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Effect of Ginseng Ethanol Extract on Lactate Dehydrogenase-5 in Rat Brain with Age

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인삼 Ethanol Extract가 쥐의 뇌 Lactate Dehydrogenase-5에 미치는 영향

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Abstract

Attempts were made to see if feeding of ginseng ethanol extract could affect properties of rat brain lactate dehydrogenase such as specific activity, heat stability, Km for substrate, inactivation by 3-bromopyruvate and trypsin, and immune response. The following results were obtained.

Specific activity of LDH was observed to reach maximum in 5 month after birth and then decrease steadily. However, that of LDH from rat fed with ginseng ethanol extract was found in rat fed with ginseng ethanol extract. 3-bromopyruvate was shown to inactivate LDH-5 from old rat fed. Inactivation of LDH-5 by trypsin was remarkable in old rat fed. Km value for pyruvate in old rat fed was remarkably decreased. Cumulative results suggest that ginseng ethanol extract could affect conformational change of LDH responsible for altered properties through unknown mechanism.

Introduction

Many studies reporting the presence of defective enzyme molecules[1,4] and alterations in the synthesis or degradation of protein, or both, in senescent animal cells[5,6] have been published; but considerable confusion in these aspect of cellular aging is apparent from a review of the literature. Orgel[7] has first proposed that defective enzymes may accumulate during senescence from a loss in the ability of aging cells to synthesize proteins with exact fidelity. This error catastroph view of againg implies that the loss in fidelity of protein synthesis restults in the accumulation of defective enzymes to such an extent that the cell is no longer able to carry out

vital physiological functions, which ultimately leads to senescence and finally death. Other workers have suggested that defects in enzyme structure may occur as the result of processes occurring at postsynthetic levels, including conformational alterations and derivative formation[8,9,10]. Finally, according to previous papers[6,11] senescent cells may have a decreased ability to selectively recognize and remove defective protein molecules; in this view, defective enzymes may accumulate as a result of a loss in fidelity of the intracellular degradative process. Whether rates of bulk protein synthesis increase, decrease, or remain the same during cellular aging is also not clear[5,6,11,12,13]. Only a few of studies were devoted to aging of enzymes from mammalian tissues, some examples being mouse and rat liver aldolase[3,14], glucose-6-phosphatase[15], rat liver superoxide dismutase[16], rat heart enolase[17], and rat muscle phosphoglycerate kinase[18]. Some enzymes, on the other hand, were found to retain their full activity in old animals[19,21].

While the partial loss of enzymatic activity is common to all enzymes that are affected by aging, change in other properties are less consistent. In some cases, a component with increased heat sensitivity was found[16,22]. Studies on values of Km[16,23], amino acid sequence[7,24,25], reactivity of sulfhydryl group toward reagent[24,25,26], out isozyme pattern[27], UV absorption[28] and circular dichroism spectra[29] were carried out.

Lactate dehydrogenase has five isozymes, H_4,H_3M, H_2M_2, H_1M_3 and M_4. These are composed of two seperate genes. The isozyme pattern is characteristic for each tissue. For example, H_4 is found predominantly in tissue which are essentially aerobic, such as the heart and adrenal cortex, and M_4 is rich in tissues that are essentially anaerobic, such as the skeletal muscle and the adrenal medulla. The turnover number and Km values of H_4 for pyruvate are lower than those of M_4[30]. Also, H_4 is inhibited by 0.33 mM oxalate[31] and high pyruvate, whereas M_4 is not[30]. The cells of brain, heart, and skeletal muscle are postmiotic, that is, they do not divide after certain period of growth of the animal and might be expected to show the maximum effect of age on the activity of the enzymes.

In the present paper, we prepared rat lactate dehydrogenase from brain with ages and observed if the effect of ginseng ethanol extract on enzymes properties such as activity, heat instability, Km for substrate, the extent of inhibition by 3-bromopyruvate, immunological response, and hydrolysis by trypsin in connection with previous paper[32] in which the activities of liver enzymes from rat were observed by feeding ginseng ethanol extract.

Materials and Method

Materials

Ginseng ethanol extract was prepared by method as described elsewhere[32] and used.

The feeding of ginseng ethanol extract was done by method as described previously.[32] Antibody of lactate dehydrogenase was prepared by usage of rabbits (New Zealand,male). Pyruvate, NAD^+ NADH, lactate, phenazine methosulfate, nitrobluetetrazolium, bovine serum albumin, DEAE-sephacel, NAD^+-agarose, 3-bromopyruvate, bromophenol blue, phenylmethyl sulfonyl fluoride, trypsin were obtained from Sigma. Acrylamide, N′, N′-methylene-bisacrylamide, ammonium persulfate, TEMED, commassie brilliant blue R-250 were products of Bio Rad. Cellulose polyacetate membrane was purchased from Gelman Science. The rest of chemicals were of

analytical grade.

Preparation of lactate dehydrogenase

The rats were killed by dislocation of the neck and brain was removed immediately, washed in cold 0.1 M patassium phosphate buffer (pH 7.4), and weighed. A 10% homogenate (W/V) of brain tissue was prepared in ice-cold 0.1 M phosphate buffer with Teflon pestle. The homogenate was centrifuged at 14,000 xg in refrigerated centrifuge for 30 min. The supernatant fraction was collected and suitably diluted with 0.1 M phosphate buffer, pH 7.4, for the assay of lactate dehydrogenase. All procedures were carried out at 0°-4°C.

