EFFECTS OF HUMAN BLOOD PLASMA ON THE CLEARING REACTION BY THE LIPOPROTEIN LIPASE I OF PSEUDOMONAS SP. M-12-33

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In 1966 ARIMA and his co-workers[1,2] reported the isolation of microorganisms which produce lipoprotein lipases with properties very similar to, if not identical with, those of the lipoprotein lipases of post-heparin plasma and other animal tissues in regard to the substrate specificities and behaviors against the specific inhibitors. The following examination[2] found that *Pseudomoas* sp. M-12-33 and *Mucor javanicus* IAM 6108 were superior microbes for the production of lipoprotein lipases.

Effects of culture conditions on the production of lipoprotein lipases by these microbes were examined[3,4] and the obtained lipoprotein lipases were successfully purified to give electrophoretically or ultracentrifugally homogeneous preparations[5,6]. All the purified lipoprotein lipases were shown to be lipoproteins themselves[5,7]. These results seem to support the conclusion that these microbial lipoprotein lipases are really lipoprotein lipases but not ordinary lipases.

Extensive studies have been done on the characteristics and physiological roles of animal lipoprotein lipases[8,9,10]. It seems probable that the lipoprotein lipase has an important function in the lipid metabolism *in vivo*, though there are considerable contradictions in the results obtained by different research groups by using only crude enzyme preparations available.

KORN[11] demonstrated that hydrolysis of triglyceride emulsions by a lipoprotein lipase, an ammonium hydroxide extract of acetone powder from rat heart, occurred only when triglycerdie emulsions had been exposed to human serum high density lipoproteins. He claimed that a true substrate, which he called "activated substrate", was formed by the interaction of serum lipoproteins and triglycerides. SCANU and PAGE[12] confirmed KORN's work and SCANU[13] further showed that apoprotein-phospholipid complexes derived from human serum high density lipoprotein could also activate triglyceride emulsions to a true substrate for the lipoprotein lipase obtained from chicken adipose tissue.

BIER and HAVEL[14] examined the role of serum lipoproteins in the formation of enzyme-substrate complex and found that very low density lipoproteins, whoes triglycerides are a substrate for lipoprotein lipases, had 13 times the activity of high density lipoproteins as estimated per unite weight of component proteins. This observations seem to suggest that one or more of the major apoproteins of very low density lipoproteins, present as a minor constituent of high density lipoproteins, may be required for the activation process. CHUNG, SCANU, and REMAN[15] examined the effect of various phospholipids on the lipoprotein lipase of rat adipose tissue, and showed that phospholipids appear to be important determinants in the action of lipoprotein lipase *in vitro* and that a certain degree of specificity of phospholipids in their action apparently dependent on both polar and non-polar moieties of these components.

The purpose of present experiment is to make clear unambiguously the role of human blood plasma in the clearing reaction by lipoprotein lipases, because it must be important to elucidate the mechanism of clearing reaction by using the pure lipoprotein lipase **I** of *Pseudomonas* sp. M-12-33, which is now made available by the present authors.

In this report two functions of human blood plasma will be described, one is an essentiality

of high density lipoproteins for the activation of triglyceride emulsion to a true substrate for lipoprotein lipase and the other is a function of plasma albumin as an acceptor of free fatty acids liberated by the action of lipoprotein lipase.

Materials and Methods

Estimation of Lipoprotein Lipase Activity.

The turbidity method[16] was employed to estimate the lipoprotein lipase activity throughout the present study.

Determination of Protein Concentration.

Protein was estimated by the absorbance at 280 mμ with a Hitachi photoelectric spectrophotometer Model 139 by using bovine serum albumin as a reference protein.

Preparation of the Pure Lipoprotein Lipase I.

The pure lipoprotein lipase **I** was prepared by gel filtration with Sephadex G–200 followed by ion-exchange chromatography on DEAE-cellulose as reported by NARASAKI *et al.*[5] from a crude enzyme powder of *Pseudomonas* sp. M–12–33. The lipoprotein lipase **I** was further purified by chromatography on DEAE-Sephadex A–50 and lyophilized to be stored in a desiccator at −20°C.

Disc Electrophoresis.

Discontinuous electrophoresis in 7.5% polyacrylamide gels was carried out according to the method of ORNSTEIN and DAVIS[17-19] by the use of an Emuesu disc electrophoretic apparatus Type–8 (Emuesu Kiki Co., Ltd., Osaka). Electrophoresis was performed at pH 8.3 with the Tris-glycine buffer system and at a constant current of 5 mA per gel for 40 min. Gels were stained with Amido schwarz for 1 hr and destained electrolytically with 8% acetic acid at a constant current of 10 mA per gel for 1 hr.

