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APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 27-2-90

SPRUSON & FERGUSON

LODGED AT SUB-OFFICE
20 FEB 1986
Sydney

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

CONVENTION APPLICATION FOR A STANDARD PATENT

53810/86

We, ABBOTT LABORATORIES, of 14th Street and Sheridan Road, North Chicago, Illinois 60064, United States of America hereby apply for the grant of a standard patent for an invention entitled:

"FLUORESCENCE POLARIZATION ASSAY FOR AMYLASE"

~~"HYDROLASES, REAGENTS FOR USE IN THE ASSAY AND METHOD FOR MAKING THE REAGENTS"~~

which is described in the accompanying complete specification.

DETAILS OF BASIC APPLICATION

Number of Basic Application:-
705,268

Name of Convention Country in which Basic Application was filed:-
United States of America

Date of Basic application:-
25 February 1985

PATENT OFFICE 100 LODGED AT SUB-OFFICE 20 FEB 1986 Sydn 50 AUSTRALIA ONE HUNDRED DOLLARS FIFTY DOLLARS

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DATED this NINETEENTH day of FEBRUARY 1986

ABBOTT LABORATORIES

By: M.J. Anderson

Registered Patent Attorney.

PATENT OFFICE 20 AUSTRALIA TWENTY DOLLARS Sydn 18 SUB-OFFICE PATENT OFFICE 5 AUSTRALIA FIVE DOLLARS

TO: THE COMMISSIONER OF PATENTS
AUSTRALIA

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AUSTRALIAN PATENT OFFICE

SBR:JMA:116W

4233

Spruson & Ferguson

AUSTRALIA
CONVENTION
STANDARD
& PETTY PATENT
DECLARATION
SFP4

COMMONWEALTH OF AUSTRALIA

THE PATENTS ACT 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a patent for an invention entitled:

Title of Invention: "FLUORESCENCE POLARIZATION ASSAY FOR MACROMOLECULAR HYDROLASES, REAGENTS FOR USE IN THE ASSAY AND METHOD FOR MAKING THE REAGENTS"

Full name(s) and address(es) of Declarant(s)

I/~~We~~ JOSEPH M. BERNIK, ASSISTANT SECRETARY

of ABBOTT LABORATORIES,

~~of~~ 14th Street & Sheridan Road
North Chicago, Illinois 60064
U.S.A.

do solemnly and sincerely declare as follows:–

LODGED AT SUB-OFFICE
15 MAY 1986
Sydney

Full name(s) of Applicant(s)

1. ~~I am/We are the applicant(s) for the patent~~

(or, in the case of an application by a body corporate)

1. I am/~~We are~~ authorised by ABBOTT LABORATORIES

the applicant(~~s~~) for the patent to make this declaration on its/~~their~~ behalf.

2. The basic application(~~s~~) as defined by Section 141 of the Act was/~~were~~ made

Basic Country(ies) in The United States of America

Priority Date(s) on February 25, 1985

Basic Applicant(s) by Mark Raymond Shaffar

3. ~~I am/We are the actual inventor(s) of the invention referred to in the basic application(s)~~

(or where a person other than the inventor is the applicant)

Full name(s) and address(es) of inventor(s)

3. Mark Raymond Shaffar

of 3365 C Beacon Street, No. 15, North Chicago, Illinois 60064, United States of America

~~(respectively)~~

is/~~are~~ the actual inventor(~~s~~) of the invention and the facts upon which the applicant(~~s~~) is/~~are~~ entitled to make the application are as follows:

Set out how Applicant(s) derive title from actual inventor(s) e.g. The Applicant(s) is/are the assignee(s) of the invention from the inventor(s)

The said applicant is the assignee of the actual inventor

4. The basic application(~~s~~) referred to in paragraph 2 of this Declaration was/~~were~~ the first application(~~s~~) made in a Convention country in respect of the invention (~~s~~) the subject of the application.

