CHAPTER IV

Purification of Native and UV irradiated DNA Specific Deoxyribonuclease (acid DNase) from Old Rat Brain.
CHAPTER - IV

PURIFICATION OF NATIVE AND UV IRRADIATED DNA SPECIFIC
DEOXYRIBONUCLEASE (ACID DNase) FROM OLD RAT BRAIN

INTRODUCTION:

All biological reactions in the body are catalyzed by enzymes. They are therefore essential for various functions of the body. Hence structural alterations in enzyme molecules during aging may alter the functional ability of an organism (Reiss, 1977). A considerable amount of data has accumulated which show that the levels of certain enzymes decrease and of a few other increase and several other enzymes do not show any change with age. A number of enzymes have been found to be ‘altered’ in old animals (Sharma, et al., 1978, 1980a, b; Rothstein, 1977, 1979). The common result of altered enzymes is a reduced specific activity of the enzyme based upon activity/unit of antiserum or activity/mg pure enzyme. The enzymes may become 'altered' by either errors in sequence or modifications after their synthesis and during 'cell life' (Sharma & Rothstein, 1980), Rothstein (1984) have provided substantial evidence that the modification involving only a change in conformation after synthesis is responsible for the observed alterations in properties of 'old' enzymes.

Previous studies from this laboratory from chick and rat brains showed that the acid DNase activity was highest during early embryonic stages and decreased during postnatal ages.
Chanda et al., (1975) showed similar type of results in rat cerebellum. Subsequent studies of Subrahmanyam (1989) on acid DNase in brain cells showed the presence of this enzyme in nuclei of neurons, astrocytes and oligodendrocytes in substantial amounts. The specific activities of acid DNase in nuclear fraction decrease with age in neurons. A decrease or increase in enzyme activity could be due to various reasons. The enzyme protein may be synthesized in altered amounts which means the protein synthetic machinery vis-a-vis the enzyme may be affected at the genetic level or at the translation level. Alternatively the enzyme protein synthesized may be the one that is, altered, either in its primary structure or in conformation due to post translational modification as a result of which the catalytic activity of the enzyme may be changed.

The present work deals with the purification of acid/NUV DNase from old rat brain, in order to gain some insight into the reasons for its decreased activity in aging rat brain. Some physico chemical properties of purified enzyme were studied. The results indicate that conformational change in the 'old' enzyme molecules may be the reason for the decreased specific activity.

MATERIALS AND METHODS:

The general materials and methodology for assay of acid/NUV DNase is described in Chapter II and III.
IMMUNOTITRATION:

The monospecific antibodies of pure young acid/NUV DNase (Fig. 23 & 24) was used to ascertain the levels of DNase at different ages by immunotitration. The acid/NUV DNase preparations were incubated with increasing amounts of antiserum at 4 C. The final volume of reaction was then brought to 0.5 ml with 0.01 M sodium acetate buffer pH 5.0. The incubation was carried out for 24 hrs after which the reaction mixture was centrifuged for 30 min. at 5000 xg. A control experiment was run simultaneously using non-immune serum.

PURIFICATION OF NUV/ACID DNase FROM OLD RAT BRAIN:

Brains of 2 year old albino rats were taken and homogenized in ice cold double distilled water containing 10 mM PMSF to make 10% homogenates. The procedure for enzyme extraction, purification, enzyme and protein assay was similar as described in Chapter II and III.

POLYACRYLAMIDE GEL ELECTROPHORESIS:

PAGE, SDS-PAGE, staining and destaining of the gel was carried out essentially as described in Chapter III.

DETERMINATION OF N-TERMINAL AMINOACID:

The N-terminal aminoacid of old rat brain DNase was
determined by the procedure of Chang (1983). The procedure was described in Chapter III.

RESULTS

Immunotitration of pure acid/NUV DNase obtained from young rat cerebrum against the antiserum prepared to young purified DNase was shown in Fig. 25. The results indicated that the pure young enzyme (2 µg) required 40 µl antiserum to inhibit 50% of its activity. The initial experiment was conducted to find out a suitable amount of antiserum which could be directly used for the immunotitration acid/NUV DNase activity present in brain homogenates of different ages.