Materials.

A crude lipoprotein lipase powder of *Pseudomonas* sp. M–12–33 was produced by Amano Seiyaku Co., Ltd., Nagoya, by the procedure of NARASAKI *et al.*[3] Human normal blood was obtained from a blood bank, Takamatu, and centrifiuged at 12000 rpm for 20 min to obtain plasma. The blood plasma was then taken into test tubes and stored in a freezer at −20°C. Wheat albumin was extracted from wheat germ (Nissin Seihun Co., Ltd., Sakaide), and purifed by fractional precipitation with $(NH_4)_2SO_4$ and chromatography on DEAE-Sephadex A–50. Olive oil was a specific preparation for lipase assay (Nakarai Kagaku Yakuhin Co., Ltd., Kyoto). Egg white albumin was a product of Nutritional Biochemical Co., U.S.A. Sephadex G–200 and DEAE-Sephadex A–50 were the products of Pharmacia Fine Chemicals, Sweden. DEAE-cellulose was obtained from Brown Co., U.S.A. Bovine serum albumin was Bovine Albumin Powder, Fraction V, of Armour Pharmaceutical Co., England.

Results and Discussion

Activation of an Olive Oil-Emulsion with Various Proteins.

A 10% olive oil-emulsion (0.2 ml) was activated with 9.8 ml each of human blood plasma, 5% bovine albumin, 5% egg white albumin, or 5% wheat albumin at 35°C for 30 min to be used as a substrate for the lipoprotein lipase **I** (5γ/ml, or 420 units/ml).

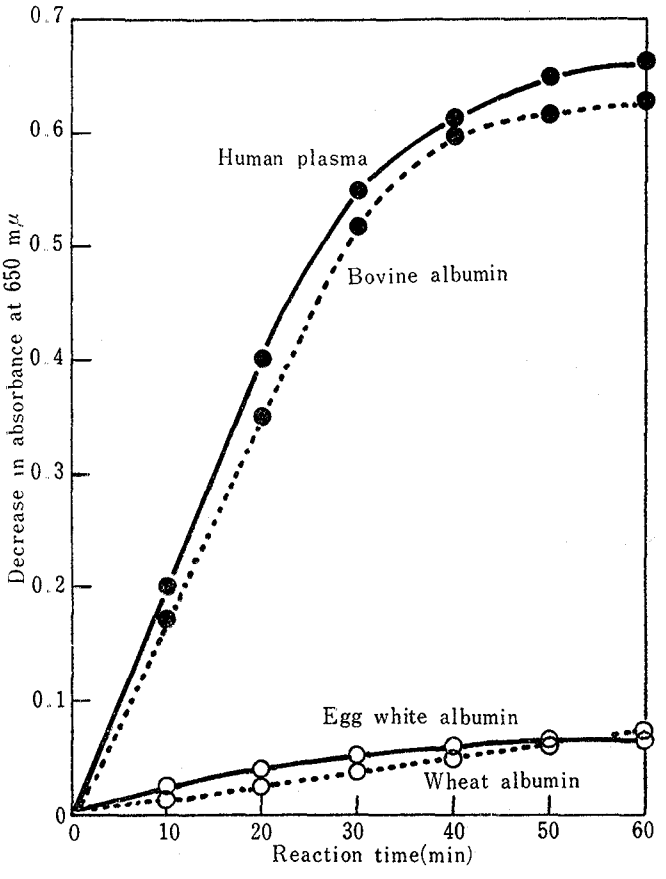

Fig. 1. Activation powers of various proteins

As shown in Fig. 1, egg albumin and wheat albumin had actually no activation power, but bovine albumin showed the same activity as that of human blood plasma. Therefore, it may be said that an albumin fraction of blood plasma contains all the factors required for the clearing of an olive oil-emulsion by the lipoprotein lipase **I**.

Isolation of Active Factors in the Human Normal Blood Plasma by Fractional Precipitation with Ammonium Sulfate.

The human blood plasma was dialyzed against deionized water through a cellulose tubing (Visking Co., U.S.A.) for 2 days at 2°C and the formed precipitate was removed by centrifuging at 12000 rpm for 20 min. The active factors were all recovered in the supernatant solution and the precipitate had no activity.

