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해양 미생물을 이용한 생리활성 물질 탐색을 위한 미량 분석 방법의 개발

Development of Microanalysis Methods for Screening of Biologically Active Substance Using Marine Microorganism

1994. 3

한 국 해 양 연 구 소

제 출 문

한국해양연구소장 귀하

본 보고서를 "해양 미생물을 이용한 생리활성물질 탐색을 위한 미량 분석 방법의 개발"과제의 보고서로 제출합니다.

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᠎# 요 약 문

I. 제목

해양 미생물을 이용한 생리활성물질 탐색을 위한 미량 분석 방법의 개발

II. 연구의 목적 및 중요성

해양미생물들은 여러 가지 다양한 생리학적 특징들을 갖고 있는 데 이들을 이용하여 새로운 생리 활성을 갖는 물질들을 얻거나 인류에 유용한 천연 약용물질들을 얻으려는 시도가 있어왔다. 이러한 신규 생리활성 물질을 찾기위한 screening 이 효과적으로 수행되기 위해서는 우선 가능한 한 많은 검색대상균주를 확보하는 일이고 두번째가 매우 효율적이고 감도가 높은 분석 방법을 사용하는 일이 대단히 중요하다.

III. 연구 내용 및 범위

본연구에서는 인류의 순환기 계통 장애의 주원인으로 작용하는 cholesterol 대사에 작용하는 효소 (cholesterol esterase)와 고지혈증 치료제로 개발되고 있는 Eicosapentaenoic acid (EPA)의 검색 방법을 해양 발광 세균의 생체발광 반응을 이용한 고감도 분석방법을 개발하였다.

혈액내 지질감소, 관상동맥, 심장질환의 억제, 암세포의 선택적 파괴, 류머치스성 관절염 치료등의 효능을 보이고 있는 Eicosapentaenoic acid (EPA) 등 n-3 계열의 고도불포화 지방산들은 추출, 정제되여 의약 및 건강 식품으로 이용되고 있다. 그 원료로 주로 사용되어온 등푸른 생선에서 추출한 어유이외에 EPA를 생산하는 미생물을 이용하여 발효 공정을 통해 대량생산을 꾀하려는 시도가 대두되고 있다.

IV. 연구 개발 결과 및 활용에 대한 건의

1. 세균의 생체발광 반응을 이용한 cholesterol esterase 활성도 측정법

생리학적으로 매우 중요한 생체내 cholesterol 의 대사에 관여하는 효소중의 하나인 cholesterol esterase (cholesteryl ester hydrolase EC 3.1.1.3) 의 활성도의 발광형 측정법을 개발하였다. 측정을 위해 그자체로는 발광하지 않으나 myristic acid 존재하에서 발광하는 발광세균의 돌연변이 균주 (*Vibrio harveyi* mutant M-17)를 이용하여 cholesterol myristate를 기질로 사용하였고 그 반응 산물인 myristic acid를 이 측정 체계를 이용하여 정량하였다. 이 활성도 측정방법을 이용하여 Bovine pancreas 유래의 cholesterol esterase의 반응 특성을 조사함으로써 이 방법이 기존의 방사성 동위원소에 의한 측정보다 매우 신속, 간편하고 안전하며 동일한 수준의 효소 활성 측정 감도를 갖고 있음을 확인하였다.

2. 해양 발광 세균을 이용한 EPA 생산 검색법

Eicosapentaenoic acid (EPA)를 생산하는 미생물의 신속한 검색을 위하여 해양 세균의 생체 발광 현상을 이용하는 방법을 개발하였다. *Photobacterium leiognathi* 는 사용된 균주중 선택성, 반응성, 안정성에서 가장 뛰어난 검색용 균주로 판명되었으며 고도 불포화 지방산 존재하에서 5 분이내에 생체 발광의 95 % 가 저해됨을 photometer를 사용하여 확인하였다. 검색 대상 균주로 부터 유리 지방산을 분리하는 과정에서 기존의 비누화 반응 대신에 phospholipase A_2 를 사용한 효소 가수분해법을 사용하여 반응시간을 2 시간에서 10 분으로 단축할수 있었다. 또한 다수의 시료를 동시 분석하기 위하여 96 microwell plate에서 반응을 시키고 이를 photometer대신 Polaroid film을 사용한 camera luminometer를 이용하여 효율적으로 처리할 수 있었다.

본연구 결과 개발된 cholesterol esterase 분석법은 체내 cholesterol 대사의 효율성과 기능을 검정하는 진단 시약으로 개발 가능하며 EPA검색법은 다량의 검색대상 균주에서 신속하게 EPA생산 여부를 1 차 검정하는 방법으로 사용이 가능하다.

Summary

I. Title

A Study on the EPA production Using Marine Microorganism

II. Objectives and Significance

Marine microorganisms show various kinds of biologically unique properties, and marine biotechnology has been developped to usinf these marine microorganisms to obtain novel biologically active substances, such as new medicine and materials. The most important factors for the succesful screeing of these biologically active substances are, firstly to obtain as many microorganisms as possible from ecologically diverse environment and secondly, to develop very effective screening and assaying method in labor and cost.

III. Contents and Scope

In this project, new highly sensitive and rapid assay methods for the assay of cholesterol esterase which catalyzes the fatty acid esterification of cholesterol and for the detection of eicosapentaenoic acid (EPA) produced from marine microorganisms were developped using the bacterial bioluminescence of luminous marine bacteria.

IV. Results and Suggestions

1. A New Screening Method for the Microbial Production of Eicosapentaenoic acid Using Bioluminescence of Marine Bacteria

For the rapid screening of eicosapentaenoic acid (EPA) producing bacteria, the bioluminescence of marine bacteria was employed. *Photobacterium leiognathi* was chosen as the best reporting species for this method in selectivity, reactivity, and stability. Polyunsaturated fatty acids, ausentic or prepared from EPA positive strain, show strong inhibition of *in vivo* bioluminescence up to 95 % in 5 minutes which can easily detected with photometer. For the preparation of free fatty acid from target bacteria, enzymatic hydrolysis with phospholipase A_2 was method of choice to decrese reaction time (10 minutes) compared with chemical saponification method (2 hours). For the processing of large number of sample at a time, the use of camera luminometer using Polaroid film and 96 microwell plate was demonstrated.