Declared at North Chicago, Illinois this 12th day of February 19 86

ABBOTT LABORATORIES

.....
Signature of Declarant(s)

(12) PATENT ABRIDGMENT (11) Document No. AU-B-53810/86
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 596574

(54) Title
FLUORESCENCE POLARIZATION ASSAY OF HYDROCASES

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(71) Applicant(s)
ABBOTT LABORATORIES

(72) Inventor(s)
MARK RAYMOND SHAFFAR

(74) Attorney or Agent
SPRUSON & FERGUSON

(57) Claim

1. A method of determining amylase concentration in a sample, comprising:

a) adding to the sample an amylase substrate coupled to a fluorophore, said amylase substrate capable of being cleaved by amylase wherein the size of said amylase substrate is substantially reduced to provide a polarization response which is substantially different from the polarization response provided by said amylase substrate prior to being cleaved, and an albumin;

b) incubating the test solution for a period of time;

c) passing plane polarized light through the test solution of step b); and

d) detecting the fluorescence polarization response from step c) as an indication of the presence of amylase in the sample.

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FORM 10 SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

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COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE:

Class 53810/86 Int. Class

Complete Specification Lodged:

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Related Art:

Name of Applicant: ABBOTT LABORATORIES

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Complete Specification for the invention entitled:

"FLUORESCENCE POLARIZATION ASSAY FOR AMYLASE"

~~HYDROLASES, REAGENTS FOR USE IN THE ASSAY AND METHOD FOR MAKING THE REAGENTS~~"

The following statement is a full description of this invention, including the best method of performing it known to us

SBR:JMA:116W

BACKGROUND OF THE INVENTION

Technical Field

This invention relates to a method of determining amylase concentration in a sample, especially in biological fluid samples such as whole blood, serum, plasma or urine.

Background Art

There are many situations in which it is desirable to analyze for macromolecular hydrolase activity. In the past, assays for such hydrolases have typically involved the use of a plurality of enzymes and substrates as reagents. Such assays have presented difficulties in that the stability of the various enzymes and substrates, combined in a single reagent solution, is dependent upon conflicting requirements for conditions such as temperature, pH, salinity and the like. For example, the substrates may be more stable in relatively low pH solutions and the enzymes may be more stable in higher pH solutions, such that a serious problem results from the lack of availability of a mutually acceptable environment for the substrate and for the enzymes. Other potential difficulties in conducting such assays are presented by the possibility of cross-reactivity between the various enzymes required for the analysis.

One especially advantageous application for a macromolecular hydrolase assay is the quantitative determination of a polysaccharide hydrolase, such as alpha amylase. Alpha amylase is an enzyme which hydrolyses alpha-glucosidic linkages in starch

and related polysaccharides. Several disease states show elevated amylase, including pancreatitis, peptic ulcer, obstruction of the pancreatic duct, pancreatic carcinoma, acute alcohol ingestion or poisoning and salivary gland diseases such as mumps. According to one prior art procedure, the activity of alpha amylase is assayed by measuring the degree of light absorption of NADH (reduced nicotinamide adenine dinucleotide) at a wavelength of 340 nm, after reacting a sample with phosphorylase, phosphoglucomutase, glucose-6-phosphate dehydrogenase and a limit dextrin.

Another application for a macromolecular hydrolase assay is the quantitative determination of endoproteases such as trypsin. Trypsin is an endoprotease found in the pancreas. Typically, trypsin is assayed by using a chromogenic substrate such as N-α-Benzoylarginine ethyl ester (BAEE). Since BAEE is a relatively small synthetic substrate, assays conducted in this manner are not extremely specific.

While small synthetic substrates are relatively simple to make, they tend to lack specificity due to their size. Large synthetic substrates tend to be more specific for a given enzyme, but can be extremely difficult to manufacture. A detailed knowledge of the chemistry of the cleavage of the enzyme is required and, once this knowledge is obtained, the synthesis itself may be complex.

Alternatively, trypsin has been analyzed by placing a sample on a gelatin film. A positive value of trypsin is reported if the gelatin liquifies. This latter method suffers from the drawback that it tends to be merely a qualitative type of analysis. A further assay method applicable to some proteases is the commonly used radioactivity labelling method. A major drawback of this method is that the unreacted substrate

must be separated from the reacted substrate before the test results can be taken by measuring the radioactivity.