Fig. 26 shows immunotitration of acid/NUV DNase activity in homogenates of young and old rat brains. The initial activity was adjusted to 5 units in both young and old samples before the addition of antiserum. These results point out that the old enzyme required more antiserum per unit of activity compared to young enzyme. In other words, the old enzyme required 370 µl of antiserum to inhibit 50% of its activity, whereas young enzyme required 130 µl of antiserum to inhibit 50% of its activity. These results suggest the presence of considerably higher concentration of enzyme antigen per unit of enzymatic activity in old compared to young enzyme. The acid/NUV DNase was purified from old rat brain by employing the same procedure followed for the purification of young enzyme.
Fig. 25 Immunotitration of purified acid DNase obtained from young rat cerebrum against the antiserum prepared against the same enzyme purified from young brain. A fixed amount of acid/NUV DNase (10 units) was mixed with different amounts of antiserum in 1 ml of 0.01 M sodium acetate buffer pH 5.0 and the mixture was incubated at 4°C for 24 hr. The immunocomplex was separated by centrifugation at 5,000 x g for 30 min. The acid/NUV DNase activity was assayed in supernatant.
Fig. 26 Immunotitrlation of acid DNase obtained from 'young' and 'old' rat brain homogenate against the antiserum prepared to young purified DNase. The acid/NUV DNase from both young and old brain homogenates were subjected to immunotitrlation by mixing with different amounts of young acid/NUV DNase antiserum and the mixtures were incubated for 24 hrs at 4°C. The initial activity was adjusted to 5 units in both 'young' and 'old' samples before the addition of antiserum. The acid/NUV DNase activity was determined in the supernatant. o-o 'young' acid/NUV DNase e-e old acid/NUV DNase (incubation time overnight at 4°C) x-x acid/NUV DNase activity in the presence of control serum and each point represents average of 3 experiments.
Fig. 27 shows the elution profile of old acid/NUV DNase from sephadex G-100. The elution profile is similar to that of young acid DNase (See Fig. 2, Chapter III). The active fractions were pooled and loaded on to the affinity column of DNA bound Ecteola-cellulose. Fig. 28 shows the elution profile of acid/NUV DNase from affinity column. The elution profile is similar to that of young enzyme (see Fig. 3 Chapter III). The active fractions were pooled and concentrated and used for further studies.

The purification schedule of old rat brain acid/NUV DNase was shown in Table 11. As can be seen 110 fold purification with ZV, yield was achieved. It may be noted that the specific activity of enzyme from old brain is only one fourth of that from young brain in the initial extracts, whereas after the affinity chromatography step the enzyme preparation from old brain exhibited only one eighth of the specific activity as that of corresponding young enzyme. It can be noted from Table 2 that the young enzyme showed a specific activity of about 4840 while purified preparation from old brain showed a specific activity of 614 only.

The final preparation showed a single band on SDS-polyacrylamide gel electrophoresis (Fig. 29). The molecular weight of old NUV DNase was found to be 62 KD (Fig. 30) by plotting Rf against the corresponding molecular weight of marker proteins on semi-log graph paper.
Fig. 27 Elution profile of 'old' acid/NUV DNase activity from Sephadex-G-100: The 95% ammonium sulphate precipitate containing the activity was dialysed and loaded on 1.5 Cm x 84 Cm column of sephadex G-100. The column was preequilibrated with 10 mM sodium acetate buffer pH 5.0 and developed with the same buffer at a flow rate of 15 ml per hour. 2 ml fractions were collected. Fractions 15 to 25 were pooled for further purification. Elution profile was monitored by taking OD at 280nm of each fraction (x-x) An aliquot of each fraction was taken for the enzyme assay (0-0) Activity with native DNA as substrate.
Fig 28 Elution profile of 'old' Acid/NUV DNase from DNA bound Ecteola cellulose affinity column: The active fractions from the sephadex G-100 column were loaded on 2 cm x 7 cm affinity column. The column was preequilibrated and developed with 10 mM sodium acetate buffer pH 5.0. The unbound proteins were washed out. The bound proteins were eluted by NaCl stepwise gradient in the buffer. The concentrations of NaCl used were 0.05, 0.1, 0.2 and 0.3M. Other details are shown in Fig.27.
Table - 11