2. A New Bioluminescent Assay of Cholesterol Esterase Using Luminescent Marine Bacterium Vibrio harveyi mutant M-17.

A new bioluminescent assay method for the activity of cholesterol esterase (cholesteryl ester hydrolase EC 3.1.1.3) was developed using bioluminescent marine bacteria. A dark mutant of *Vibrio harveyi* (desinatd as M-17) was used for this assay, which does not emit high level of light in itself, but in the presence of myristic acid it gives normal level of light as wild type cell. Cholesterol myristate and *Bovine pancreas* cholesterol esterase were used for this demonstration and the product, myristic acid was quantitated with *V. harveyi* mutant M-17. Down to 5 nM of myristic acid produced can be detected. It was proved that this method is rapid, reliable, and easy to perform and safe.

The assay method of cholesterol esterase can be used to determine the intracellular and blood cholesterol level and degree of cholesterol metabolism, and the assay for EPA can be applied directly to the preliminary screening of epa producing microorganisms.

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A New Bioluminescent Assay of Cholesterol Esterase Using Luminescent Marine Bacterium *Vibrio harveyi* mutant M-17

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A new bioluminescent assay method for the activity of cholesterol esterase (cholesteryl ester hydrolase, EC 3.1.1.13) was developed using bioluminescent marine bacteria. A dark mutant of *Vibrio harveyi* (designated as M-17) was used for this assay. It does not emit high level of light in itself, but in the presence of myristic acid it gives normal level of light as wild type cell. Cholesterol myristate and *Bovine·pancreas* cholesterol esterase were used for this demonstration and the product, myristic acid, was quantitated with *V. harveyi* mutant M-17. Down to 5 nM of myristic acid produced could be detected. It was proved that this method was rapid, reliable, and easy to perform and safe.

KEY WORDS □ cholesterl esterase, bioluminescence, *Vibrio harveyi* mutant M-17

Cholesterol esterase (sterol ester acylhydrolase, EC 3.1.1.13) is one of the most important enzymes in cholesterol metabolism, catalyzing the reversible hydrolysis of cholesterol alkyl ester (10). The presence of cholesterol esterase is almost ubiquitous and can be found in any cell where membrane and cholesterol exist. In the cell, cholesterol is usually stored in the form of cholesterol alkyl ester, and the free cholesterol is produced from the cholesterol alkyl ester pool through hydrolysis catalyzed by cholesterol esterase (8). The cholesterol esterase is also used for cellular cholesterol quantitation together with cholesterol oxidase. The usual assay system for cholesterol esterase is using radioisotope-labelled fatty acid and quantitizing the released free fatty acid after separation and isolation with TLC, which needs lots of labors such as extraction, chromatographic separation, radioisotope quanti tation (5).

The bioluminescent system of luminescent bacteria, such as *Vibrio harveyi*, uses long chain aliphatic (fatty) aldehyde, reduced flavin mononucleotide ($FMNH_2$), and molecular oxygen as substrates catalyzed by bacterial luciferase producing blue-green light peaked at around 490 nm (4). Luminescent bacteria need other enzyme systems than bacterial luciferase for light production *in vivo*, that is the suppling system of long chain aldehyde from fatty acid moiety of membrane phospholipid, which is composed of three enzymes, acyl transferase, acyl carrier protein and reductase (11). First, acyl transferase takes myristic acid moiety from phospholipid to acyl carrier protein using ATP, and then the reductase reduces this myristoyl group to myristic aldehyde using NADPH. One of the *Vibrio harveyi* mutants (named as M-17) has defective acyl transferase due to a point mutation in Gly^{57} residue to Glu and so it cannot produce high level of bioluminescence *in vivo* but, while in the presence of exogeneous myristic acid ($C_{14:0}$), it can produce normal level of light as wild type cell (9). The light intensity of *V. harveyi* mutant M-17 induced by addition of exogeneous myristic acid is linearly correlated with the amount of myristic acid and can be used for myristic acid quantitation method (12). In this report, a new luminescence assay system for cholesterol esterase was demonstrated using commercially available cholesterol myristate as substrate and cholesterol esterase from *Bovine pancreas*, and luminescent bacterium *V. harveyi* mutant M-17.

MATERIALS AND METHOD

Cholesterol esterase from *Bovine pancreas* was purchased from Sigma Chemical Co. One unit of cholesterol esterase was defined to hydrolyze 1.0 μmole of cholesterol oleate per minute at pH 7.0 and 37°C. Cholesterol myristate and myristic acid were also from Sigma Chemical Co.

Cholesterol myristate was dissolved in chloroform, washed with 5 % NaCl solution and distilled water to remove any contaminated free myristic acid, and then mixed with same amount of ethanol for better suspension when added into buffer.

Luminous bacterium *V. harveyi* mutant M-17 was originally produced by Cline and Hastings through random mutagenesis with nitrosoguanidine (2) and was a kind gift of Professor Tu (University of Houston). This bacterium was grown in 50 m*l* of sea water complete media (5 g bactotryptone, 3 g yeast extract, 3 m*l* glycerol, 75 % aged sea water per liter, pH 7) at 25°C up to $O.D._{660nm}=1.5$. Cells were harvested by centrifugation (Sorvall RC-5C) using SS-34 rotor at 10,000 rpm for 5 min and then washed twice with cold 0.1 M phosphate buffer (pH 6.5, containig 3 % NaCl). The harvesting time is very important because the myristic acid-induced bioluminescence begins to decline after reaching the maximum value at late logarithmic phase of growth. The washed cell pellet was resuspended in cold 0.1 M phosphate buffer (pH 6.5, containing 3 % NaCl) to final $O.D._{600nm}=10$ and stored on ice, and 0.1 m*l* of this suspension was diluted to 1 m*l* with 0.1 M phosphate buffer (pH 6.5, containing 3 % NaCl) at room temperature just before each measurement. This cell suspension gave reproducible result for about 3 hours.