Yet another application is the quantitative determination of lipases. In the past, lipases have typically been analyzed by synthetic chromogenic substrate or turbidity clarification methods. Such techniques are not without drawbacks and these methods suffer from problems of stability, purity and characterization of the substrate, as well as problems of specificity and reproducibility.

Accordingly, there presently exists a need for a simple and accurate means to quantitatively analyze fluids for amylase.

SUMMARY OF THE INVENTION

The present invention is directed to: analytical methods, for conducting a fluorescence polarization assay to quantitatively determine the presence of amylase.

According to a broad form of this invention there is provided a method of determining amylase concentration in a sample, comprising:

a) adding to the sample an amylase substrate coupled to a fluorophore, said amylase substrate capable of being cleaved by amylase wherein the size of said amylase substrate is substantially reduced to provide a polarization response which is substantially different from the polarization response provided by said amylase substrate prior to being cleaved, and an albumin;

b) incubating the test solution for a period of time;

c) passing plane polarized light through the test solution of step b); and

d) detecting the fluorescence polarization response from step c) as an indication of the presence of amylase in the sample.

The synthesis methods for making the reagents comprise coupling a substrate capable of being cleaved by amylase with a fluorophore capable of producing a fluorescence polarization response. The general formula for such a substrate can be given by $P-(x-F)_n$, wherein P stands for the amylase; wherein x stands for a stable linking group which can be chosen from the group consisting of alkyls, aryls, amides, amines, thioamines, carbonates, thiocarbonates, carbonyls, sulfonates, imides, esters, thioesters, ethers, thioethers; wherein F stands for a fluorophore; and wherein n stands for the number of fluorophores and linking groups on the substrate.

Further objects and attendant advantages of the present invention will be best understood with reference to the following detailed description and Examples.

DETAILED DESCRIPTION OF THE INVENTION

The fluorescence polarization assay of the present invention provides many advantages over macromolecular hydrolase assays which have heretofore been known or proposed. Several of these advantages result because only a single substrate is required in the assay system of the invention, while

further advantages result from the preferred use of a natural, rather than a synthetic substrate.

Since only one substrate is required in the assay system of the present invention, problems attendant to stabilization of the assay system are minimized. As has been discussed above, in other assay systems the several substrates and enzymes used often have mutually incompatible requirements for stability. A concomitant advantage is that only a single substrate need be made for use in the assay system.

The assay of the present invention preferably uses a natural, rather than a synthetic, substrate. The preparation of a natural substrate is a relatively simple task, because it is not necessary to chemically determine the structure of a synthetic substrate that will be specific for amylase, nor is it necessary to map out and execute a complex synthetic procedure directed to producing such a substrate. An important result of using a single substrate, which is itself easily prepared, is relatively low reagent cost. Still further, assays conducted in accordance with the present invention are highly sensitive, and extremely reproducible.

Natural substrates are also especially advantageous because they tend to produce even better assay specificity by comparison with synthesized materials.

The assay of the present invention operates according to the principles of fluorescence polarization. According to these principles, if a fluorophore is excited by a plane-polarized beam of light, it will in turn emit polarized light. The degree of polarization of the emitted light will be inversely related to how much the molecule containing the fluorophore has rotated between the time of excitation and emission. The time it takes for a molecule to rotate through an angle of approximately 68.5 degrees has been defined as the rotational relaxation time of the molecule. The rotational relaxation time is relatively small (on the order of about one nanosecond) for small molecules (e.g., such as fluorescein), and large (on the order of about 100 nanoseconds) for large molecules (e.g., such as immunoglobulins). The rotational relaxation time of a molecule is primarily dependent on its volume, so that the observed polarization of its fluorescence in solution gives a direct indication of its size.

Still another advantage of the assay of the present invention is that it is a homogenous assay, i.e., it is not necessary to separate the cleaved products from the unreacted substrate before taking the fluorescence polarization reading.

