CHAPTER II
4.1.0.0. INTRODUCTION

In the first chapter it is demonstrated that either vit. E and/or Se deficiency induces oxidative stress in female albino rats. Vitamin E and/or Se deficiency also induced GSTs in lung and liver tissues significantly. In order to identify the isozymes of GSTs induced, GSTs were purified by affinity chromatography and further separated on SDS-PAGE. The subunit composition of GSTs, however, was analyzed on RP-HPLC.

Earlier differences in tissue distribution and expression of GST isozymes have been documented mainly by comparison of SDS-PAGE and immunoblotting (Tu et al., 1983; Hayes and Mantle, 1986). However, there were and still are problems with cross-reactivity of GST antibodies and quantification by immunoblotting. Development of an HPLC technique by Ostlund et al. (1987) significantly improved the ability to identify and quantify GST proteins, and has been used extensively (Ketterer et al., 1988, Hayes et al., 1990). In the present study the HPLC technique is modified so that all known subunits can be separated with essentially baseline resolution.

4.2.0.0. RESULTS

Female wistar strain albino rats at weanling stage were fed with -E+Se, +E-Se, -E-Se and +E+Se diets for a period of 13 weeks. After the dietary regimen, animals were killed and liver and lung tissues excised after perfusion. Cytosolic GSTs from tissues were purified by GSH linked agarose column chromatography. Fig. 26 represents the typical chromatogram of affinity purification of rat liver and/or lung cytosolic GSTs.

4.2.1.0. Hepatic GSTs
4.2.1.1. Affinity chromatography

Purification of GSTs by GSH-affinity chromatography was carried out five
Fig. 26: Affinity purification of rat liver and/or lung cytosolic GSTs

Absorbance at 280 nm

Activity with CDNB (μmoles/mL)

Fraction No. (3 mL each)
times separately for each group using pooled liver tissue (12 g) from respective groups. Table 5 to 8 shows the typical purification profiles of GSTs from liver tissues of -E+Se, +E-Se, -E-Se and +E+Se animals. As shown in the profiles, increase in specific activity was observed in liver crude extracts (10,000 X g) of +E-Se (49%) and -E-Se (77%) animals when compared to that of +E+Se animals. Similarly the specific activity levels in the cytosolic fractions (105,000 X g supernatant) of +E-Se and -E-Se animals also were much higher compared to +E+Se animals. As a result, the overall purification fold of +E-Se (33%) and -E-Se (43%) animals decreased when compared to -E+Se and +E+Se animals. This observed increase in the specific activity levels in the crude extracts as well as cytosolic fractions and decrease in the final fold purification are indicative of induction of GSTs in the liver tissue of +E-Se and -E-Se animals. Also the total affinity purified GST protein levels were much higher in +E-Se (50%) and -E-Se (77%) animals than the levels found in +E+Se animals. No appreciable differences in specific activity (37 U/mg protein) and % yield (~70%) were observed in the affinity purified liver GSTs of animals fed on vit.E and/or Se supplemented and deficient diets. The comparison of total activity, total protein and yield at the final step of liver GST purification for all the dietary groups is represented in table 9.

4.2.1.2. SDS-PAGE and western blot analysis of GSTs from cytosolic extracts

The affinity purified liver GSTs were resolved into 3 subunits on SDS-PAGE (Fig. 27, lane 1&6) with molecular weights of 25.6, 27 & 28 kDa, which were designated as Ya, Yb and Yc respectively, as per the nomenclature of Mannervik et al (1985). In order to determine whether increased GST activity in +E-Se and -E-Se was due to an increased amount of GST protein, equal amounts of liver cytosol protein (20 μg) was loaded and separated on SDS-PAGE (Fig. 27). Western blotting analysis was performed after electrophoresis using polyclonal antibodies raised against affinity purified liver GSTs (Fig. 28A), rat liver Yc (Fig. 28B), rat liver Yb (Fig. 28C) and rat liver Ya subunits (Fig. 28D).
### TABLE 5: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON -E+Se DIET

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 X g</td>
<td>837.5</td>
<td>2417</td>
<td>0.346</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>105,000 Xg</td>
<td>743.0</td>
<td>1279</td>
<td>0.581</td>
<td>88.73</td>
<td>1.68</td>
</tr>
<tr>
<td>Affinity pooled</td>
<td>563.6</td>
<td><strong>15.4</strong></td>
<td>36.6</td>
<td>67.3</td>
<td>105.8</td>
</tr>
</tbody>
</table>

Each value is the mean of five individual observations.
One unit is defined as one umole of thioether formed/min.