For a typical luminescent assay of cholesterol esterase, 10 μl of cholesterol myristate stock solution was added into 1 m*l* of 50 mM phosphate buffer (pH 7.0) containing 0.01 % (w/v) sodium taurocholate. The reaction was started by addition of given amount of cholesterol esterase and incubated at 37°C water bath. For a light emission measurment, an aliquot of 20 μl of reaction mixture was taken at every 5 minutes and then added into bacterium cell suspension (1 m*l*) and vortexed for 5 seconds. To stop the cholesterol esterase reaction, 20 μl aliquots might be taken and put into a test tube and then dipped in boiling water, but the 50-fold dilution in enzyme, substrate and detergent, the temperature change from 37°C to room temperature (about 25°C), and the change in pH (from 7.0 to 6.5) can stop the reaction almost completely. The maximum light intensity (Io) was measured with the photomultiplier photometer equipped with Hamamatsu R-447 phototube with Mitchell-Hasting type pre-amplifier (7) and Hewlet-Packard high voltage power supply and recorded with strip chart recorder. The calibration was done using continuous light source of Hastings and Weber (3). One light unit (LU) was defined as 2×10^9 quanta per second.

RESULTS AND DISCUSSION

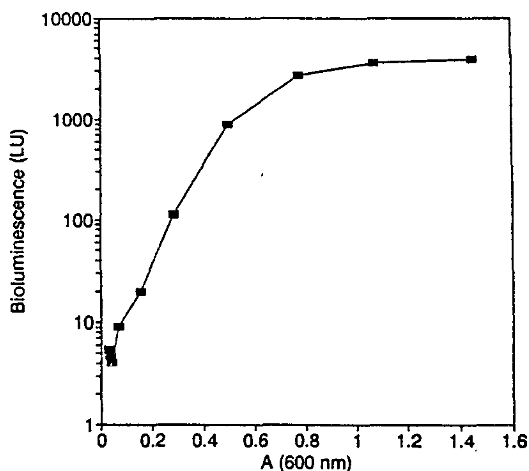

Fig. 1. *Growth curve of Vibrio harveyi mutant M-17 in sea water complete media.*
Maximum light intensity (Io) in the presence of myristic acid (10μM) was plotted against A_{600nm} showing the light-producing activity per cell.

Luminous bacterium *V. harveyi* dark mutant M-17 was cultured in sea water complete medium, and the growth was followed by measuring absorbance at 600 nm and the luminescence activity was measured with 1 m*l* aliquots in the absence and presence of 10 μM of myristic acid. The level of bioluminescence without myristic acid was about 3 LU ml^{-1} A_{600nm}^{-1} and suggested that little amount of myristic acid was present in the cell. The free myristic acid may be produced by the action of some lipase such as phospholipase A. The luminescence activity induced by addition of myristic acid was maximal just before the growth reached stationary phase and the intensity was about 3500 LU ml^{-1} A_{600nm}^{-1} and then began to decline in stationary phase (Fig. 1). For the best result, the cell should be harvested and washed with buffer at this point ($A_{600\,nm}$ about 1.5). The washing and cold storage of cell was important because this process could eliminate any extracellular hydrolase which might hydrolyze cholesterol myristate during the light measurement.

Using the bacterial suspension in phosphate buffer, the dependence of the light intensity on the concentration of added myristic acid was measured, and a standard curve was obtained, which was linear within the range from 5 nM to 10 μM final concentration of myristic acid (Fig. 2). Higher concentration of myristic acid than 10 μM did not give higher light intensity at same cell density. A suspension with higher cell density would give higher light intensity, but the

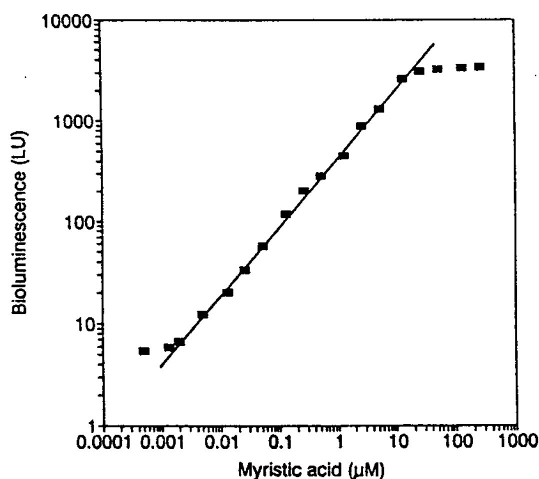

Fig. 2. *Standard curve of myristic acid concentration and initial light intensity (Io) using M-17 suspension.*
Various concentrations of myristic acid standard solution were prepared in absolute ethanol, 10 μl of these solutions was added into 1 ml of M-17 suspension in 0.1 M phophate buffer (pH 6.5), and the initial maximum light intensity was measured.

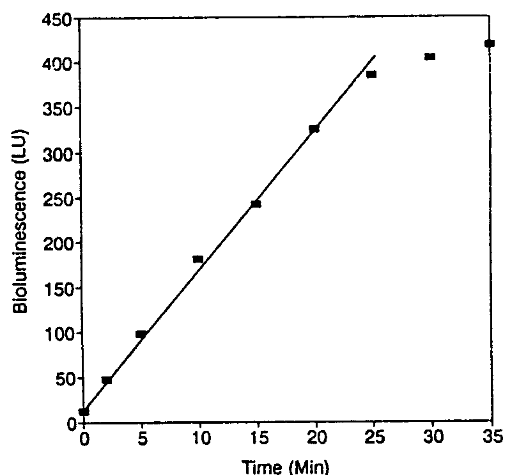

Fig. 3. *Time dependent myristic acid production from cholesteryl myristate by cholesterol esterase assayed using M-17.*
To 1 ml of 50 mM phosphate buffer (pH 7.0) containing 0.01 % sodium taurocholate, 10 μl of 10 mM cholesteryl myristate solution was added in chloroform:ethanol = 50:50 solution, and the reaction was started with addition of about 30 mUnit of cholesterol esterase. The reaction mixture was incubated at 37°C and 20 μl aliquots were taken to assay as described in Materials and Methods.

background light signal from the cell without any exogeneous myristic acid also increased and so there was no real advantages for the sensitivity.