According to the present invention, amylase concentration can be analyzed by providing a substrate capable of being cleaved by amylase, where the substrate is labelled with a fluorophore. By sequentially exciting the reaction mixture with vertically and then horizontally polarized light and analyzing only the vertical component of the emitted light, the polarization of fluorescence in the reaction mixture can be determined very accurately. When the substrate is intact, the fluorophore is part of a very large molecule, so the fluorescence polarization response yields a high value. When the substrate has been cleaved by the hydrolase enzyme, the fluorophore is part of a smaller molecule, and the fluorescence polarization response attributable to that fluorophore moiety will be substantially reduced. If much enzyme is present, the rate of fluorescence depolarization will be substantial, while if little or no enzyme is present, the amylase substrate will be cleaved relatively slowly or not at all, and the rate of fluorescence depolarization will be correspondingly slow.

The principles of the present invention are applicable to alpha and beta amylases.

A wide variety of fluorophores can be used in the method of the present invention. As previously indicated, the choice of the fluorophore will depend upon amylase and reagent system employed. Representative of the classes of fluorophores useful are fluoresceins, rhodamines, flavins, coumarins, naphthalenes, acridines, anthracenes, polynuclear fused hydrocarbons, stilbenes, anthranilic acids, aminostyrylpyridines, quinolines, salicylic acids, cyanines, oxonols, phenanthidines, fluorescamines, as well as derivatives and salts thereof. Illustrative of specific fluorophores that can be used include, for example, eosin, rhodamine, aminonapthalene sulfonate, acriflavin, fluorescein, dihydroxybenzoic acid, hydroxyquinoline, NADH, riboflavin, brilliant sulfaflavin, quinine, naphtholsulfonic acid, thioflavin, coumarin, acridine orange, 8-anilino-1-naphthalene sulfonic acid, oxazine, umbelliferone, acridine, resorufin, and derivatives and salts thereof. In the presently preferred embodiments, fluoresceins are used; specifically 4,6-dichlorotriazin-2-(yl)amino fluorescein (DTAF) and fluorescein isothiocyanate (FITC) have been found to be especially effective and advantageous.

The selection of the fluorophore to be used, as will be well understood by those skilled in the art, will depend upon such factors as: (1) the excitation and emission wavelengths of the fluorophore and of the instrument employed; (2) the linking functionality between the fluorophore and the substrate; (3) the rotational relaxation time of the free label; (4) the quantum yield; and (5) the extinction coefficient.

In addition, the proper selection of wavelengths of excitation and emission of the fluorophore is important for the reduction of interference from various components in the sample under analysis, such as hemoglobin and bilirubin. Accordingly, the fluorophore should be chosen such that its excitation and emission wavelengths do not overlap the absorbance spectrum of such substances as hemoglobin and bilirubin.

The linking functionality affects the choice of fluorophore in that certain substrates have functional groups known to react with certain fluorophores. For example, DTAF reacts strongly with the amine moiety on lysine groups; accordingly, when lysine groups are readily available on the substrate, DTAF may be the fluorophore of choice. Other examples will be readily ascertainable to those skilled in the art. Sometimes connecting groups, such as aliphatic chains, can be used to link the respective linking functionality of the fluorophore and the substrate. A list of types of exemplary linking groups has been previously set forth.

The rotational relaxation time is calculated by the Perrin equation, which is well known to those skilled in the art. This is an important factor because the accuracy of the assay will depend upon detecting differences in fluorescence polarization response over a period of time. If the cleaved fluorophore substrate produces a polarization response not appreciably different from that produced by the intact fluorophore-substrate, then it will be difficult to detect a significant depolarization response. The quantum yield and extinction coefficient of the fluorophore are important because they affect the minimum degree of substitution of the fluorophore onto the substrate, so that a sufficient fluorescence

intensity can be observed for a working concentration of substrate. If the quantum yield and extinction coefficient are poor, then an insufficient fluorescence intensity for an accurate measurement of the fluorescence polarization of a solution may be observed. A poor quantum yield necessitates a high degree of substitution on the substrate.

