PATENT SPECIFICATION (11) 1 571 265

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(21) Application No. 14182/78 (22) Filed 11 April 1978
(31) Convention Application No. 820 072
(32) Filed 28 July 1977 in
(33) United States of America (US)
(44) Complete Specification published 9 July 1980
(51) INT CL³ C12Q 1/40; C08B 37/00
(52) Index at acceptance
G1B BR
C3U 2F 4C3

(19)

(54) AMYLASE DETERMINATION

(71) We, AMERICAN HOSPITAL SUPPLY CORPORATION, One American Plaza Evanston, Illinois 60201, United States of America, a corporation organised and existing under the laws of the State of Illinois, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to methods of determining amylase activity in samples, such as biological fluids, and more particularly to a method for the determination of amylase activity based on the cleavage of chromophore derivatives of oligosaccharides.

One of the most widely studied and accepted procedures in clinical chemistry is the determination of serum and urine α-amylase which is used for the diagnosis of pancreatic disease.

During the past twenty-five years various amylase methods have been developed for use in the clinical laboratory. Some of the methods, i.e. saccharogenic method, involves complicated methodology which makes its routine use prohibitive. Other methods, i.e. turbidometric and viscosimetric methods for the determination of α-amylase activity are dependent on changes in the physical properties of the substrate, which may be influenced to a considerable degree by other factors present in the serum. Today, one of the most widely used methods for α-amylase determination is the starch-iodine method. With this method only a specific portion of the substrate is measured and the enzyme does not work under substrate saturation conditions. Further, the presence of serum proteins may interfere with the starch-iodine reaction.

In addition to the above difficulties associated with the mentioned method, a further difficulty is encountered because the aforementioned methods can only be used to determine a rather limited range of α-amylase activity. Also, some of the methods cannot be used for accurate determination of either sub-normal or highly elevated α-amylase levels.

It has been shown that α-amylase activity can be determined based on the solubilization of colored starch grains by α-amylase. It has also been shown that by using cross-linked soluble starch marked with a dye marker under alkaline conditions, α-amylase activity could be determined. However, these reported procedures require elevated incubation temperature, prolonged incubation time and oftentimes double dialysis to achieve adequate sensitivity.

Synthetic fluorogenic and chromogenic starch substrates have also been used to determine amylase activity in biological fluids. In these methods a chromophore is covalently bound to a water insoluble, cross-linked starch. Amylase acts upon the substrates to release water-soluble fragments which are measured spectroscopically after first separating the water-insoluble residue.

In another assay involving a synthetic substrate (Nature, 182 (1958) 525—526) a p-nitrophenol derivative of maltose is used. The p-nitrophenol replaces the anomeric hydroxyl group of maltose. Amylase causes cleavage of the substrate to produce p-nitrophenol which can be monitored at 410 nm. However, the assay is 16 hours long and maltase also cleaves the substrate. In addition, since this derivative has no endo-α-1,4 bond its specificity as an amylase substrate is subject to considerable questions.

The method according to the invention is distinguished from the known prior methods by either greater simplicity, greatly decreased incubation time or greater sensitivity.

In accordance with the present invention, there is provided a process for determining the α-amylase content of a sample, comprising adding a chromogenic oligosaccharide substrate to a solution containing a measured amount of sample and α and β glucosidase and determining amylase activity by the release of chromophore.

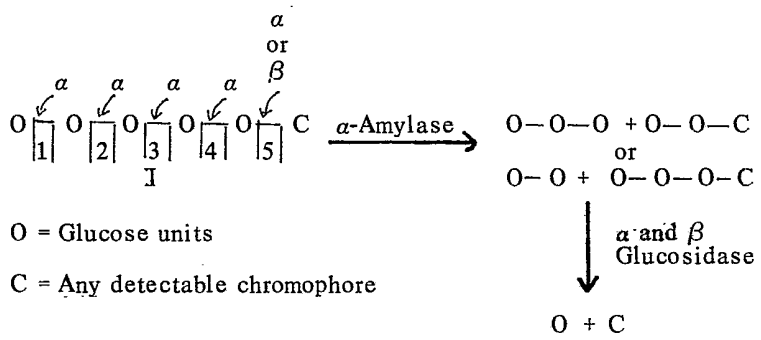
To provide greater sensitivity, preferably

the substrate is an aligosaccharide having 4 to 10 glucose units. Oligosaccharides of this chain length are resistant to cleavage by glucosidase and contains endo-α-1,4 linkages which are required for amylase activity. Cleavage of these 1,4 bonds by amylase produces smaller fragments which are acted upon by α-glusosidase and/or β-glucosidase to liberate chromophore. The amount of chromophore liberated is thus proportional to amylase activity.

The chromophore may be p-nitrophenol. Oligosaccharides having 4 to 10 glucose units and tagged with p-nitrophenol have sufficient chain length to resist cleavage by maltase and possessing an endo 1,4 bond can react rapidly with amylase.

