United States Patent [19]

Komatsu

[11] **3,898,130**

[45] **Aug. 5, 1975**

[54] **RAPID ENZYMATIC HYDROLYSIS OF TRIGLYCERIDES**

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[22] Filed: **Mar. 18, 1974**

[21] Appl. No.: **451,735**

[52] **U.S. Cl.** ..... **195/30;** 195/63; 195/103.5 R

[51] **Int. Cl.** **C12d 13/02**

[58] **Field of Search** 195/30, 63

[56] **References Cited**

UNITED STATES PATENTS

3,703,591 11/1972 Bucolo et al. ..... 195/103.5 R

OTHER PUBLICATIONS

Alford et al., Journal of Lipid Research, Vol. 5, pp. 390–394, July 1964.

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[57] **ABSTRACT**

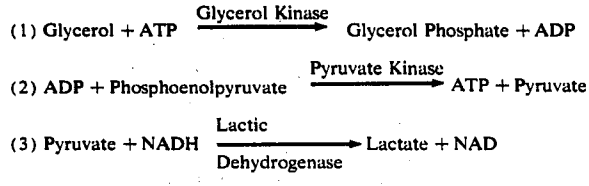
A process and product for rapidly liberating glycerol from its esterified form as a fatty acid ester, for example, when present in an aqueous media such as serum, wherein a combination of a pancreatic lipase and a microbial lipase, particularly *Candida lipase*, are mixed with the fatty acid ester in the presence of a bile salt.

30 Claims, No Drawings

RAPID ENZYMATIC HYDROLYSIS OF TRIGLYCERIDES

BACKGROUND AND SUMMARY

An effective enzymatic test for the determination of serum triglycerides turns mainly on the development of a rapid and complete process for the hydrolysis of the triglycerides, since various procedures for measuring the glycerol formed by such a reaction are well known. Thus, as described by F. H. Kreutz in Klin. Wochshr. 40:362 (1962), glycerol may be readily determined by combining the following reactions:



In this combination of reactions, 1 mole of NADH is oxidized for each mole of glycerol phosphorylized; therefore, a change in the optical density at 340 nm is a direct measure of the amount of glycerol in the assay.

U.S. Pat. No. 3,703,591 discloses that the preliminary step, that of hydrolyzing the triglycerides to form glycerol, may be carried out enzymatically using a mixture of a lipase and a protease. While it has long been known that certain lipases may alone be used to hydrolyze triglycerides, and that the enzymatic activity might possibly be enhanced by the presence of a bile salt, prior procedures using only lipases (with or without bile salts) have been unsatisfactory because of the long duration of the hydrolysis step and because even then incomplete hydrolysis is the usual result.

Therefore, an important aspect of this invention lies in the discovery that a complete and surprisingly rapid hydrolysis of triglycerides may be achieved by using a combination consisting only of certain lipases along with a bile salt, thereby dispensing with the requirement of including a proteolytic enzyme such as chymotrypsin as one of the reactants. More specifically, 100 percent hydrolysis may be accomplished in 3 to 5 minutes using a combination of *Candida lipase* and pancreatic lipase with a bile salt such as sodium taurodeoxycholate. The glycerol so produced may then be assayed by any of a number of known methods, one such method having already been described above.

Other objects and advantages of the invention will be apparent as the specification proceeds. Additional references disclosing the state of the art are: R. G. H. Morgan and N. E. Hoffman, Biochim. Biophys. Acta, 248:143 (1971); R. L. Ory, J. Kiser and P. A. Pradel, Lipids, 4:261 (1968); H. Brockerhoff, J. Bio. Chem. 246:5828 (1971); P. Desnuelle, Enzymes, 7:575 (1972); patent 2,527,305.