Spectrophotometric assay of lactate dehydrogenase

The method of assay of lactate dehydrogenase was that of Kornberg[33]. One unit of activity is defined as the amount of enzyme required to bring about transformation of 1 μ mole pyruvate to lactate per minute, and specific activity as units per miligram of protein. Km was determined in the reaction mixture at various concentrations of pyruvate by Lineweaver—burk plot.

Protein determinations

Protein was determined by the procedure of Lowry et al.[34] using serum albumin as a standard.

Heat inactivation studies

The enzyme solution was heated in water bath at 50°C. Aliquots of the enzyme solution were assayed for activity at 10 min intervals after cooling.

Electrophoresis on cellulose polyacetate membrane and activity staining

Cellulose polyacetate membrane was soaked in 0.05 M Tris-barbital buffer (pH 8.8) containing 0.35% EDTA, on which the enzyme solution was applied and electrophoresis was carried out for 30 min at 250 V. Localization of lactate dehydrogenase activity was performed as described by Dietz and Lubrano[35] and scanned at 525 mn by the use of Helena Laboratory Quick Scan Densitometer in order to calculate ratio of lactate dehydrogenase isoenzyme.

Storage

The enzyme solution was stored at 4°C in the presence of 1% phenylmethyl sulfonyl fluoride ethanol solution to inhibit proteolysis as suggested by Grant et al.[36] and without phenylmethyl sulfonyl fluoride, and the enzymatic activity was checked at 2 day intervals.

Preparation of lactate dehydrogenase-5 (M_4)

Crude enzyme solution was obtained from rat brain as described elsewhere[3] and in turn protein in supernatant precipitated between 40 and 70% ammonium sulfate saturation. The precipitated protein was dissolved in 10 mM Tris-HCl (pH 7.4) containing 1 mM 2-mercaptoethanol. The enzyme (M_4) was isolated by chromatography on DEAE-Sephacel Column (2.5 x 10cm) by collecting fractions at rate of 2 ml/5 min with the same buffer, and the rest of isozymes with 0-0.4 N NaCl linear gradient. The enzyme (M_4) isolated was dialyzed against

the same buffer for one night and was placed on NAD-agarous column (1.5×3 cm) previously equilibriated with the same buffer, washed with same buffer and eluted 0-0.5 mM NADH linear gradient by.

The fraction containing enzymatic activity were pooled, and used as enzyme source.

Rabbit anti-enzyme serum

The serum was prepared by immunizing a rabbit by repeated intradermal and intramuscular injection of enzyme preparation (mg/ml) from 5 month old rate in emulsion with an equal volume of Freund's complete adjuvant[37]. Removal of lactate dehydrogenase in serum was done in water bath at 60°C for 3 min and in turn antibody was used.

Inhibition of lactate dehydrogenase-5 (M_4) by 3-bromopyruvate

Various amounts of 3-bromopyruvate known as sulfhydryl group reagent was added to the enzyme solution and kept at 30°C for 30 minutes, and withdrawn, and assayed for the enzymatic activity as described above.

Inhibition of lactate dehydrogenase-5 (M_4) by trypsin

50 μl of 0.05 M Tris-HCl (pH 7.4) buffer containing 0.001 M $MgSO_4$ was added to the enzyme solution (13 μg) and mixed with 10 μl of 4 μ trypsin (specific activity, 278 unit/mg), and incubated at 37°C for 10 min intervals, and assayed for enzymatic residual activity[29].

Result and Discussion

The life span of an animal may be broadly divided into two periods: growth and senescence.

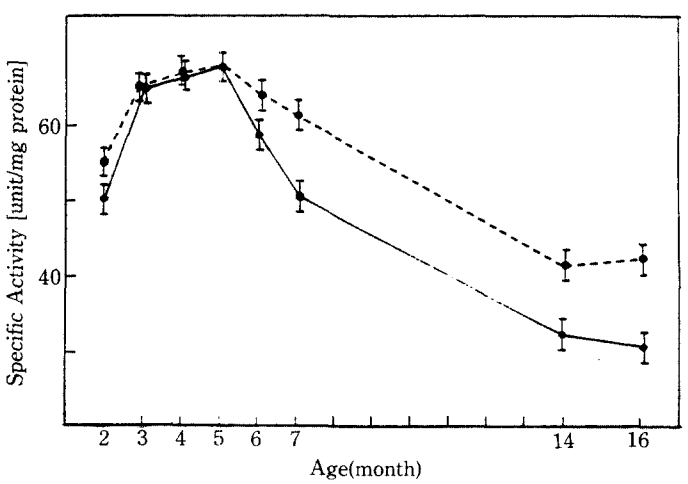

Fig. 1. Specific activity of brain LDH from rat administered with or without ginseng ethanol extract on time course.
•—•; with ginseng ethanol extract
•------•; with ginseng ethanol extract
*One unit; 1 μmole NAD^+ formed/min.