Cholesteryl myristate was chosen as a substrate in this work, because the produced myristic acid gave the highest response in bioluminescence assay with *V. harveyi* mutant M-17 (Table 1). Although cholesteryl myristate is not so good substrate as cholesteryl oleate and, in this work, 1 unit of cholesteryl esterase which can hydrolyze 1 μmole of cholesteryl oleate per minute was found to hydrolyze only 0.13 μmole of cholesteryl myristate per minute, which is expressed as the sensitivity index of cholesterol myristate indicating the highest multiplication of bioluminescence response by substrate reactivity to cholesterol esterase. Other cholesterol alkyl esters such as cholesterol palmitate could be also used for this assay, but the produced palmitic acid gave about 5 % of maximum light intensity of myristic acid and low sensitivity index (Table 1).

Table 1. *Comparison of substrate specificity for cholesterol esterase and bioluminescence reaction with M-17 of cholesterol fatty acid ester due to the fatty acid moiety.*

Fatty acid	Reactivity to cholesterol esterase[a] (%)	Bioluminescence with M-17[b] (%)	Sensitivity index[c]
$C_{14:0}$	13	100	1,300
$C_{16:0}$	28	6	168
$C_{16:1}$	ND	2.5	ND
$C_{18:0}$	44	0.2	8.8
$C_{18:1}$	100	0.05	5
$C_{18:2}$	110	0.01	1.1

[a] from Hernandez and Chaikoff (12) except $C_{14:0}$
[b] measured with saturating concentration of each fatty acid
[c] a × b;
ND, not determined

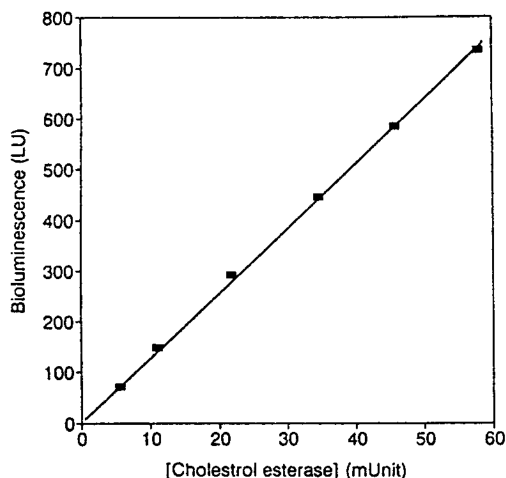

Fig. 4. *Various amounts of cholesterol esterase activity in the presence of 0.1 mM of cholesteryl myristate in 50 mM phosphate buffer (pH 7.0 at 37°C) using 20 minutes fixed time assay procedure.*

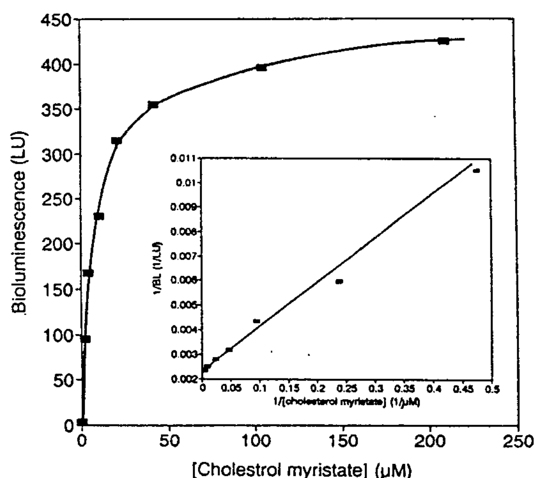

Fig. 5. *Luminescent assay of cholesterol esterase activity performed at various concentrations of cholesterol myristate.*

30 mUnit of cholesterol esterase was used in 50 mM phosphate buffer (pH 7.0 at 37°C)and 20 minutes fixed time assay was used. The inset figure represents the double reciprocal plot of the cholesterol esterase activity graph.

The hydrolysis of myristic acid from cholesteryl myristate by cholesterol esterase was followed using this bioluminescence method (Fig. 3), and the curve of light intensity was linear until 20 minutes and then the slope began to decline. A 20 minutes of fixed time assay was suitable for large number of analysis at a time. Cholesterol esterase activity versus the light intensity showed good linearity with this luminescent assay down to 5.6 mUnit which was the amount of hydrolyzing 5.6 nmol of cholesterol oleate per minute at 37°C (Fig. 4). Cholesterol esterase activity versus increasing concentration of cholesterol myristate showed typical hyperbolic saturation curve, and from the double reciprocal plot, the K_m of cholesterol myristate for cholesterol esterase was 14 μM (Fig. 5).

The usual cholesterol esterase assay system is quantitizing the released free cholesterol or free fatty acid after the enzymatic hydrolysis. The most popular method is using cholesterol fatty acid ester with radioisotope-labelled fatty acid moiety, in which method the reaction mixture is extracted with organic solvent after the reaction, free fatty acid is separated with thin layer chromatography, scraped off, and the radioisotpote activity is measured with scintillation counter. Recently, an alternative spectrophotometric assay using color-developing substrate, cholesteryl-3-glutaric acid-resorufin ester and measuring the absorbance change at 572 nm (extinction coefficient=60 cm^{-1} mM^{-1} at pH 6.8) or fluorescence change at 583 nm was developed (6). The big structural difference of artificial substrate from the real substrate and its bulky resorufin residue decreases its reactivity as a substrate of cholesterol esterase, and it is necessary to hydrolyze the initially formed resorufin-3-glutaric acid ester to the glutaric acid and free resorufin which shows color or fluorescence. The detection limit of resorufin with spectrophotometer is about a few μM range. This cholesteryl-3-glutaric acid-resorufin ester is about 20,000 fold expensive than choleterol myristate used in this assay (1).

This bioluminescence assay of cholesterol esterase gives a very straightforward analysis system without any complicated extraction and separation procedures. There is very little background signals leading to higher sensitivity and the luminous bacteria can be easily cultured and stored which can be directly used as assaying reagent with relatively little cost and labor. The chemicals used are relatively inexpensive and easily available and are not radioisotope-labelled compounds giving almost negligible environmental contamination problem. The weak point of this luminescence assay using M-17 is that this system is also sensitive to long chain aliphatic aldehydes such as decanal or tetradecanal and gives false light signal when the assay system is contaminated with long chain aldehyde. Especially unpurified crude enzyme from the plant source can have such problems. For this case, inclusion of 1 mM of hydroxy lamine can trap contaminated aldehyde and solve this

problem. Also the stability of M-17 suspension is not very high and gives reproducible results for up to 4 hrs. so new bacterial suspension should be prepared in every assay, but the reproducibility from batch to batch is quite high if proper care for the harvesting is performed.