Purification of Acid/RNase from 'old' Rat Brain

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol. (ml)</th>
<th>Units/ (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total pro.</th>
<th>Total act.</th>
<th>Sp. act</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain homogenate</td>
<td>320</td>
<td>50</td>
<td>8</td>
<td>2560</td>
<td>16000</td>
<td>6.2</td>
<td>100</td>
</tr>
<tr>
<td>60% Supernatant</td>
<td>270</td>
<td>35</td>
<td>2.5</td>
<td>675</td>
<td>9450</td>
<td>14</td>
<td>59</td>
</tr>
<tr>
<td>95% Ppt</td>
<td>30</td>
<td>102</td>
<td>6</td>
<td>180</td>
<td>3060</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>13</td>
<td>69</td>
<td>1.39</td>
<td>18</td>
<td>897</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>Affinity Chromatography</td>
<td>2</td>
<td>166</td>
<td>0.27</td>
<td>0.54</td>
<td>332</td>
<td>614</td>
<td>2</td>
</tr>
</tbody>
</table>

Specific activity is expressed as $\mu$g of acid soluble DNA-P liberated/2 hrs/mg protein. Other details are as in Table 2.
Fig. 29 10% SDS-PAGE.

Lane 1 to 4 Affinity Chromatography fraction
Lane 5 Sephadex G-100
Lane 6 Ammo. Sul. fraction.
Lane 7 Brain homogenate
Lane 8 Std. Proteins, BSA Egg albumin,
Glyceraldehyde-3-PHosphate dehydrogenase & Chymotrypsin.
Fig. 30  Molecular Weight Determination by SDS-PAGE: Proteins of known mol.wt. and the purified enzyme fraction were run on SDS-PAGE on separate lanes. Rf values of the proteins were calculated and plotted against the corresponding mol.wt. The mol.wt. of purified enzyme fraction band was determined from the plot by its Rf value.
**Fig. 31** N-terminal amino acid of pure old acid/NUV DNase was determined by using DABITIC method (Chang, 1983). The details are described in Materials and Methods.

**Fig. 31a** A line drawing of Fig 31.

(e) Internal marker (G) Glycine.
The N-terminal amino acid of old NUV/acid DNase was found to be glycine (Fig.31) by DABITIC method which is same as that observed with young enzyme.

Thermal inactivation studies were performed by assaying acid/NUV DNase activity at different temperatures. Acid/NUV DNase in 0.01 M sodium acetate buffer pH 5.0 from young (2-5 µg protein) and old (10-15 µg of protein) rat brain was incubated in a water bath at 55°C and 65°C and the enzyme activity was assayed at different time intervals. Initial activity of enzyme was adjusted to 10 units in both ages. Fig. 32 shows the effect of the incubation at 55 and 65°C on the stability of purified DNase. Pre-incubation of purified NUV DNase "young" or "old" at the temperatures mentioned for different time periods, resulted in considerable loss of activity. It is also clear from Fig. 32 that the loss of activity is more in case of old enzyme compared to young. Thus, it would appear that aging rat brain may contain partially denatured DNase molecules.

The inactivation of young and old acid/NUV DNase by pronase E (Streptomyces griseus) was shown in Fig. 33. The initial activity of acid/NUV DNase was adjusted to 100 units/100 µl for both the enzyme preparations. To this 1.5 units of pronase E (sp.acty 6 units/mg) was added and incubated in 10 mM Tris HCl pH 7.5 at 37°C. Aliquots of 10 µl were withdrawn at different time periods. Proteolysis was terminated by the addition of 4 µl of 0.01 M PMSF in acetonitrile and residual enzyme activity was determined. Fig. 33 shows the pronase E digestion studies of young and old
Fig 32 Effect of preincubation at different temperatures for varying periods on the purified acid/NUV DNase prepared from 'young' and 'old' rat brain. Each point represents the average of three experiments.
Fig. 33 Inactivation of DNase by pronase E: (○-○) 'young' DNase (●-●) old DNase. In the control experiment (enzyme with no protease present) there was no significant change in the activity over the indicated time period. Each point represents average of three experiments.
acid/NUV DNase. These results suggest that the old enzyme was more susceptible to pronase E digestion compared to its young counterpart.

**DISCUSSION:**

The acid/NUV DNase old rat brain was purified to apparent homogeneity, where pure enzyme showed a single band on SDS-polyacrylamide gel electrophoresis. The molecular weight of enzyme was calculated to be 62,000 daltons.