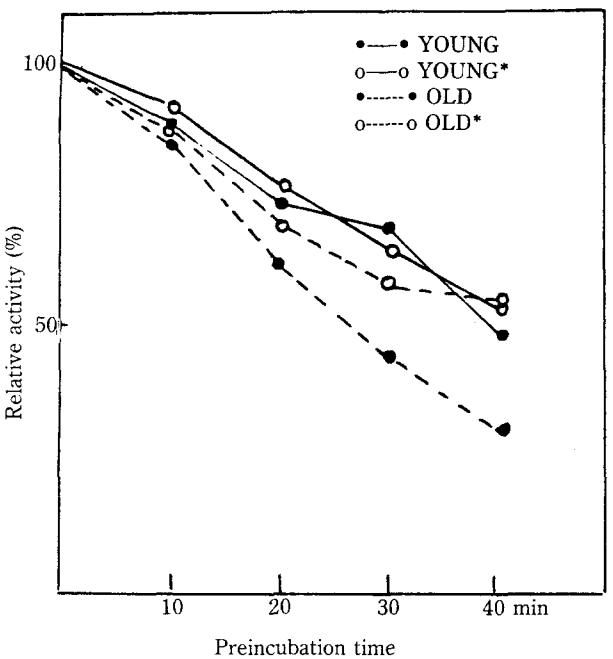

Fig. 2. Heat stability of brain LDH from old and young rats administered with or without ginseng ethanol extract (50±2°C).
*; Ginseng ethanol extract adminstered group.

In this set of experiment, 2, 3, 4, 5, 6, 7, 14 and 16 old represent the various stages. According to previous paper[39], the 30-week old rat represents the peak of the growth period and the 74-and 96-week old rats represent the senescence phase. Thus, the changes in LDH studied here cover the life span of the animal almost (Fig. 1). On the basis of the specific activity of in the present paper, 4-month old rats are referred to as young rats whereas 14-month old rats are referred to as old rats for our experimental convenience.

Figure 1 shows that specific activity of brain lactate dehydrogenase (LDH) is shown to increase steadily by 5 month and thereafter decrease constantly. However, specific activity of lactate dehydrogenase obtained from brain of old rats fed with ginseng ethanol extract (GEE) is observed to be higher than those of rat without (GEE). Such trends are in good agreement with our previous report[32]. Whether rates of bulk of protein synthesis increase, decrease or remain the same during cellular aging is also not clear[5,6,12,38,40]. However, it has been well documented that synthesis of serum albumin by rodent liver cells is increased during aging process[5]. Ambiguous as mechanism of GEE on specific activity of LDH *in vivo*, the extract is clearly shown to retard decline in specific activity to some extent.

The heat-stability diagram (Fig. 2) show that there seems to be in order of decreasing heat stability: young LDH(GEE); young LDH; old LDH(GEE); and old LDH. But there is clearly no big difference in specific activity of LDH except those from old rats fed with GEE and old rats without GEE. Such trends as order of decreasing heat stability between young and old rats are in agreement with that of enolase[29], which is claimed to converse as followings: young enolase—

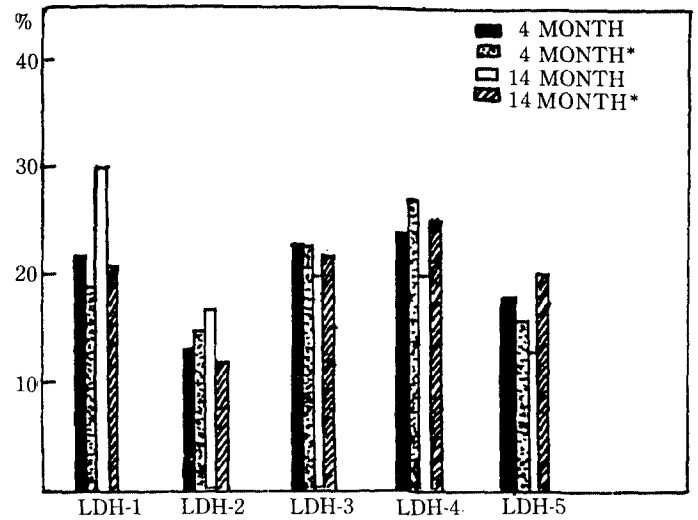

Fig. 3. Electrophoretic profile of the percent distribution of each isozyme of the rat administered with or without ginseng ethanol extract.
*; Ginseng ethanol extract administered group.