DESCRIPTION

It is preferred that the enzymatic hydrolysis of triglycerides by the coactive lipases be undertaken in the presence of the components of the three additional reaction systems represented in the equations given above so that all of such reactions may be undertaken simultaneously in a single operative procedure or, if desired, in a two step procedure in which one of the components needed for the conversion of glycerol, such as the glycerol kinase, is added to all of the other components after hydrolysis has occurred and an initial reading of optical density has been made. Since the various components necessary for the enzymatic conversion of glycerol have already been indicated and are well known in the prior art, and since the proportions of such components are also well known, a detailed discussion herein is believed unnecessary. It is believed sufficient to state that the combination of lipases and bile salt may be part of an assay mixture which includes one or more of those components known for use in the enzymatic conversion of glycerol and the concurrent oxidation of NADH in a colorimetric or spectrophotometric test.

The system responsible for the enzymatic hydrolysis of triglycerides comprises a coactive mixture of pancreatic lipase, a microbial lipase, and a bile salt. All three components are essential for effective hydrolysis. A variety of pancreatic lipases having activity within the range of about 10 to 100 lipase units per milligram (mg), and preferably within the range of 20 to 80 units per mg, are believed suitable, an example being the pancreatic lipase sold under the designation "PL3" by Worthington Chemical Company, of Freehold, New Jersey. The microbial lipase is more specifically a *Candida lipase* which may, for example, be obtained from the cultured broth of *Candida cylindracea*. Such *Candida lipase* should have activity within the range of 30 to 800 lipase units per mg, and preferably within the range of 200 to 800 lipase units per mg. Other *Candida lipases* are believed to be equally effective when used in combination with the pancreatic lipase and bile salt of the triglyceride-hydrolyzing system.

While both pancreatic and *Candida lipases* must be present, it has been found that the proportional amounts of those constituents, measured in terms of lipase units, may be varied considerably in accordance with selected time requirements for completion of hydrolysis. One lipase unit of activity is the amount sufficient to release one micromole of acid per minute at 25°C. from an olive oil emulsion containing gum acacia and 15 mg per milliliter (ml) sodium taurocholate at a pH of about 8.0. Such definition of a lipase unit, and the procedure on which it is based, are well established and are disclosed more fully in Worthington Enzyme Manual, p. 63 (1972).

More specifically, the amount of pancreatic lipase in the reaction mixture should be at least 0.14 lipase units for each microliter of body fluid (blood serum or plasma) having a triglyceride value within the range of 0 to 500 mg per 100 ml (mg percent) in order to achieve complete hydrolysis within 12 minutes. On the same basis, the amount of *Candida lipase* in the mixture should be at least 0.28 units, and the amount of bile salt should be at least 0.002 mg, for each microliter of body fluid. Where shorter reaction times are required or desired, the amounts of such constituents must be in-

creased. Thus, for complete hydrolysis within 3 to 5 minutes, at least 1.2 pancreatic lipase units, 0.54 *Candida lipase* units, and 0.02 mg of bile salt, are required for each microliter of body fluid. Stated differently, in a reagent combination having a volume of 1 milliliter, the values (for hydrolyzing 50 microliters of serum or plasma having a triglyceride value of 0 – 500 mg percent within 12 minutes) should be at least 7 pancreatic lipase units, 14 *Candida lipase* units, and 0.1 mg bile salt; or at least 60 pancreatic lipase units, 27 *Candida lipase* units, and 1.0 mg bile salt (for the hydrolysis of 50 microliters of such serum or plasma within 3 to 5 minutes).

Since speed in completing an assay may be important, particularly in connection with clinical diagnostic tests, a maximum time period for completion of hydrolysis in a clinical test has been arbitrarily set at approximately 12 minutes. It is to be understood, however, that in other tests where longer time periods are more acceptable, lower concentrations or amounts of the respective lipases and bile salt may be used.

As previously indicated, a bile salt is an essential component of the system. While alkali metal salts of taurocholic, taurochenodeoxycholic or taurodehydrocholic acid may be used, particularly effective results have been achieved with alkali metal salts of taurodeoxycholic acid. The salt of taurodeoxycholic acid, in admixture with *Candida lipase* and pancreatic lipase under optimum conditions, has been found to produce faster results at lower concentrations than the other bile salts.