Table I shows that the active factors were collected in a fraction precipitating with 40–60% saturated $(NH_4)_2SO_4$ at an yield of 98%. This fraction contained 58% of protein in the dialyzed plasma consisting mainly of albumin and α-lipoprotein, as revealed by disc electrophoresis. Thus, the activation power was concentrated into this fraction 1.7-fold.

Purification of the Concentrated Active Factors by Chromatography on DEAE-Sephadex A-50.

The active fraction obtained by fractional precipitation with $(NH_4)_2SO_4$ (40 ml, protein=

Table 1. Isolation of active factors in the human normal blood plasma by fractional precipitation with ammonium sulfate.

Fractions pptg. with $(NH_4)_2SO_4$ (% satn.)	Protein* (mg/ml)	Yield of Activity (%)	Relative activity (%/protein base)
0	50	100	100
0–20	1.3	0	0
20–40	11.0	2	10
40–60	29.0	98	170
60–80	5.0	0	0
80–100	3.7	0	0

* The precipitated proteins were dissolved in deionized water amount to the volume of the original blood plasma.

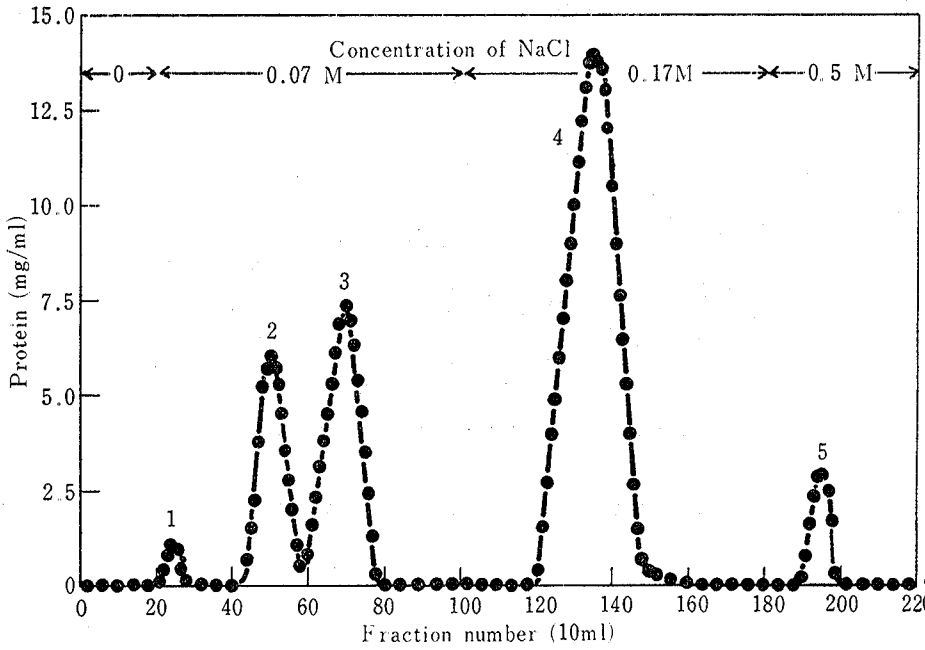

Fig. 2. Chromatogram on DEAE-Sephadex A-50.

Table 2. Purification of the concentrated active factors by chromatography on DEAE-Sephadex A-50.

Fractions	Recovery of protein (%)	Recovery of activity (%)	Relative activity (%/protein base)
Initial	100	100	100
1	1.0	–	–
2	15.5	1	6
3	19.5	4	20
3	60.0	92	150
5	4.0	3	75

80 mg/ml) was dialyzed against 0.02 *M* phosphate buffer at pH 6.6 and then put on a column of DEAE-Sephadex A–50 (200 ml, buffered at pH 6.6 with 0.02 *M* phosphate buffer). The column was then washed with NaCl-containing 0.02 *M* phosphate buffers to obtaine 5 fractions.

The results are given in Fig. 2 and Table II.

The active factors were concentrated into the fraction 4. This fraction showed about 4 times as high activity as that of the feeded active fraction of human normal blood plasma. The fraction 4 contained albumin and α-lipoprotein, the fraction 3 consisted practically only of albumin, and the fraction 5 contained α-lipoprotein, respectively.

On the basis of these results, it may be presumed that the active factors are included in albumin and α-lipoprotein fractions of the normal blood plasma.

Separation of Active factors of the Normal Blood Plasma by Ultracentrifugation.