Maltotetraose is the smallest oligosaccharide readily susceptible to specific cleavage by amylase. Compounds other than p-nitrophenol derivatives could also be used to develop colorimetric, fluorometric and ultraviolet procedures. The p-nitrophenol derivative of an oligosaccharide mixture (of 4 to 10 glucose units) was prepared by acetylation, followed by reaction with p-nitrophenol in the presence of stannic chloride and subsequent deactylation with sodium methoxide in methanol. The reaction system for determination of amylase consists of the derivatized oligosaccharides, α and β glucosidase, and phosphate buffer. The reaction may be monitored continuously by the change in absorption at 405 nm. Purified hog pancreatic amylase gave linear rates with no lag phase. Preliminary testing with patients' samples gave good correlation with classical amylase methods.

The reaction of the above outlined invention can best be described by reference to the following reaction diagram:



α-amylase is known to act preferentially on the endo-1,4 bonds of the oligosaccharide molecule such as bonds 2,3 above. α and β glucosidase, on the other hand, prefer exo-bonds such as bonds 1,5 above. However, the α and β glucosidase does not act on carbohydrate molecules longer than 4 glucose units in length. It therefore becomes apparent that a chromophore molecule esuch as (I) above should be acted upon by amylase at both the 2 and 3 position. The reaction will result in two possible combinations of products depending on the attack point of the amylase, i.e. endo bonds 2 and 3.

To overcome the problems associated with prior amylase assay procedures, it has been found that controlled molecular weight oligosaccharides (4—10 glucose units) can be substituted for maltose. Since amylase acts preferably on endo bonds to form smaller fragments, therefore, α and β glucosidase are included in the assay conditions to release the chromophore from the smaller polysaccharide fragments. Specifically, the p-nitrophenol and the 4-methylumbelliferone derivatives, are suitable substrates.

The following examples serve to illustrate a method of preparing the p-nitrophenol substrate required for the inventive method and also the inventive amylase determination method:

Example I.

Synthesis of Substrate.

a. Acetylation of Oligosaccharide.

A suspension of fifty grams of oligosaccharides (DP-5 to DP-10) in 50 ml of pyridine was diluted with 200 ml of acetic anhydride. The mixture was then heated in a water bath (95C) with stirring for 3 hours. While still warm the mixture was poured in 1500 ml of ice water. An oil was formed which eventually solidified with stirring. The solid was washed with water, filtered and dried. Recrystallization of the crude material

from 400 ml of ethanol yielded 65 grams of a white solid.

b. Reaction of p-Nitrophenol with Acetylated Oligosaccharides.

To a refluxing mixture of 40 grams of acetylated oligosaccharides and 7.5 grams of p-nitrophenol in 120 ml of benzene and 60 ml of chloroform (ethanol free) was added 3 ml of stannic chloride. After refluxing for 15 minutes another 3 ml of stannic chloride was added, followed by 15 minutes of heating. The reaction mixture was then diluted with ethyl acetate (200 ml), and extracted with water and then with saturated sodium bicarbonate to remove the p-nitrophenol. After drying over magnesium sulfate the solvent was removed. The yellow solid can be purified by eluting with ethyl acetate-heptane (4:1) on silica gel. Isolated 13 g of a white solid.

c. Deactylation of Oligosaccharides.

To 7 grams of the p-nitrophenol derivative of the oligosaccharides in 60 ml of methanol and 25 ml of methylene chloride was added 8 ml of a sodium methoxide in methanol solution (prepared from 40 mg of sodium). After 2 days at 0C, the white precipitate was isolated by centrifuging the reaction mixture, washed with methanol-methylene chloride (1:1) containing several drops of acetic acid, decanted, and then washed twice with ether. The white solid was dried in vacuo to yield 2 grams of material.

Elemental analysis: Nitrogen 0.64%.

Example II.

Determination of Amylase Activity.

a. Endpoint Method.

An assay mixture comprising:

1) 0.8 ml Buffer solution (potassium phosphate Buffer 2×10^{-1}M containing 5×10^{-1}M Nacl and Substrate at concentration of 4 mg/ml Buffer,
2) 0.02 ml α Glucosidase 5 mg/ml,
3) 0.02 ml β Glucosidase 5 mg/ml,
4) 0.05 ml Sample,

is incubated for exactly 10 minutes at 37° C. After exactly 10 minutes the reaction is stopped by the addition of 3.0 ml of 0.1 N Sodium Hydroxide. The amount of p-nitrophenol released which is proportional to amylase activity is then recorded with time at 405 nm.

Figure I is a comparison graph of elevated Serum Samples from the upper limit of normal to eleven time the upper limit of normal range of 60—150 somogy units/di by a Turbidimetric Method as plotted against the absorbance of each sample as obtained by the endpoint method.

Figure II shows the absorbances seen with the Endpoint Method as compared with absorbances obtained by the Iodometric Method.

Listed below are the values plotted from Graphs I and II, which are shown in the accompanying drawings. As can be clearly seen, linear relationships were obtained on both graphs.