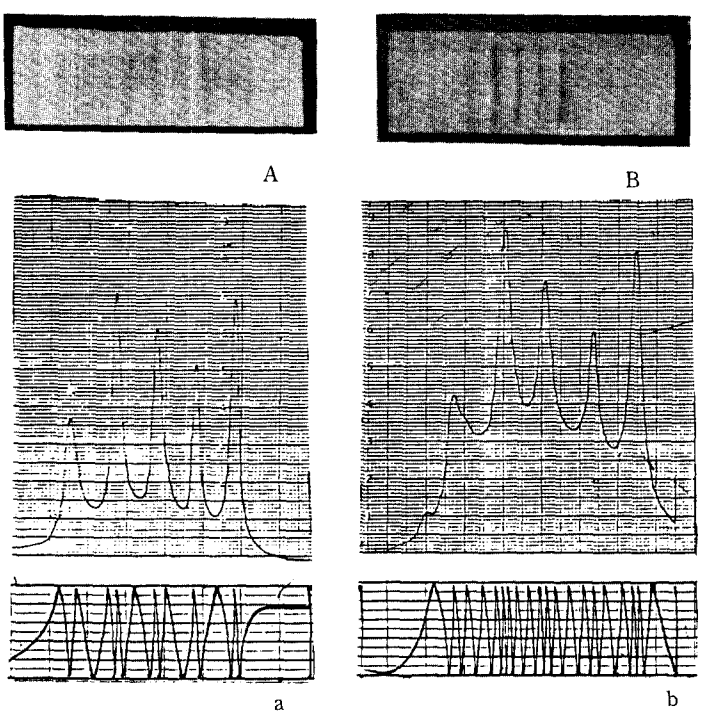

Fig. 4-1. Electrophoretic patterns of LDH from young rat brain on cellulose polyacetate membrane and their densitometic profiles.
(A,a), LDH from 4 months old rat brain; (B,b), LDH from 4 months old rat brain administered with ginseng ethanol extract.

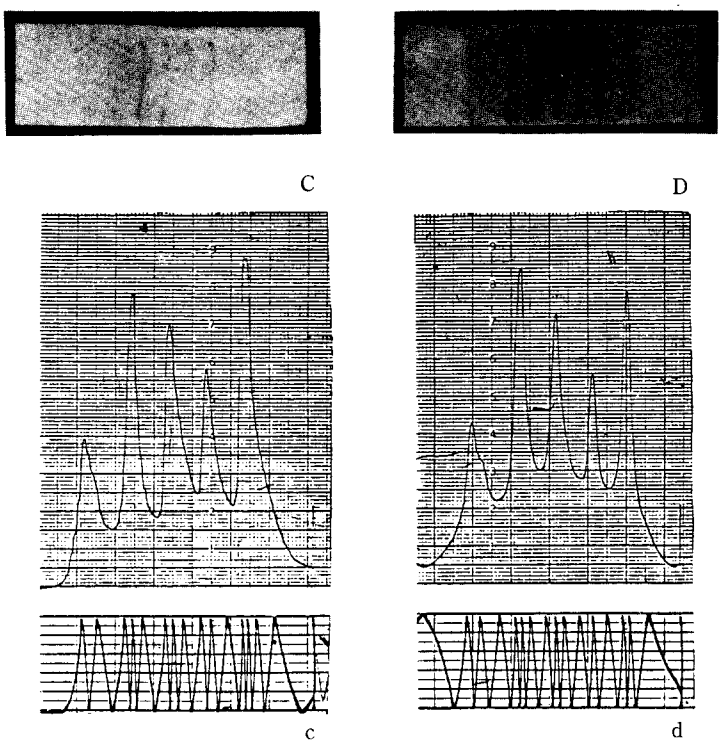

Fig. 4-2. Electrophoretic patterns of LDH from old rat brain on cellulose polyacetate membrane and their densitometric profiles. (C,c), LDH from 14 months old rat brain; (D,d), LDH from 14 months old rat brain administered with ginseng ethanol extract.

stable old enolase—unstable old enolase—inactive old enolase. However, phosphoglycerate kinase from old rats exhibits temperature resistance compared to that of young rats[18], in contrast to enolase. Isozymes of LDH diagram drawn from Figs. 3, 4, and 5 shows that relative amount of LDH-5 (M_4) is clearly decreased with age and that of LDH-5 is increased in case of old rats fed with GEE. According to previous paper, the ratio of H_4 to M_4 is increased due to decrease of M_4 and not due to increase of H_4 with age[39]. Thus, the capacity of the brain of older animal to work in anaerobic conditions may be lower than that of the young. This is particularly true of the heart, which is aerobic and in which the increase in H:M ratio is the highest. The synthesis of more M subunits by heart cells in culture and their inhibition in the presence of oxygen [33] seems to support the above conclusion. Anyhow, GEE could have rats retain LDH-5 and get such behaviors as described above.

Fig. 5-1 shows the isolation of LDH-5 from young LDH isozymes by DEAE-Sephacel chromatography appearing between no 7 and 40 fractions which appear between no 10 and 18 fractions on NAD-agarose column. Bands of isolated isozymes formed by cellulose polyacetate membrane electrophoresis show clearly each band (Fig. 5-2) Bands (above) show isozymes of LDH except LDH-5 and band (bottom) shows LDH-5.