Substrates should be chosen with a view toward how well they are cleaved by amylase. It is important that the cleavage occur such that the size of the substrate will be considerably reduced by the hydrolase in a relatively short period of time. The presently preferred substrate for an assay for alpha amylase in accordance with the invention, for example, is potato amylose, which produces good results when coupled to the fluorophore moieties DTAF and FITC described supra. Other substrates that have produced good results in this assay are amylopectin, potato starch, Litner's soluble starch, and Maltrin® 040, 050, 100, 250, 500 and 550 (Maltrin is a registered Trade Mark), obtainable from the Grain Processing Corporation, Muscatine, Iowa. Other useful substrates are starches such as wheat starch, rice starch, corn starch and dextrin.

A suitable enzyme calibration procedure involves preparing a series of solutions containing varying amounts of enzyme, and then obtaining an enzyme activity value from assay of these solutions by a reference method. The calibrated enzyme solutions are then analyzed by the fluorescence polarization procedure set forth above. The polarization values are plotted against the enzyme activity values to construct a standard curve.

The assay parameters are optimized by balancing the variables of the procedure. The sample size incubation parameters of the assay are optimized by

balancing the variables of assay span (expressed as millipolarization between the lowest and the highest calibrator), sample matrix interference, assay throughput, incubation time and precision. The presently preferred optimal sample size is twenty-five microliters per two milliliters of reaction volume. An optimal throughput is obtained by keeping the incubation time to about five minutes or less. For precision with a coefficient of variation of less than 5%, an assay span of greater than 50 millipolarization units is preferred. The substrate concentration in the reagent mixture is optimized by balancing the assay span, the photometer intensity and the sample matrix interference.

An assay for amylase conducted in accordance with the preferred procedure set forth herein has proven highly accurate. A correlation study was performed comparing a commercially available amylase test (Agent®, (Registered Trade Mark) from Abbott Laboratories, North Chicago, Illinois) to the results obtained from the preferred procedure, using 36 patient samples with varying levels of amylase. The correlation study gave good results, demonstrating the accuracy of an amylase assay conducted in accordance with the invention.

Of course, it should be understood that the presently preferred procedures and assay parameters are set forth only by way of illustration and not limitation, and can be greatly varied, as will be apparent to one skilled in the art, to suit particular applications. Accordingly, although the presently most preferred assay conditions involve an assay temperature at or about 35°C, incubation times at or about 5 minutes, a pH at or about 7.5 and a substrate concentration at or about 50 micrograms per millimeter, these conditions can be varied widely. Thus, for example, the assay temperature can be kept within the

range of about 15°C to about 45°C; the incubation time can be selected from between about a second to about several hours, depending upon the desired ~~sensitivity~~ sensitivity of the assay (although incubation times selected from between about 1 minute to about 1 hour are preferred); the pH can be maintained in a range from about 3 to about 11, depending upon the enzyme activity at the specified pH; and the substrate concentration can be in the range from about 0.01 nanograms/ml to about 300 mg/ml in the final test solution, depending upon the solubility and quantum yield of the fluorophore.

Further, the preferred fluorescence depolarization assay of the invention can be varied, for example, by testing the fluorescence polarization response not necessarily at intervals commencing from immediately after the time of incubation. For example, a single reading can be taken at a time after incubation and compared with a reading from a control containing substrate but no enzyme. Yet another technique may be configured wherein sample and reagent are combined and preincubated for a period of time before the initial reading is taken. The solution can then be incubated further for a set interval of time before the second reading is taken. The fluorescence polarization response between the two time points is then used.

It is therefore intended that the foregoing detailed description and the following Examples be regarded as illustrative rather than limiting, and that the scope of the invention be defined solely by the appended claims, including all equivalents thereof.

Example 1

The following Example sets forth a preferred procedure, in order to illustrate one method of making a substrate coupled to a fluorophore moiety according to

the invention. One gram of potato amylose is dissolved in 10 ml of dimethyl sulfoxide (DMSO). Two drops of pyridine, 700 microliters of dibulytindilaurate and 10 mg of fluorescein isothiocyanate (FITC) are added. The solution is heated to about 100°C for about 3 hours, and then allowed to cool. After cooling, the labelled substrate is purified by adding 100 ml of ethanol (EtOH) to precipitate the starch. The mixture may be further cooled to facilitate precipitation. The precipitated starch is filtered and redissolved in 10 ml of DMSO, and then reprecipitated with 100 ml of ethanol. Finally, the precipitated starch is filtered, dried and the product stored.