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초 록: 세균의 생체발광 반응을 이용한 Cholesterol Esterase 활성도 측정법

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생리학적으로 매우 중요한 생체내 cholesterol 의 대사에 관여하는 효소중의 하나인 cholesterol esterase (cholesteryl ester hydrolase EC 3.1.1.13) 의 활성도의 발광형 측정법을 개발하였다. 측정을 위해 그자체로는 발광하지 않으나 myristic acid 존재하에서 발광하는 발광세균의 돌연변이 균주 *Vibrio harveyi* mutant M-17을 이용하여 cholesterol myristate를 기질로 사용하였고 그 반응 산물인 myristic acid를 이 측정 체계를 이용하여 정량하였다. 이 활성도 측정방법을 이용하여 *Bovine pancreas* 유래의 cholesterol esterase의 반응 특성을 조사함으로써 이 방법이 기존의 방사성 동위원소에 의한 측정보다 매우 신속 간편하고 안전하며 동일한 수준의 효소 활성 측정 감도를 갖고 있음을 확인하였다.

A New Screening Method for the Microbial Production of Eicosapentaenoic Acid Using Bioluminescence of Marine Bacteria

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해양 발광 세균을 이용한 EPA 생산 검색법

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Abstract : For the rapid screening of eicosapentaenoic acid (EPA) producing bacteria, the bioluminescence of marine bacteria was employed. *Photobacterium leiognathi* was chosen as the best reporting species for this method in selectivity, reactivity, and stability. Polyunsaturated fatty acids, authentic or prepared from EPA positive strain, show strong inhibition of *in vivo* bioluminescence up to 95% in 5 minutes which can be easily detected with photometer. For the preparation of free fatty acid from target bacteria, enzymatic hydrolysis with phospholipase A_2 was method of choice to decrease reaction time (10 minutes)compared with chemical saponification method (2 hours). For the processing of a large number of samples at a time, the use of camera luminometer using Polaroid film and 96 microwell plate was demonstrated.

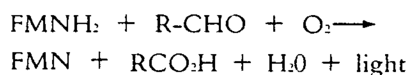
Key words : Eicosapentaenoic acid (EPA), Bioluminescence, Phospholipase A_2.

Introduction

It has been reported that n–3 series polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) are effective for the prevention and cure of thrombosis, atherosclerosis, and subsequent blood circulation disease by lowering plasma cholesterol and triglycerols (Kelly 1991). These fatty acids were also known to have a high nutritional value in the diet of many economically important mariculture species, increasing the overall health and growth as well as the resistance to disease in scallops and salmonoid fish (Bell *et al.* 1991). These PUFAs are contained in the fish oil of blue backed fish such as herring and

mackerel, but the primary producer of these fatty acids in fish is known to be marine microalgae, accumulated in the fish body through the food chain (Chohen 1986). Fish oil has a long history as principle source of production of EPA in the field of industry and health, but there are some difficulties in the purification of EPA because of the highly complicated fatty acid composition of fish oil. In particular, the content of PUFA other than EPA is quite high and a large amount of unwanted by–products due to autooxidation during transportation and storage of caught fish, is also produced. So there has been some attempt to produce EPA from EPA producing microalgae by a fermentation process instead of fish oil, but the cultivation of microalgae require strictly controlled growth conditions such as light, oxygen and carbon dioxide levels, which can result in considerable expense. Recently, EPA producing bacteria were isolated from the intestine of these blue backed fish (Yazawa *et al.* 1988). The physiological function of EPA for these bacteria and the symbiotic relationship between these EPA producing bacteria and host fish is not very clear, but the host fish can absorb EPA produced by bacteria in its intestine. Moreover, EPA is produced as the sole PUFA in these bacteria for some unknown reason and the amount of other PUFA is almost negligible, which make these bacteria quite a promising resource of PUFA through biotechnological process such as fermentation or more importantly genetic engineering because the much smaller genome size of bacteria than eukaryotic microalgae permits the genetic manuplation more easily. The process to isolate EPA producing bacteria consists of collecting as many kinds of bacteria as possible from probable samples such as the intestinal materials of marine animals (sampling) and test the existence of EPA as their membrane fatty acid (screening), and usually at least a few thousands bacteria should be isolated and screened. The usual screening method for this purpose is using thin layer chromatography (TLC) and gas chromatography (GC), but the whole process of screening using GC and TLC is quite time and effort consuming and a new rapid and reliable method for the screening is necessary.

In marine environments, there are many bioluminescent organisms, such as luminescent fish, squid, shrimps, dinoflagellates, and bacteria. At present, three genera of culturable luminous bacteria have been reported, *Photobacterium*, *Vibrio* and *Schwanella*. These luminous bacteria emit blue–green light (λ_{max} =490*nm*) using reduced flavin ($FMNH_2$), molecular oxygen (O_2) and long chain aliphatic aldehyde such as myristic aldehyde (tetradecanal, $C_{13}H_{27}CHO$) with catalysis of so called bacterial luciferase (Hastings *et al.* 1986).



$$FMNH_2 + R\text{-}CHO + O_2 \longrightarrow$$
$$FMN + RCO_2H + H_2O + light$$

Myristic aldehyde was generated from myristic acid (C14 : 0) by fatty acid reductase complex in luminous bacteria using NADPH and ATP *in vivo* condition by fatty acid reductase complex. This fatty acid reductase complex is composed of three different polypeptides, namely acyl Co–A synthtase, acyl transferase, and reductase. The acyl transferase unit has very high substrate specificity toward myristic acid, and other satu-

rated fatty acid, such as lauric acid (C12 : 0), palmitic acid (C16 : 0), and stearic acid (C18 : 0) show activity less than 1% of myristic acid to be converted into corresponding aldehydes. Unsaturated fatty acids, such as palmitoleic acid (C16 : 1), oleic acid (C18 : 1), linoleic acid (C18 : 2), linolenic acid (C18 : 3) show some inhibition against myristic acid reduction, and as a result the addition of these unsaturated fatty acid into the cell suspension show decreased *in vivo* bioluminescence (Ulizer and Hastings 1980). This decrease of light intensity by exogeneous fatty acid is very quick and can be used for the rapid detection of unsaturated fatty acid in the reaction mixture. In this report, it was confirmed that the higher the unsaturation degree in fatty acid, the higher is the inhibition degree of *in vivo* bioluminescence and a new, rapid screening methods for EPA was developed.

