7.2) in a 500 ml. Erlenmeyer flask was inoculated with one loopful of *Bacillus megaterium* B—0779 FERM—P No. 5662 from a bouillon agar slant and shake cultured at 28°C. for 24 hours. The seed culture thus prepared was transferred into a medium (20 litres) comprising 1% dextrin, 1% yeast extract powder, 0.1% $K_2HPO_4$, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% silicone SA G—471 (anti-foamer) (previously sterilised at 120°C. for 20 min., pH 7.2) in a 300 litre tank and submerge cultured at 28°C. for 45 hours, 300 r.p.m., 15 $m^3$/min. aeration. Cultured cells were collected by centrifuging at 5000 r.p.m. for 10 minutes. The wet cells were treated with a solution of 0.1% lysozyme and 5 mM EDTA in Tris-HCl buffer (pH 7.5, 4 litres) at 37°C. for 60 minutes. The solution of solubilised cells was centrifuged (5000 r.p.m., 10 min.) to separate the supernatant (11.8 U/ml., 3.2 litres). Ammonium sulphate was added to the supernatant solution up to 80% saturation and centrifuged (15000 r.p.m., 10 min.).

The precipitate was dissolved in 10 mM Tris-HCl buffer (pH 7.5, 220 ml.) and centrifuged (15000 r.p.m., 10 min.). To the supernatant solution (200 ml., 123.5 U/ml.) was added 10% calcium chloride solution (20 ml.) and again centrifuged (15000 r.p.m., 10 min.). Ammonium sulphate was added to the supernatant (200 ml., 85 U/ml.) and fractions of 51—63% ammonium sulphate saturation were collected and centrifuged (15000 r.p.m., 10 min.). The precipitate was dissolved in 10 mM Tris-HCl buffer (pH 7.5, 20 ml., 417 U/ml.) and applied to a column of "Sephadex" G—25. The eluate was lyophilised to give purified maltose dehydrogenase (370 mg., 20 U/mg.).

CLAIMS

1. An assay method for amylase activity, comprising assaying a substrate decomposed by the enzyme amylase, wherein the substrate used is a glucose polymer having a modified reducing terminal glucose residue or is a cyclo glucose polymer (cyclodextrin).

2. An assay method according to claim 1, wherein the decomposed substrate is assayed by treating with maltose dehydrogenase and NAD or NADP.

3. An assay method according to claim 1 or 2, wherein the substrate used is a glucose polymer with a modified reducing terminal glucose residue and a degree of glucose polymerisation of more than 5 or is a cyclo glucose polymer.

4. An assay method according to claim 3, wherein the substrate is a glucose polymer with a modified reducing terminal glucose residue derived from amylose, amylopectin, starch or a starch hydrolysate.

5. An assay method according to any of the preceding claims, wherein the modified reducing terminal glucose residue is an etherified reducing terminal.

6. An assay method according to any of claims 1 to 4, wherein the modified reducing terminal glucose residue is an esterified reducing terminal residue.

7. An assay method according to any of claims 1 to 4, wherein the modified reducing terminal residue is a gluconolactone or a gluconic acid residue or a derivative thereof.

8. An assay method according to any of claims 2 to 7, wherein the assay is performed by measuring the amount of reduced NAD or reduced NADP.

9. An assay method according to any of claims 2 to 7, wherein the assay is performed by a colorimetric assay in which reduced NAD or reduced NADP is reacted with a reduced form of a hydrogen transport colorimetric reaction reagent.

10. An assay method according to claim 9, wherein the reduced form hydrogen transport colorimetric reaction reagent is a reagent comprising a tetrazolium salt and diaphorase.

11. An assay method according to claim 9, wherein the reduced form hydrogen transport colorimetric reaction reagent is a reagent comprising a tetrazolium salt and phenazinemethosulphate.

12. An assay method according to any of the preceding claims, wherein the assay is performed by assaying the amylase activity in the sample which has been pretreated with $\alpha$-glucosidase or kinase in the presence of $Mg^{++}$ and ATP.

13. An assay method according to claim 12, wherein the kinase is hexokinase.

14. An assay method according to any of claims 2 to 13, wherein the maltose dehydrogenase used is an enzyme produced by culturing a maltose dehydrogenase-producing micro-organism of the genus *Bacillus* in a nutrient medium and isolating the enzyme from the culture mass.

15. An assay method according to claim 14, wherein the maltose dehydrogenase-producing micro-organism of the genus *Bacillus* is a micro-organism of *Bacillus megaterium.*

16. An assay method according to claim 15, wherein the *Bacillus megaterium* is the strain *Bacillus megaterium* B—0779 (FERM—P No. 5662).

17. An assay method according to claim 1 for amylase activity, substantially as hereinbefore

described and exemplified.

18. Maltose dehydrogenase, whenever obtained from *Bacillus megaterium* B—0779 (FERM—P No. 5662).

19. A test kit for carrying out the assay method according to any of claims 1 to 17, substantially as hereinbefore described.

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