The fractions 3, 4, and 5, which were obtained by chromatography on the DEAE-Sephadex A–50 column, were combined and concentrated to give 50 mg/ml of protein. A density of the concentrated active fraction was brought to 1.21 with NaBr and NaCl. Then the solution was centrifuged in a Hitachi preparative ultracentrifuge Model 40–P, with the use of a RP–40 rotor at an average centrifuging force of 105400×g for 24 hr at 15°C. The top fraction was collected, and diluted with two volumes of a NaBr-NaCl solution of density 1.21, and then centrifuged twice in a RP–40 rotor at 105400×g for 24 hr at 15°C. The washed top fraction was dialyzed

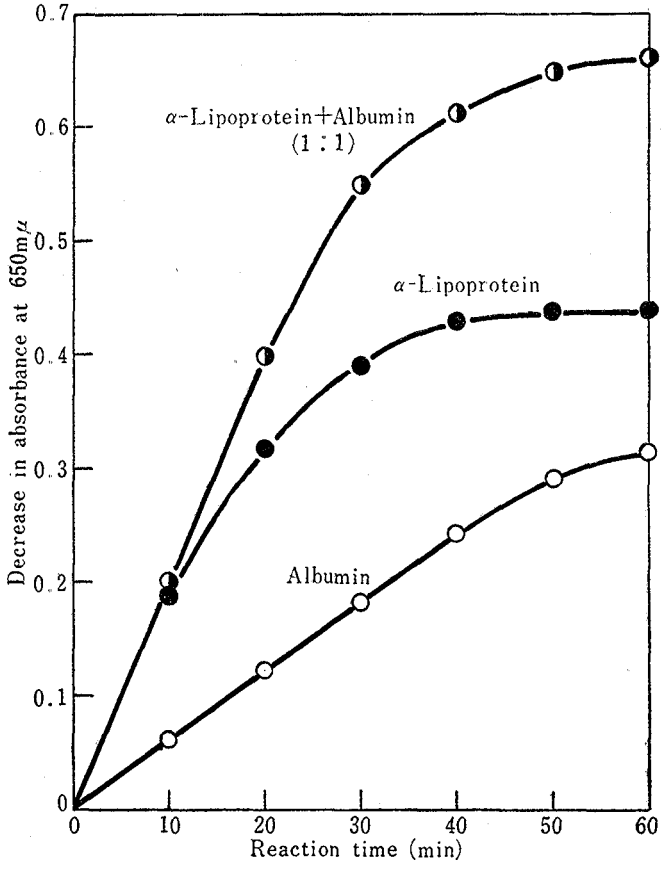

Fig. 3. Activation powers of α-lipoprotein and albumin.

against deionized water and concentratrated to give 5 mg/ml of protein to be used as an α-lipoprotein fraction of the human normal blood plasma. The bottom fraction was dialyzed against deionized water and then concentrated to give 50 mg/ml of protein to be used as an albumin fraction. The both fractions were proved to be practically homogeneous by disc electrophoresis, corresponding to α-lipoprotein and albumin, respectively.

Fig. 3 shows a mixture of the α-lipoprotein and the albumin fraction was most active, followed by the α-lipoprotein fraction. The albumin fraction showed only 1/6 activity of the α-lipoprotein fraction. Possibility that the albumin fraction is contaminated with α-lipoprotein could not be excluded in the present experiment. Therefore, whether the albumin fraction has some activation power or the observed activity of the albumin fraction is the result of contamination of α-lipoprotein is not yet clear.

An apparent increasing falling off of the activity of the α-lipoprotein fraction with the increasing reaction time seems to indicate that an inactivation of the lipoprotein lipase **I** was brought about with free fatty acids liberated during the clearing reaction. This inference was further strengthened by a addition test of oleic acid to the reaction mixture.

Thus, it can be concluded that α-lipoprotein is essential for the activation of triglyceride emulsions to a true substrate for lipoprotein lipases and albumin is required for protecting lipoprotein lipases from the inactivation with free fatty acids liberated during the clearing reaction.

Summary

The role of human normal blood plasma in the clearing reaction was examined by the use of the pure lipoprotein lipase **I** of *Pseudomonas* sp. M-12-33.

The active factors in the human normal blood plasma were separated into the α-lipoprotein and the albumin fractions by combining chromatography and ultracentrifugation.

The experiments suggest that α-lipoprotein is essential for the activation of triglyceride emulsions to a true substrate for lipoprotein lipases and albumin is required for protecting lipoprotein lipases from the inactivation with free fatty acids liberated during the clearing reaction by lipoprotein lipases.