By column chromatography and electrophoretogram of LDH, clear differences in enzymatic

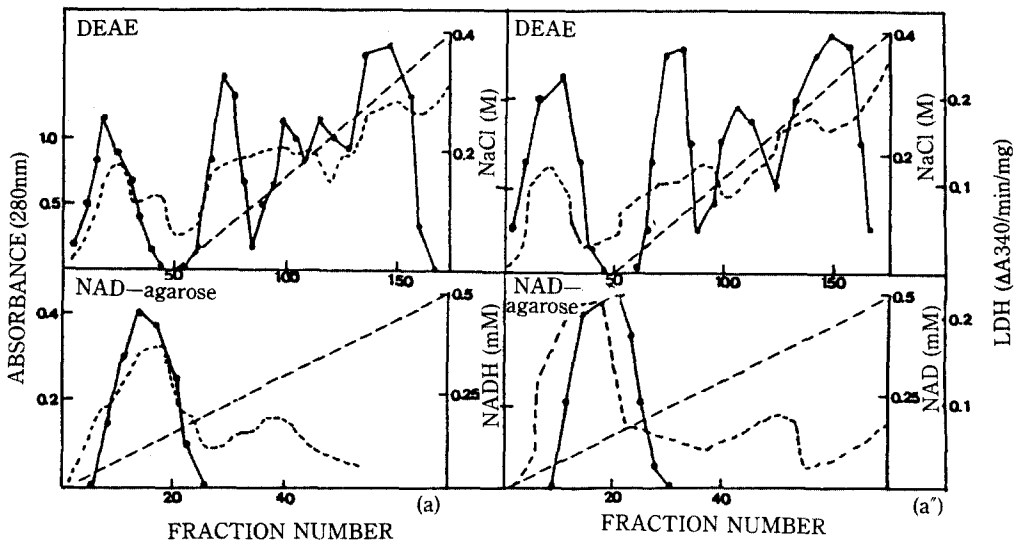

Fig. 5-1. Purification of LDH-5 from young rat brain.
(a) Young rat.
(a") Young rat administered with ginseng ethanol extract.

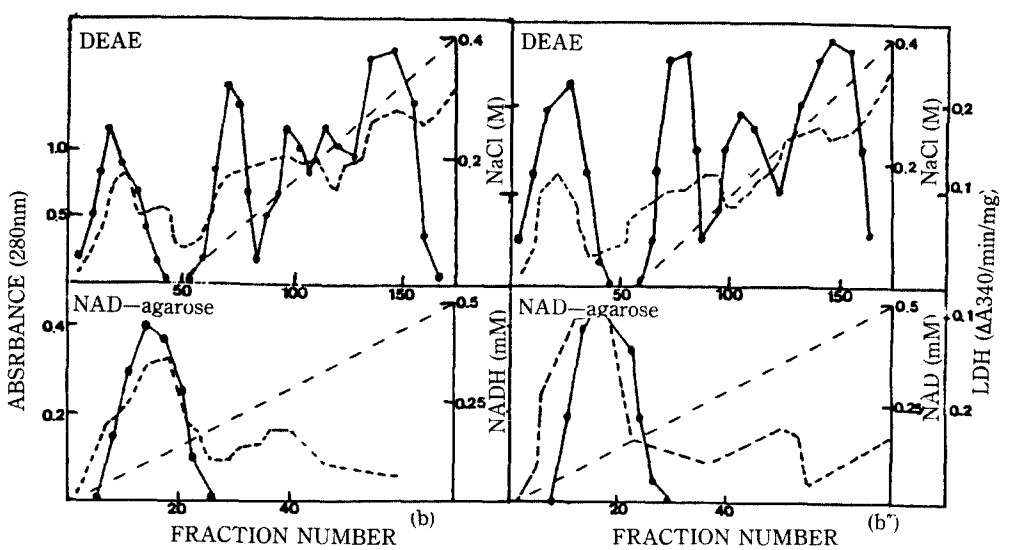

Fig. 6-1. Purification of LDH-5 from old rat brain.
(b) Old rat.
(b") Old rat administered with ginseng ethanol extract.

Fig. 7 shows that activity ot all LDH-5 decreases with increase in amount of antibody prepared by LDH-5 from young rat and LDH-5 from young rat is more sensitive than that from old rat. And it also shows that activity of LDH-5 from rat fed with GEE is more decreased than that

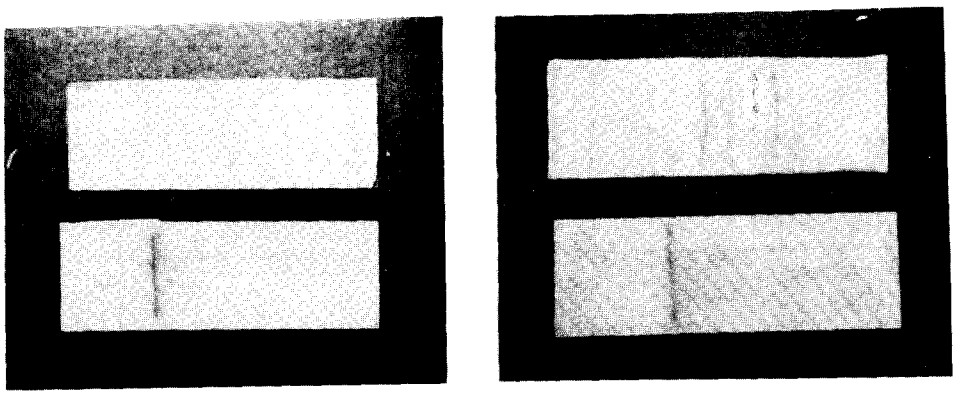

Fig. 5-2. Electrophoretogram of purified LDH-5 from young rat brain on cellulose polyacetate membrane.
(a) Young rat.
(a") Young rat administered with ginseng ethanol extract.

activity between young rat fed with GEE and without were not observable. On activity of LDH isozymes from old rat fed with GEE and without, almost similar trends were observed (Figs. 6-1 and 6-2).