Materials and Method

Materials : Aliphatic fatty acids, aldehydes, and phospholipase A_2 were purchased from Sigma Chemical Co. Bacto–Trypton, bacto yeast extract, and bacto agar were from Difco Chemical Co. All other chemicals and solvents used were of analytical grade. Luminous bacteria *Photobacterium leiognathi, Photobacterium phosphoreum, Vibrio harveyi, Vibrio fischeri* were the kind gift of Prof. Tu (University of Houston, Houston, TX).

Fatty acid preparation : Bacteria to be screened for EPA production were grown in ZoBell agar medium containing 70% aged sea water. About 20±5*mg* of bacterial cell were taken from the agar plates with a calibrated loop (20±5*mg*), and suspended in 0.4 *ml* of Folch solution (chloroform–methanol =2 : 1 (v/v) mixture, Folch *et al.* 1957), and then sonicated for 1 min. in an ice–water bath. The cell debris was removed by centrifuge with HST–8 microcentrifuge (Han–il Co., Korea) at 14,000 rpm for 5 min. To the supernatant, 0.2*ml* of 0.88% KCl solution was added and mixed with Vortex, and then phases were separated by centrifugation. The organic phase was transfered to a new vial and solvent was removed under vaccum with Speed–Vac (Heto Co., Denmark). To the dried vial with lipid residue, 0.2*ml* of 0.1 M phosphate buffer (pH 7.0) with 0.01% Triton X–100 was added and mixed with Vortex. To this lipid suspension, 10 μl of phospholipase A_2 solution (50 unit per *ml*) was added and the whole reaction mixture was incubated at 35℃ for 10 min. 0.1*ml* of this mixture was used as the free fatty acid source to examine the presence of PUFA. EPA producing bacterium B1513MA (*Aeromonas* spp.) isolated by this laboratory (Cho *et al.* 1992a) was used as the EPA positive type species and *E. coli* HB101 was used for negative type species. For nonenzymatic production of free fatty acid, total lipid were extracted with identical Folch extraction and then saponified with 5% KOH solution in ethanol. After incubation at 50℃ for 2 hours, water and ether were added and the mixture was centrifuged to separate organic phase from aqueous phase. The ether was evaporated and the residue was redissolved in ethanol.

Preparation of reporting bioluminescence bacteria : Luminous bacterium *Photobacterium leiognathi* was grown in 10*ml* of Sea Water Complete media (5*g* bactotryptone, 3*g* yeast ex-

tract, 3*ml* glycerol, 75% aged sea water, per liter pH 7.0) overnight at 25℃ up to O.D.$_{660\ nm}$=2 and the emission of light was visible without dark adaptation. Cell was harvested with centrifuge (Sorvall RC–5C) with SE–12 rotor at 10,000 rpm for 10 min. and then washed twice with cold 0.1 M phosphate buffer (pH 7.0) containig 3% NaCl. The cell pellet was resuspended in 0.1 M phosphate buffer (pH 7.0) with 3% NaCl to final O.D.$_{660\ nm}$=10 and stored at 4℃.

Screening: For light emission measurement, 10μl of final suspension was dissolved in 0.2*ml* of standard buffer and add 10μl of fatty acid sample. The light intensity was measured for 5 min. with photomultiplier photometer equipped with Hamamatsu R–447 photomultiplier phototube and Hewlett–Packard high voltage power supply. The photometer was calibrated with the standard of Hastings and Weber (1962). For *P. leiognathi*, the light intensity of 0.2*ml* is about 6–8 at 500 volt of dynode voltage which is correspond to 6 X 10^{14} *q/sec.*

For large number of assays, 96 microwell plate was used. 10μl of fatty acid extract from target bacteria were added to each well of plate, and then add 200μl of luminous bacteria suspension using multidispensor. The whole plate was gently shaken to mix it well and put into a dark box in a dark room, after 3 to 5 minutes the plate was examined with dark adapted eye or photographed with Polaroid camera using Polaroid type 667 black and white film (ASA 3000). Without PUFA, a luminous bacteria suspension will give fully induced bioluminescence. However, if the target bacteria produce a significant amount of PUFA (such as EPA), the bioluminescence will be inhibited by PUFA to any lower level. The more PUFA was produced, the more inhibition of bioluminescence will be observed. These results were compared with Hewlett–Pakard HP5890 GC with Supelco Omegawax 320 capillary column (30*m*) or Ultra–1 capillary column (0.32*mm* internal diameter and 30*m* length) with FID detector.

Results and Discussion

Four kinds of luminous bacteria, *Photobacterium leiognathi, Photobacterium phosphoreum, Vibrio harveyi,* and *Vibrio fischeri Y–1* were examined for reactivity (How fast the light intensity change by added fatty acid), selectivity (effect of EPA compared to other fatty acid) and stability (reproducibility of data) for EPA screening with *in vivo* bioluminescence inhibition by exogeneous fatty acids and *P. leiognathi* turn out to be the most suitable reporting reagent (Table 1). The suspension of luminous bacterium *P. leiognathi* gives stable light intensity for at least 30 min. at room temperature, and when PUFA sample is added, a prompt inhibition of bioluminescence emission resulted. In Fig. 1, the decrease of light intensity with time by the addition of EPA into the suspension of *P. leiognathi* were shown. After 5 min., the light intensity decreases to 6% of original value. The effects of a series of free fatty acids on the bioluminescence intensity of *P. leiognathi* are shown in Fig. 2. In this work, the octadecatetraenoic acid (18 : 4) and EPA (20 : 5) show the highest inhibition degree of more than 95% and α–linolenic acid (18 : 3, n–6) and γ–linolenic acid (18 : 3, n–3) give the next high inhibition, arachidonic acid (20 : 4) and

DHA (22 : 6) give a lesser degree of inhibition. The degree of inhibiton by oleic acid (18 : 1), and linoleic acid (18 : 2) is about 10 – 15%. Fully saturated fatty acids, palmitic acid (16 : 0), stearic acid (18 : 0), arachidic acid (20 : 0), and docosanoic acid (22 : 0) give slightly stimulated light intensities.