According to a presently preferred procedure for conducting the assay, three reagents are prepared, which, for convenience, will be referred to as A, B and C: A = 300 mg/ml fatty acid-free bovine serum albumin; B = 10 mg/ml fluorescein labelled amylose in 85% DMSO with 0.1 M NaCl; and C = 3.6 M NaCl. A buffer solution, hereinafter referred to as "TDx® buffer," (TDx is a registered Trade Mark) comprises 0.1 M sodium phosphate buffer with 0.01% bovine gamma globulin and 0.10% sodium azide, at pH 7.5. It should also be noted that in this preferred embodiment, the assay can be advantageously performed on an Abbott TDx Fluorescence Polarization Analyzer. Twelve and one-half microliters of C reagent are mixed with 25 microliters of A reagent into 962.5 ml of TDx buffer. Plane polarized light is passed through the mixture and the horizontal and vertical intensities are read. Then 12.5 microliters of C reagent are added to the solution, along with 25 microliters of B reagent, 20 microliters of sample and an additional 942.5 microliters of TDx buffer, to provide a test solution. The test solution is permitted to incubate for 5 minutes at 35°C. Plane polarized light is passed through the solution, and the

fluorescence polarization is measured. The final polarization response is calculated and compared to polarizations from a calibration curve.

Example 2

Three grams of amylose from sweet potato were added to a solution of approximately 2 mg of DTAF in approximately 100 ml of TDx buffer. The amylose did not completely dissolve. After periods of 5 and 30 minutes, 20 microliters were removed and diluted to 1 ml. Then 20 microliters of the diluted mixture were further diluted in 1 ml of buffer. This solution was added to a cuvette and the fluorescence intensity and net millipolarization were read at gain 5 on the Abbott TDx Fluorescence Polarization Analyzer. Five minutes after the coupling reaction was initiated, the fluorescence polarization was observed to rise, indicating a coupling of the DTAF to amylose. Ten microliters of a 10 milligram per milliliter bacterial amylase solution were then added to the cuvette containing the DTAF coupled amylose. The polarization then immediately dropped due to the hydrolysis of the amylose into small fragments, thus demonstrating the principle of this technique.

Example 3

The substrate from Example 2 was further prepared by adding approximately 100 ml of EtOH and 100 ml of acetone to precipitate the fluorescein labelled amylose. The material was then filtered in a buchner funnel, using approximately 50 ml EtOH to wash away free DTAF. The amylose was dried and resuspended in water. The solution was then filtered to remove insoluble amylose.

Example 4

To 1 ml of TDx buffer, 90 microliters of saturated NaCl solution, 25 microliters of substrate

from Example 3 and 25 micrograms of sample were added. The solutions were mixed and incubated at 35°C. At various incubation times the fluorescence polarization was read on the TDx analyzer. The samples used were Sigma normal and elevated enzyme. These samples contained approximately 1700 and 4500 Sigma units per liter of amylase, respectively. The following data was obtained:

INCUBATION TIMES

	2 minutes	7 minutes	12 minutes	18 minutes
no enzyme	214 mp	214 mp	213 mp	212 mp
Sigma normal	213 mp	193 mp	179 mp	165 mp
Sigma elevated	202 mp	167 mp	150 mp	138 mp

The data demonstrates that the porcine amylase (in the Sigma controls) cleaved the substrate.

Example 5

One gram of sweet potato amylose was dissolved in 10 milliliters of dimethyl sulfoxide (DMSO). Two drops of pyridine, 700 microliters of dibutyltindilaurate and 10 mg of FITC were added to the solution. When dissolved, the solution was heated at 100°C on a steam bath for three hours. After cooling, 100 ml of ethanol were added to precipitate the amylose. The precipitated amylose was filtered using a sintered glass funnel. The unreacted FITC in the ethanol filtrate was discarded. The labelled amylose was then redissolved in DMSO and reprecipitated two more times.