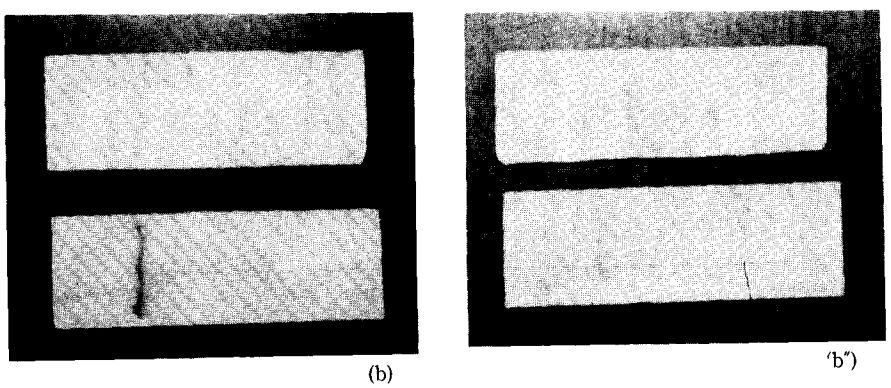

Fig. 6-2. Electrophoretogram of purified LDH-5 from old rat brain on cellulose polyacetate membrane.
(b) Old rat.
(b") Old rat administered with ginseng ethanol extract.

of rat without GEE. As shown in Fig. 8, Km value for pyruvate increased with age. However, GEE was observed to decrease remarkably Km value of LDH-5 from old rat whereas no effect in case of LDH-5 from young rat. Fig. 9 shows inactivation of LDH-5 by bromopyruvate. As in case of Km value for pyruvate, activity of LDH-5 from old rat fed with GEE was higher than that of old rat. Fig. 10 shows inactivation of LDH-5 by trypsin. Clearly, LDH-5 from old rat fed with GEE was observed to be easily attacked by trypsin and inactivate probably.

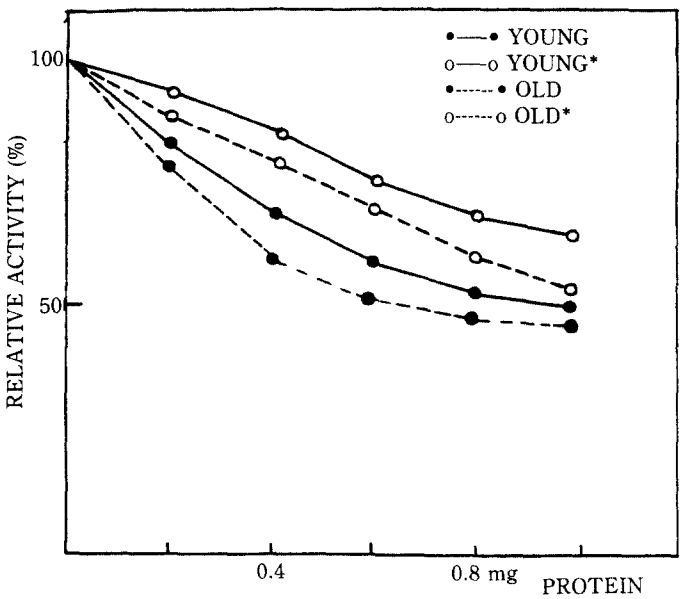

Fig. 7. Immunotitration of LDH-5 from young and old rats administered with or without ginseng ethanol extract. The activities of each enzymes were adjusted to the same level.
Each datum represents the average value for two preparations. Values agreed within 5%.
*; Ginseng ethanol extract administered group.

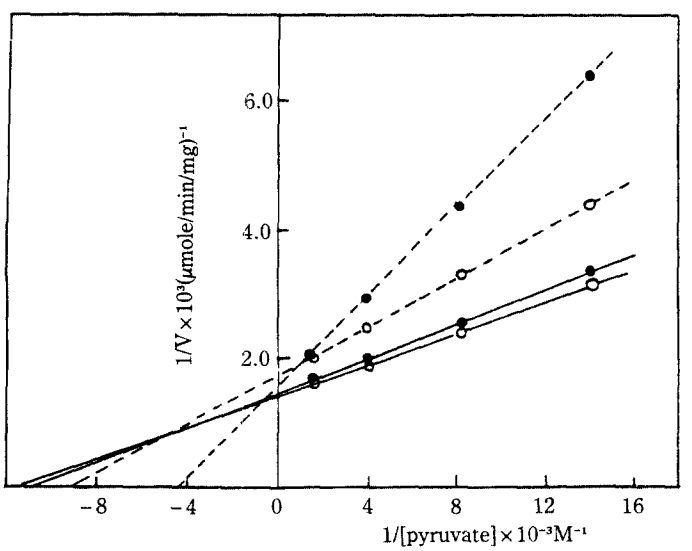

Fig. 8. Lineweaver-Burk plot for the determination of Km for LDH-5.
•—•, Young enzyme; o—o, Young enzyme*
•------•, Old enzyme; o------o, Old enzyme*.
*; Ginseng ethanol extract administered group.