Table 1. Reactivity, selectivity and stability of luminous bacterium for EPA screening.

Luminous bacterium	Reactivity	Selectivity	Stability
Vibrio harveyi B_{392}	+	−	++
Vibrio fisheri Y-1	++	+	+
Photobacterium phosphoreum A-13	++	+	+
Photobacterium leiognathi SL-2	++	++	++

++ : very good, +:good, −:poor

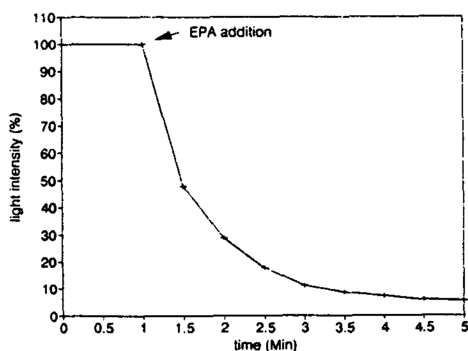


Fig. 1. Decrease of *in vivo* bioluminescence intensity of *P. leiognathi* by addition of EPA (0.1*ng* in ethanol) measured with photomultiplier photometer at 600 volt dynoid voltage.

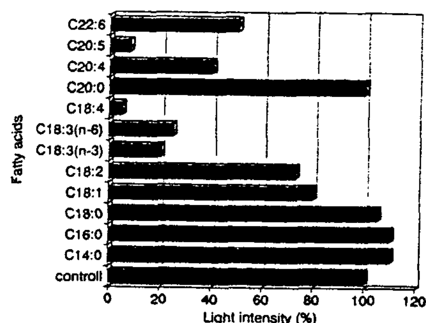


Fig. 2. Effects of various free fatty acids on the bioluminescence of *P. leiognathi*. Light intensity values are taken at 5 min. after the addition of fatty acid solution in ethanol (about 0.1*ng*).

Fig. 3. An example of photographic detection of EPA production using *P. leiognathi* bioluminescnece in 96 microwell. Photograph was taken with polaroid type 667 film (ASA3000). Lanes 1 and 12 are controls and other lanes contain extracts of EPA producion (dark spots) and nonproducing bacteria (bright spots).

The process of light inhibition can be easily detected with the dark adapted human eye in a dark room or recorded with a camera luminometer or Polaroid camera in a dark

room. A microwell plate (96 microwells per plate with a capacity of 0.3*ml* in each well) can accomodate enough luminous suspension to be easily detected with high sensitive Polaroid film. In Fig. 3, the use of a microwell plate for the detection of PUFA was demonstrated from a large number of samples with luminous method. Lanes 1 and 12 are controls which contain 0.2*ml* of luminous bacterium suspension containing 1% ethanol instead of fatty acid extract and bright spots are added with the extract of EPA negative species whereas dark spots are EPA positive species. The difference between the fatty acid compositions of typical EPA positive and negative species were shown in gas chromatogram as a comparison (Fig. 4).