METHOD COMPARISON DATA

in somogy units/di

Patient Sample	X Upper Limit of Normal	Turbidimetric Method	Iodometric Method	pnp End Point Method
1	1X	172	166	192
2	2X	291	300	245
3	3X	421	417	478
4	4X	540	534	528
5	6X	910	858	919
6	11X	1659	1717	1629

WHAT WE CLAIM IS:—

1. A process for determining the α-amylase content of a sample, comprising adding a chromogenic oligosaccharide substrate to a solution containing a measured amount of sample and α and β glucosidase and determining amylase activity by the release of chromophore.

2. A process according to claim 1, wherein potassium phosphate is added to the solution as buffer.

3. A process according to claim 1 or 2,

wherein the substrate is an oligosaccharide having 4—10 glucose units.

4. A process according to any of claims 1 to 3, wherein the chromophore is p-nitrophenol.

5. A process according to any of claims 1 to 3, wherein the chromophore is 4-methylumbelliferone.

6. A process according to any preceding claim, wherein the chromophore is a colorimetric, fluorescent or U.V. absorbing substance.

7. A process according to claim 1, wherein the substrate has been prepared by a method substantially as described herein in Example I.

8. A process according to claim 1, substantially as described herein in Example II.

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Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1980.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

FIG. 1

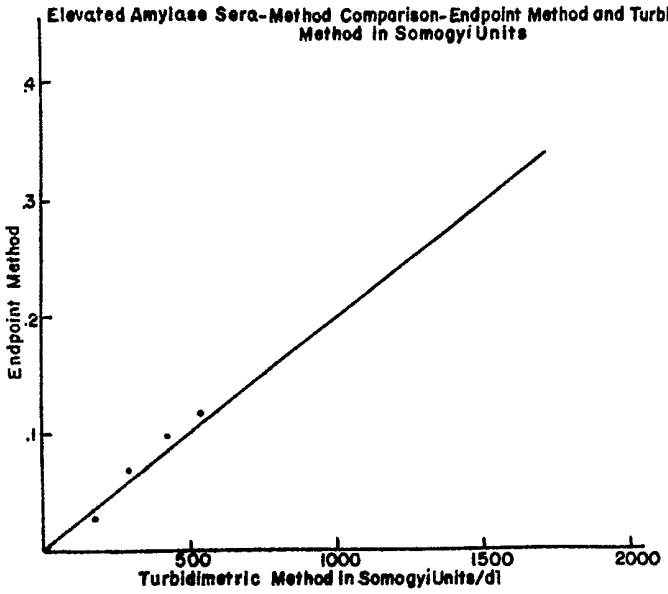
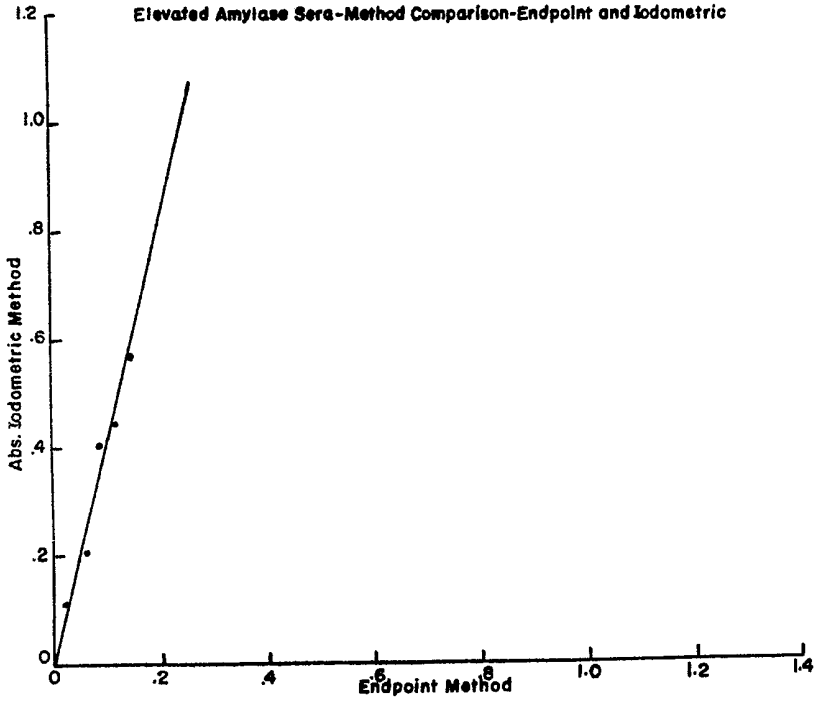

Elevated Amylase Sera-Method Comparison-Endpoint Method and Turbidimetric Method in Somogyi Units
Endpoint Method
.4
.3
.2
.1
500
1000
1500
2000
Turbidimetric Method in Somogyi Units/dl

FIG. 2


Elevated Amylase Sera-Method Comparison-Endpoint and Iodometric
Abs. Iodometric Method
1.2
1.0
.8
.6
.4
.2
0
.2
.4
.6
.8
1.0
1.2
1.4
Endpoint Method