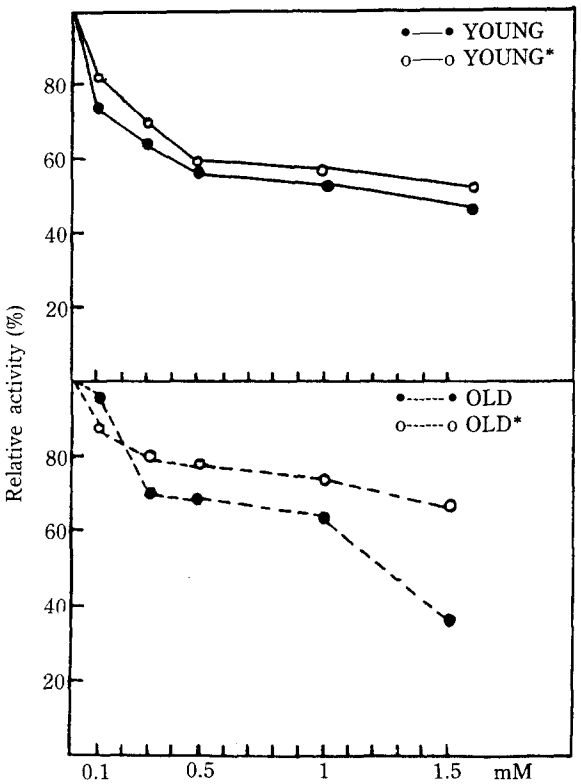

Fig. 9. Kinetic inactivation of LDH-5 activity by bromopyruvate. The LDH assays were performed as described under Material and Methods at varing levels of bromopyruvate.
*; Ginseng ethanol extract administered group.

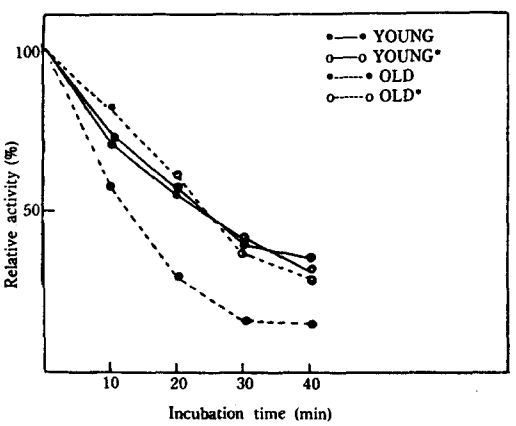

Fig. 10. Inactivation of LDH-5 by trypsin.
In the control experiment (enzyme with no trypsin present), there was no significant change in activity over the indicated time period.
*; Ginseng ethanol extract administered group.

During experimental period, there was no reduction in enzymatic activity suggesting that there might be no proteolysis of LDH.

The cause of the age-related changes in many enzymes[3,16,23,41,42] has not yet been determined. In case of old enzymes, there appear to be no gross changes in amino acid composition, nor any substitution which result in change of charge. Beyond any resonable doubt, proteolysis is not factor. Mixing of young and old homogenates[3] or allowing crude homogenates to stand for several hours[41] does not change the properties of the various enzymes so far studied. Similarly, the presence or absence of protease inhibitors does not affect the respective properties of young or old enolase[29] or isocitrate lyase[41]. In case of LDH, phenymethylsulfonyl fluoride did not affect the activity of LDH (Fig. 3).

If the alteration of old enzyme is due solely to a postsynthetic modification, as proposed by previous papers[18,41], then the change in protein conformation may result from an enzyme-mediated actions, a kinetically determined change in folding of protein or passive accumulation of some small component which can affect the function of the enzyme. The results presented in this paper suggest that conformational difference between LDH from young rat and that from old rat is responsible for altered properties such as antibody response, inactivation by bromopyruvate and trypsin and Km value for pyruvate as in case of enolase[29]. If such hypothesis holds true for LDH, GEE might affect conformational changes of LDH responsible for retarding decline in enzymatic activity and other properties described above.

The data presented here suggest the idea that altered properties of LDH from old rat fed with GEE are a result of conformational changes. However, other post-translational mechanism or sequence changes might be possible. Final solution to the problem must wait the detailed sequence analysis of LDH from rat fed with GEE and without, and degree of LDH *de novo* biosynthesis.

要 約

인삼 에탄올 추출물을 투여한 쥐 (시험군)와 투여하지 않은 쥐(대조군)의 뇌 Lactate Dehydrogenase (LDH; L-Lacate; NAD reductase, E.C. 1.1.1.27)의 여러성질 즉 비활성도, 열에대한 안정도, 기질과의 반응성, 3-bromopyruvate에 의한 비활성화의 정도, trypsin에 대한 sensitivity를 나이에 따라 비교하였다. LDH의 비활성도는 대조군의 경우 생후 5개월에서 가장 높았으나 그 이후 점차 감소하였고, 시험군의 경우엔 대조군의 경우 생후 5개월에서 가장 높았으나 그 이후 점차 감소하였고, 시험군의 경우엔 대조군에 비해 비 활성도가 적게 감소하였다. Antiserum에 의한 LDH-5의 효소 활성 저하도는 시험군의 경우 훨씬 컸다. 3-bromopyruvate에 대한 효소 활성 저하도는 old age 시험군의 경우 적었다. LDH의 pyruvate에 대한 Km 값은 old age 시험군의 경우 훨씬 적었다. 인삼 에탄올 추출물이 쥐 뇌 LDH노화에 따른 성질 변화를 일으키는 comformational change에 효과가 있는 것으로 생각된다.