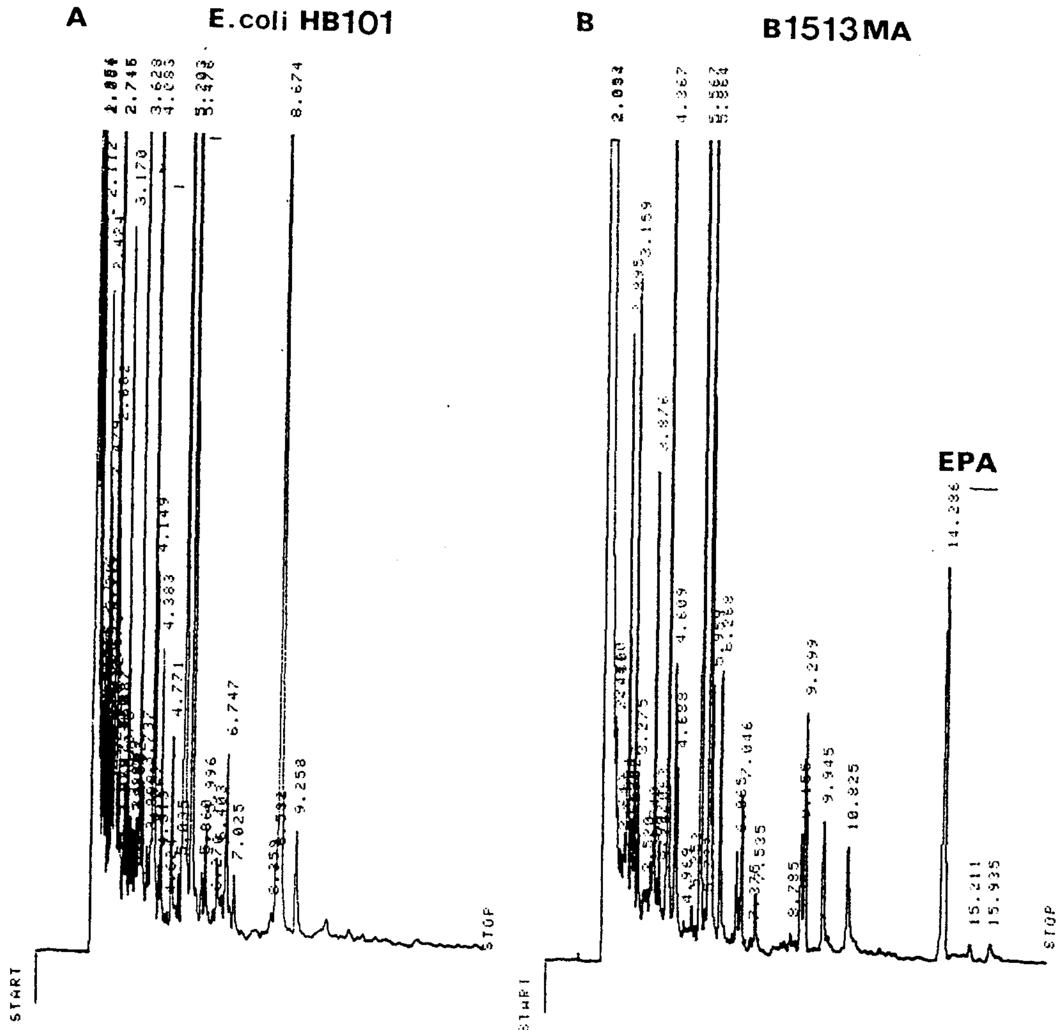


Fig. 4. Comparison of the fatty acid composition of EPA producing B1513MA and non-EPA producing *E. coli* HB101 with Hewlett-Pakard HP5890 gas-chromatography at oven temprature 195℃ and detected with FID detector at 260℃, Ultra-1 capillary column(0.32*mm* internal diameter and 30*m* length) was used.

For the preparation of fatty acid samples from bacteria, the classical Folch extraction and saponification method has been used (Cho *et al.* 1992b), but this method needs incubation at a high temperature (60℃ for 2 hours). To save this incubation time, enzymatic hydrolysis was employed. Usually, unsaturated fatty acid is located on sn-2 position in the phospholipid in the membrane, and an enzyme phospholipase A_2 (PLA_2) catalyzes the hydrolysis of this sn-2 position ester bonded fatty acid specifically. After the Folch extraction of total lipid from the cell, the solvent was evaporized and the remaining lipid (mainly phospholipid) was hydrolyzed with this phospholipase A_2. Using this enzymatic hydrolysis method, the whole extraction time can decrease from 3 hr to less than 1 hr.

Marine microorganisms, such as bacteria, actinomycetes and fungi, show very diverse and unique characteristics (Miyach *et al.* 1989) such as production of highly heat stable enzymes, biologically active substances, and novel polymers, and one of the interesting properties is the ability to produce visible light (bioluminescence) biologically. Some luminous fish or squid grow these luminous bacteria in their light organ. The exact function of luminescence for the part of bacteria is not elucidated well, but probably the emission of light permits them to be detected easily by higher organisms in the food chain, and as a result they can found much better environment for survival like the intestine or light organ, or the skin of a fish. Recently, the application of this bioluminescence of marine bacteria for microanalysis in the environment and pollution measurment has increased (Cho 1991). The luminescence assay methods are generally simple, safe, highly sensitive, reliable, and rapid and have several other advantages over previous methods ; Firstly, this analysis method is based on light generation, and there are very little background signals, leading to high sensitivity to the level of radioisotope assays. Secondly, luminous bacteria are usually quite easy to obtain and culture, and the enzyme bacterial luciferase is one of the most easily preparable enzyme to high purity with relatively little cost and labor, and it also has good thermal stability permitting long storage and operative life. Thirdly, the instruments and chemicals used were relatively inexpensive and easily available ; and finally, the substrate and reaction products of this assay are readily degradable aliphatic compounds which give almost negligible environmental contamination problems.

The immobilization of luminescence bacteria on polyacrylamide has been reported which emit light on solid state, and thermally stable *P. leignathi* can be immobilized in stable form on any supporting resin and can be used directly as biosensor, and luminous bacteria co-immobilized with phospholipase A_2 can be prepared and used directly as EPA detection sensor with the aid of fiber optics which will transport the generated light signals to a photometer.

Acknowledgement

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요 약

Eicosapentaenoic acid (EPA)를 생산하는 미생물의 신속한 검색을 위하여 해양 세균의 생체 발광 현상을 이용하는 방법을 개발하였다. Photobacterium leiognathi는 사용된 균주중 선택성, 반응성, 안정성에서 가장 뛰어난 검색용 균주로 판명되었으며 고도 불포화 지방산 존재하에서 5분이내에 생체 발광의 95%가 저해됨을 photometer를 사용하여 확인하였다. 검색 대상 균주로부터 유리 지방산을 분리하는 과정에서 기존의 비누화 반응 대신에 phospholipase A_2를 사용한 효소 가수분해법을 사용하여 반응 시간을 2시간에서 10분으로 단축할수 있었다. 또한 다수의 시료를 동시 분석하기 위하여 96 microwell plate에서 반응을 시키고 이를 photometer대신 Polaroid film을 사용한 camera luminometer를 이용하여 효율적으로 처리할 수 있었다.

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제 3 절. 일본 사가미 화학연구소와의 공동연구 추진

연구 책임자: 김 상진, 이 홍금

본 연구를 수행함과 동시에 그동안 본 연구 팀의 연구 책임자 3 인이 구 해양 미생물 연구실 시절을 포함하여 과거 수행되었던 연구 과제들 (생리 활성 물질 탐색을 위한 해양 방선균의 분리, 분류 및 보전 체계확립에 관한 연구, PE00244, 연구 책임자: 김상진, 이홍금: 해양 미생물을 이용한 EPA화합물 개발에 관한 연구, PG00160, PN00181, 연구 책임자: 김 상진, 조 기웅; 해양 미생물을 이용한 생리 활성 선도 물질 탐색 기술 개발, PN00219, 연구 책임자: 이 홍금)의 수행과정에서 수집되었던 다양한 종류의 해양 미생물과 방선균들을 이용하여 새로운 생리활성 물질을 탐색하기 위하여 이 분야에서 앞선 탐색 체계를 수립하고 있는 일본 사가미 화학 연구소와 공동 연구를 수행하기로하고 협정서를 교환하였다.

본 협정에 따라 93 년 가을 부터 방선균을 포함한 해양 미생물 균주 200 여종의 균주의 배양액과 균체를 추출하여 추출물을 제공하여 연구를 수행하고 있다.

본 협정에서는 국내에서 G-7과제로 수행중인 생리활성 선도 물질 탐색과제에서 동일한 균주들을 사용하여 탐색 수행중인 Phospholipase A2 저해제 탐색 (제일제당)은 탐색 대상에서 제외하기로 하였으며 수행중인 G-7과제와는 별도로 다양한 생리 활성 물질의 탐색을 위한 국제 공동 연구로 도출하고자 한다.