As indicated above, and as illustrated more fully by the examples set forth hereinafter, the combination of pancreatic and *Candida lipases*, in admixture with a bile salt, preferably the salt of taurodeoxycholic acid, reacts with the triglycerides in body fluids to produce complete hydrolysis in periods as short as 3 minutes. Such a combination of reactants may be used in any test requiring the rapid and complete hydrolysis of triglycerides. The process and product of this invention may, for example, be used in conjunction with a complete triglyceride assay including the three glycerol-determining reactions described at the beginning of this application and well known in the prior art. All of the components required for the complete colorimetric determination of triglycerides in body fluids may be premixed and lyophilized to provide a stable reagent set for clinical use.

EXAMPLE 1

A reagent suitable for practicing this invention may be prepared by making the following 3 ml reaction mixture:

Pancreatic lipase [Worthington PL3], 180 lipase units
Candida lipase [Worthington], 80 lipase units
Sodium taurodeoxycholic acid, 3 mg
LDH, 10 International Units (IU)
Pyruvate kinase, 10 IU
NADH, 0.75 micromoles
Phosphoenolpyruvate, 1.5 micromoles
ATP disodium, 0.5 micromoles
Magnesium chloride, 0.0067 M
Potassium phosphate buffer, 0.1 M, pH 7.0

The assay is carried out by adding an aliquot of liquid containing the triglyceride to be assayed, such as 50 microliters of serum or plasma with triglyceride values of 0 to 500 mg percent, to the above reaction mixture. Following incubation for approximately 5 minutes at a temperature between 25°C. to 37°C., the optical density is measured at 340 nm. Thereafter, 10 units of glycerol kinase is added and the mixture is again incubated at 25°C. to 37°C. for another 5 minutes. The optical density is again determined at 340 nm, and the difference in optical densities is proportional to the triglyceride content after appropriate adjustment, using conventional clinical laboratory procedures, for whatever blank reaction is produced.

EXAMPLE 2

The procedure of Example 1 was performed using the same reactants, proportions, and conditions, except that a purified lipase obtained from the cultured broth of *Candida cylindracea* nov. sp. was substituted for the *Candida lipase* of the first example. Complete hydrolysis of the triglycerides of the sample were obtained within 5 minutes in the same manner as set forth in Example 1.

EXAMPLE 3

Several reagent combinations were prepared in accordance with Example 1 except that a variety of bile salts were used. The results were tabulated below. Each reagent combination contained 0.1 mg *Candida lipase* (activity of 80 units), 3.0 mg pancreatic lipase (activity 180 units), and the amount of bile salt indicated. Times are given in minutes for completion (100 percent) of hydrolysis of serum samples having triglycerides values of 100 mg percent (i.e., 100 mg per 100 ml water) and 280 mg percent.

TIMES REQUIRED FOR COMPLETION OF HYDROLYSIS WITH ENZYME COMBINATIONS INCLUDING DIFFERENT BILE SALTS

Bile Salt (Na)	Amount (mg)	Time (min.) for 100 mg% Sample	Time (min.) for 280 mg% Sample
(1) Taurocholic	6.0	20	—
	12.0	14	—
	18.0	10	—
	24.0	5	12
	30.0	5	10
(2) Taurodeoxycholic	0.3	12	—
	1.0	7	7
	1.5	5	6
	3.0	4	5
(3) Taurochenodeoxycholic	1.5	—	14
	3.0	—	12
	6.0	3	10
(4) Taurodehydrocholic	6.0	13	—
	24.0	13	—
	30.0	10	—

While in the foregoing an embodiment of the invention has been disclosed in considerable detail for purposes of illustration, it will be understood that many of those details may be varied without departing from the spirit and scope of the invention.