References

1. Holliday, R., and Tarrant, G.M: Nature 238, 26 (1972).
2. Gershon, H., and D. Gershon: Nature 227, 1214 (1970).
3. Gershon, H., and D. Gershon: Proc. Natl. Acad. Sci. U.S.A. 70, 909 (1973).
4. Zeelon, P., H., Gershon. and D. Gershon: Biochemistry 12, 1743 (1973).
5. Ove, P., M. Obenrader, and A. Lansing: Biochim. Biophys. Acta 227, 211 (1972).
6. Bradley, M.O., L. Hayflick, and R.T. Schimke: J. Biol. Chem. 251, 3512 (1976).
7. Orgell, L.E: Proc. Natl. Acad. Sci. U.S.A. 49, 517 (1963).
8. Mennecier, F., and J.C. Dreyfus: Biochim. Biophys. Acta 364, 320 (1974).
9. Gershon, D., and H. Gershon: Gerontology 22, 212 (1976).
10. Dreyfus, J.C., H. Robinson, F. Schapira, A. Weber, J. Marie, and A. Kahn: Gerontology 23, 211 (1977).
11. Bradley, M.O., L. Hayflick, and R.T. Schimke: Exp. Cell. Res. 96, 103 (1975).
12. Beuchene, R.E., L.M. Roeder, and C.H. Barrows: J. Gerontol. 25, 359 (1970).
13. Geary, S., and J. Florini: J. Gerontol. 27, 325 (1972).
14. Weber, A., C. Gregori, and F. Schapira: Biochim. Biophys. Acta 444, 810 (1970).
15. Grinna, L.S., and A.A. Barber: Exp. Gerontol. 10, 239 (1975).
16. Reiss, U., and D. Gershon: Eur. J. Biochem. 63, 617 (1976).
17. Rothstein, M., M. Coppen, and H.K. Sharma: Biochim. Biophys. Acta 614, 591 (1980).
18. Sharma, H.K., H.R. Prasanna, and M. Rothstein: J. Biol. Chem. 255, 5043 (1980).
19. Gupta, S.K., and M. Rothstein: Arch. Biochem. Biophys. 174, 333 (1976).
20. Petel, J.K., and H.G. Lebherz: J. Biol. Chem. 254, 8179 (1979).
21. Yagil, G: Exp. Gerontol. 11, 73 (1976).
22. Bolla, R., and N. Brot: Arch. Biochem. Biophys. 169, 227 (1975).
23. Sharma, H.K., S.K. Gupta, and M. Rothstein: Arch. Biochem, Biophys 174, 324 (1976).
24. Gafni, A: Biochemistry 20, 6035 (1981).
25. Harmesh, K., H.K. Sharma, and M. Rothstein: Biochemistry 17, 2869 (1978).
26. Sharma, H.K., and Rothstein: Proc. Natl. Acad. Sci. U.S.A. 77, 5865 (1982).
27. Bhatia, C.R., and J.P. Nilson: Biochem. Genet 3, 207 (1969).
28. Demchenko, A.P., and N.N. Orlovska: Exp. Gerontol. 15, 619 (1980).
29. Sharma, H.K., and M. Rothstein: Biochemistry 17, 2869 (1978).
30. Dawson, D.M., T.L. Goodfriend, and N.O. Kaplan: Science 143, 929 (1964).
31. Plummer, D.T., and J.H. Wilkinson: Biochem. J. 87, 423.
32. Cho, Y.D., B.S. Koo, and S.J. Lee: Proceedings of the 4th International Ginseng Symoposium p37 (1984).
33. Kornberg, A. Ins. P. Colowick and N.O. Kaplan: Method in Enzymology 1, 441.
34. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall: J. Biol. Chem. 193, 265.
35. Dietz, A.A., and T. Lubrano: Anal Biochim. 244, 4406 (1967).
36. Grant, C.A., L.M. Keefer, and R.A. Bradshaw: J. Biol. Chem. 253, 2724.
37. Thorpe, R., and A. Johnston: Immunochemistry in Practice Blackwell Scientific Publications, London (1982).

38. Buetow, D.E., and P.S. Gandhi: Exp. Gerontol. 8, 243 (1973).
39. Singh, S.N., and Kanungo, M.S: J. Biol. Chem. 243, 4526 (1968).
40. Britton, G.W., and F.G. Sherman: Exp. Gerontol. 10, 67 (1975).
41. Reiss, U., and M. Rothstein: Biochim. Biophys. Res. Commun. 61, 1012.
42. Gupta, S.K., and M. Rothstein: Biochim. Biophys. Acta 445, 632 (1976).