Acknowledgement

The authors wish to thank Prof. Kei ARIMA of The University of Tokyo for his kind guidance and for his support of this work.

The crude enzyme powder used in this work was kindly supplied by Amano Seiyaku Co., Ltd., Nagoya.

The work was supported in part by the research fund donated by the Ministry of Education to T. NARASAKI (Studies on the lipoprotein lipases of microorganisms, 1965, 1966).

References

(1) ARIMA, K., NARASAKI, T., NAKAMURA, Y., TAMURA, G.: *Agr. Biol. Chem.*, **30**, 515 (1966).

(2) ARIMA, K., NARASAKI, T., NAKAMURA, Y., TAMURA, G.: *Ibid.*, **31**, 924 (1967).

(3) NARASAKI, T., TAMURA, G., ARIMA, K.: *Ibid.*, **32**, 1453 (1968).

(4) SAIKI, T., NARASAKI, T., ARAMAKI, K., TAMURA, G., ARIMA, K.: *Ibid.*, **32**, 1458 (1968).

(5) NARASAKI, T., SAIKI, T., TAMURA, G., ARIMA, K.: *Ibid.*, **31**, 993 (1967).

(6) SAIKI, T., TAKAGI, Y., SUZUKI, T., NARASAKI, T., TAMURA, G., ARIMA, K.: *Ibid.*, **33**, 414

(1969).
(7) Saiki, T., Suzuki, T., Takagi, Y., Narasaki, T., Tamura, G., Arima, K.: *Ibid.*, **33**, 1101 (1969).
(8) Robinson, D. S., French, J. E.: *Pharmacol. Revs.*, **12**, 241 (1960).
(9) Korn, E. D.: Methods of Biochemical Analysis, **VII**, 145–192, New York, Interscience Publishers Inc. (1959).
(10) Robinson, D. S.: Advances in Lipid Research, **I**, 133–182, New York, Academic Press Inc. (1963).
(11) Korn, E. D.: *J. Biol. Chem.*, **215**, 15 (1955).
(12) Scanu, A., Page, I. H.: *J. Exp. Med.*, **109**, 239 (1959).
(13) Scanu, A.: *J. Biol. Chem.*, **242**, 711 (1967).
(14) Bier, D. M., Havei, R. J.: *J. Lipid Res.*, **11**, 565 (1970).
(15) Chung, J., Scanu, A. M., Reman, F.: *Biochim. Biophys. Acta*, **296**, 116 (1973).
(16) Narasaki, T.: *Kagawa Daigaku Nogakubu Gakuzyutu Hokoku*, **20**, 49 (1968).
(17) Ornstein, L.: *Ann. New York Acad. Sci.*, **121**, Art. 2, 321 (1964).
(18) Davis, B. J.: *Ibid.*, **121**, Art. 2, 404 (1964).
(19) Nagai, Y.: *Tanpakusitu Kakusan Koso*, **11**, 744 (1966).

Pseudomonas sp. M-12-33 菌の生産するリポプロテインリパーゼ I による清澄反応における人血漿の効果

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トリグリセリド乳液のリポプロテインリパーゼによる清澄反応については，多くの研究がなされて来たが，それらの研究は，動物の心臓や脂肪組織の抽出液とか，ヘパリン静注後の血漿といった，多くの不純タンパク質を含む粗酵素による実験であるために，明確な結論を得るには疑問がある．

そこで，本研究では，*Pseudomonas* sp. M-12-33 菌の培養液から得られた純粋なリポプロテインリパーゼ I を使用して，清澄反応における人血漿の効果を検討した．

清澄反応に有効不可欠の血漿成分は，α-リポプロテインとアルブミンである事を確認できた．α-リポプロテインは，トリグリセリド乳液を活性化して，リポプロテインリパーゼの真の基質である低比重リポプロテインを生成するのに不可欠であり，アルブミンは，清澄反応によって生産される遊離脂肪酸と結合して，リポプロテインリパーゼの失活を防ぐ役割を果しているものと推論した．

本研究の経費の一部は文部省科学研究費（昭和40，41年度試験研究費，楢崎丁市，微生物のリポプロテインリパーゼに関する研究）により支払われた．

本研究を企画され終始御指導賜っている東京大学有馬啓教授に深く感謝します．

なお，粗酵素は天野製薬株式会社の御好意により提供されたものである．

(Received May 31, 1973)