I claim:

1. An enzymatic process for rapidly liberating glycerol from its esterified form as a fatty acid ester in an aqueous fluid, comprising the step of mixing said fluid with a combination of *Candida lipase*, pancreatic lipase, and a bile salt selected from the group consisting of the alkali metal salts of taurodeoxycholic, taurocholic, taurochenodeoxycholic, and taurodehydrocholic acids.

2. The process of claim **1** in which said acid is taurocholic acid.

3. The process of claim **1** in which said acid is taurodeoxycholic acid.

4. The process of claim **1** in which said acid is taurochenodeoxycholic acid.

5. The process of claim **1** in which said acid is taurodehydrocholic acid.

6. The process of claim **1** in which said bile salt is a sodium salt.

7. The process of claim **1** in which said *Candida lipase* is obtained from a culture of *Candida cylindracea*.

8. The process of claim **1** in which said *Candida lipase* in said combination provides at least 0.28 units of lipase activity for each microliter of aqueous fluid having a triglyceride value of 0 to 500 mg percent.

9. The process of claim **8** in which said *Candida lipase* provides at least 0.54 units of lipase activity for each microliter of aqueous fluid having a triglyceride value of 0 to 500 mg percent.

10. The process of claim **1** in which said pancreatic lipase in said combination provides at least 0.14 units of lipase activity for each microliter of aqueous fluid having a triglyceride value of 0 to 500 mg percent.

11. The process of claim **10** in which said pancreatic lipase provides at least 1.2 units of lipase activity for each microliter of aqueous fluid having a triglyceride value of 0 to 500 mg percent.

12. The process of claim **1** in which said combination includes at least 0.002 milligrams of said bile salt for each microliter of aqueous fluid having a triglyceride value of 0 to 500 mg percent.

13. The process of claim **12** in which said combination includes at least 0.02 milligrams of said bile salt for each microliter of aqueous fluid having a triglyceride value of 0 to 500 mg percent.

14. A reagent combination for the rapid hydrolysis of fatty acid esters to liberate glycerol therefrom, comprising a mixture of *Candida lipase*, pancreatic lipase, and a bile salt selected from the group consisting of the alkali metal salts of taurodeoxycholic, taurocholic, taurochenodeoxycholic, and taurodehydrocholic acids.

15. The combination of claim **14** in which said acid is taurocholic acid.

16. The combination of claim **14** in which said acid is taurodeoxycholic acid.

17. The combination of claim **14** in which said acid is taurochenodeoxycholic acid.

18. The combination of claim **14** in which said acid is taurodehydrocholic acid.

19. The combination of claim **14** in which said bile salt is a sodium salt.

20. The combination of claim **14** in which said *Candida lipase* is obtained from a culture of *Candida cylindracea*.

21. The combination of claim **14** in which said *Candida lipase* has at least 14 lipase units for each milliliter of reagent combination.

22. The combination of claim **21** in which said *Candida lipase* has at least 27 lipase units for each milliliter of reagent combination.

23. The combination of claim **14** in which said pancreatic lipase has at least 7 lipase units for each milliliter of reagent combination.

24. The combination of claim **23** in which said pancreatic lipase has at least 60 lipase units for each milliliter of reagent combination.

25. The combination of claim **14** in which at least 0.1 milligrams of bile salt are provided in each milliliter of reagent combination.

26. The combination of claim **25** in which at least 1.0 milligrams of bile salt are provided for each milliliter of reagent combination.

27. The reagent combination of claim **14** in which said pancreatic lipase has an activity of about 10 to 100 lipase units per milligram.

28. The combination of claim **27** in which said pancreatic lipase has an activity of approximately 20 to 80 lipase units per milligram.

29. The reagent combination of claim **14** in which said *Candida lipase* has an activity of approximately 30 to 800 lipase units per milligram.

30. The reagent combination of claim **29** in which said *Candida lipase* has an activity of approximately 200 to 800 lipase units per milligram.

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