Throughout the purification schedule the enzymes from young and old brain behaved similarly in their elution pattern and mobility in electric field. These results revealed an important fact that young and old acid/NUV DNases are similar in size and charge. These results also indicated that there is no gross difference between primary structure of young and old acid/NUV DNase.

The N-terminal amino acid was found to be glycine in both young and old acid/NUV DNase preparations. This gives an idea that altered properties of old enzyme are not the results of 'proteolysis'. This gives an additional support that there are no major differences in the primary structures of acid/NUV DNase preparations obtained from young and old rat brain. If the error catastrophe theory of Orgel (1963) were to hold good, then it is to be expected that several wrong amino acid would be incorporated into the enzyme molecules in aging brain and should behave
differently with respect to various criteria mentioned above. Thus, the present observations do not support the error catastrophe theory.

However, the present data also show that the activity of acid/NUV DNase from aging brain is markedly decreased. In fact, the extent of reduction is so marked (85%) it is not known whether there is any other enzyme suffering loss of activity at this magnitude with age. So far, purified superoxide dismutase (Reiss et al., 1976) isocitrte lyase (Reiss and Rothsteln, 1975) seem to be the enzymes most affected during aging, and even here there was only a 60% decrease in the activity. The question then arises, "What is the cause for loss of enzyme activity in acid/NUV DNase molecules from aging brain? The answer to this question is provided, at least to some extent, by some of the immunological, thermal inactivation and protease digestion studies. Immunotitration experiments with young and old acid/NUV Dnase showed that the latter requires more antiserum per unit of activity (Fig.26). This suggest that the enzyme molecules synthesized in aging brain are catalytically "defective" and therefore can be considered as "altered" or different from the molecules elaborated by embryonic brain.

The next question that would arise is what is the "difference" or "alteration" that the acid/NUV DNase molecules from old rat brain suffer from as compared to the molecules synthesized by embryonic and developing brain. Thermal stability as well as protease digestion experiments provide some clues to this question. The old enzyme was more sensitive to heat. The
increase in thermolability of the enzymes in old age have been attributed to subtle post translational modifications of amino acid side chains and not to substitution of amino acids. The same pattern of findings were reported in case of enzymes of free living nematode, *Turbatrix aceti* (Sharma, 1976; Rothstein, 1979). A significant observation of the present investigation is that the old enzyme was also more susceptible than the young enzyme to neutral protease digestion (Fig. 33). It is increasingly becoming clear now that covalent post translational modification of a protein may serve as a marker/signal for proteases to act upon (Reviewed by Stadtman, 1990). This means such of those proteins in the cell that are covalently modified would serve as better substrates for the neutral proteases leading to the accelerated turnover of such proteins. If in aging tissue a number of proteins lose their biological activity due to post translational covalent modifications (as is the conclusion of present and several other earlier studies) it must also follow by a rapid degradation of such altered molecules by the neutral proteases. Indeed our experiments with pronase E adduce support for such a contention as the acid/NUV DNase from old brain was degraded more efficiently (Fig. 33). But then why an aging tissue should accumulate such altered molecules in spite of being better substrates for degradation? Either the concerned protein is synthesized much faster than the degradation or the proteolysis is slowed down in aging tissue as a result of decreased levels of proteases. Evidence is accumulating to supports the latter possibility as it is shown by Starke-Reed and Oliver (1989) that the intracellular accumulation of catalytically inactive or less
active forms of several enzymes which occurs during aging is correlated with an age dependent decrease in the intracellular levels of neutral-alkaline proteases.

Immunological, spectral, thermal and protease digestion studies on purified enolase from *Turbatrix aceti* by Sharma & Rothstein (1978, 1980a) provide substantial evidence to suggest that the enzyme from old organism suffers conformational change possibly due to post translational modification. The same workers also provide evidence for the conformational alteration in the phosphoglycerate kinase of aging rat liver and brain (Sharma et al., 1980b). The present studies with purified acid/NUV DNase from embryonic and old rat brain also point out conformational alteration due to post translational modifications of amino acids in enzyme molecules as a possible reason for the loss of activity in the old enzyme.

It is not, however clear today precisely what is the alteration or modification that makes the enzyme from old animals less active catalytically.

This should obviously form one of the future directions of aging research.