Example 6

Approximately 50 mg of gelatin were dissolved in approximately 3 ml of water. Approximately 1 mg of DTAF was then added to the solution. The mixture was

incubated about 3 hours at room temperature. The free, unreacted fluorescein was then separated from the protein labelled fluorescein by adding 5 ml of ethanol. The precipitated protein was centrifuged and the unlabelled fluorescein was decanted as waste. This precipitation was repeated. The fluorescein labelled gelatin was redissolved in a small amount of water and diluted to 1/1000 with TDx buffer to measure the fluorescence polarization response. Since the polarization response was sufficiently high at approximately 120 millipolarization units, it was concluded that this procedure according to the invention is operable in an assay for trypsin.

Example 7

One mg of pancreatic trypsin (12,000 BAEE units) was dissolved into 1 ml of water. This enzyme stock was further diluted with water, and 100 microliters of the stock trypsin solution were mixed with 1 ml of a 1/1000 dilution of the stock substrate solution from Example 1. The solutions were incubated at 35°C and measured for their fluorescence polarization response 5 minutes after mixing. The following results showed that as little as 100 nanograms of trypsin in the cuvette can be measured by a polarization technique with labelled gelatin.

Trypsin Conc.	Millipolarization (MP)
no enzyme	123
100 nanograms (ng)	109
1 micrograms (µg)	94
10 µg	78
100 µg	68

Example 8

Two ml of lipoprotein solution (Miles Laboratories, Inc. code 82-018) were mixed with 2 ml of DMSO. Forty microliters of a 100 mg/ml solution of FITC in DMSO were then added. Two drops of pyridine were

added, along with 200 ml of dibutyltin dilaurate. The solution was placed on a steam bath for two hours.

After two hours, 10 microliters of solution were diluted in 1 ml of DMSO. This was further diluted 1/100 into TDx buffer. The millipolarization of the solution was found to be 183.

Example 9

The fluorescein-labelled lipoprotein substrate from Example 8 was diluted into 1 ml of TDx buffer, and various concentrations of lipoprotein lipase were added. The solution was then incubated at 35°C for 5 minutes before the fluorescence polarization was determined. A decrease in concentration proportional to enzyme concentration was observed. The resulting data follows.

Lipase Added	MP
none	180
65 units	122
163 units	93

It will be apparent that various modifications and changes can be made in the specific embodiments of the invention described herein, without departing from the spirit and scope thereof, as defined solely in the following claims.

The claims defining the invention are as follows:

1. A method of determining amylase concentration in a sample, comprising:

a) adding to the sample an amylase substrate coupled to a fluorophore, said amylase substrate capable of being cleaved by amylase wherein the size of said amylase substrate is substantially reduced to provide a polarization response which is substantially different from the polarization response provided by said amylase substrate prior to being cleaved, and an albumin;

b) incubating the test solution for a period of time;

c) passing plane polarized light through the test solution of step b); and

d) detecting the fluorescence polarization response from step c) as an indication of the presence of amylase in the sample.

2. The method of Claim 1 wherein said substrate is amylose.

3. The method of Claim 1 or 2 wherein said albumin is fatty-acid free bovine serum albumin.

4. The method of any one of Claims 1 to 3, wherein said fluorophore is fluorescein.

5. The process of any one of Claims 1 to 3, wherein the fluorophore is 4,6-dichlorotriazin-2-(yl) aminofluorescein, a derivative of 4,6-dichlorotriazin-2-(yl) aminofluorescein, fluorescein isothiocyanate or a derivative of fluorescein isothiocyanate.

6. A method of determining amylase concentration in a sample, substantially as herein described with reference to Example 1, Example 2, or Examples 3 and 4.

DATED this TWENTIETH day of FEBRUARY 1990
Abbott Laboratories

Patent Attorneys for the Applicant
SPRUSON & FERGUSON

MRC/268x