### TABLE 6: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON +E-Se DIET

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 X g</td>
<td>1152</td>
<td>2458</td>
<td>0.469</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>105,000 Xg</td>
<td>1035</td>
<td>1281</td>
<td>0.808</td>
<td><strong>89.8</strong></td>
<td>1.72</td>
</tr>
<tr>
<td>Affinity pooled</td>
<td>765.4</td>
<td>20.8</td>
<td><strong>36.8</strong></td>
<td><strong>66.4</strong></td>
<td>78.46</td>
</tr>
</tbody>
</table>

Each value is the mean of five individual observations.
One unit is defined as one umole of thioether formed/min.
TABLE 7: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON -E-Se DIET

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 Xg</td>
<td>1344.5</td>
<td>2409</td>
<td>0.558</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>105,000 Xg</td>
<td>1222.3</td>
<td>1268</td>
<td>0.964</td>
<td>91</td>
<td>1.728</td>
</tr>
<tr>
<td>Affinity pooled</td>
<td>919.7</td>
<td>24.79</td>
<td>37.1</td>
<td>66.31</td>
<td>66.48</td>
</tr>
</tbody>
</table>

Each value is the mean of five individual observations.
One unit is defined as one umole of thioether formed/min.

TABLE 8: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON +E+Se DIET

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 Xg</td>
<td>762.9</td>
<td>2422</td>
<td>0.315</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>105,000 Xg</td>
<td>693.6</td>
<td>1275</td>
<td>0.544</td>
<td>90.9</td>
<td>1.729</td>
</tr>
<tr>
<td>Affinity pooled</td>
<td>511.0</td>
<td>14</td>
<td>36.5</td>
<td>67.0</td>
<td>115.9</td>
</tr>
</tbody>
</table>

Each value is the mean of five individual observations.
One unit is defined as one umole of thioether formed/min.
TABLE 9: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON vit.E AND/OR Se SUPPLEMENTED AND DEFICIENT DIETS

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>-E+Se</td>
<td>564 ± 89*</td>
<td>15.4 ± 2.0*</td>
<td>36.6 ± 2.1*</td>
</tr>
<tr>
<td>+E-Se</td>
<td>765 ± 110b</td>
<td>20.8 ± 3.2b</td>
<td>36.8 ± 2.3a</td>
</tr>
<tr>
<td>-E-Se</td>
<td>920 ± 173b</td>
<td>24.8 ± 3.6b</td>
<td>37.0 ± 2.9*</td>
</tr>
<tr>
<td>+E+Se</td>
<td>511 ± 93*</td>
<td>14.0 ± 2.3*</td>
<td>36.5 ± 2.8*</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD of five individual observations. One unit is defined as one umole of thioether formed/min. The same alphabet denotes no significant difference between the two groups in each column. Significance was set at p < 0.05.
Fig. 27: SDS-PAGE of liver cytosolic proteins of rats fed on vit.E and/or Se supplemented and deficient diets

Lane: 1. 1 |ig liver affinity purified GSTs
2. 20 µg -E+Se liver cytosolic proteins
3. 20 µg +E-Se liver cytosolic proteins
4. 20 µg -E-Se liver cytosolic proteins
5. 20 µg +E+Se liver cytosolic proteins
6. 2 µg liver affinity purified GSTs
Fig. 28: Immunoblots of liver cytosol and affinity purified GSTs of rats fed on vit.E and/or Se supplemented and deficient diets separated on SDS-PAGE and probed with antibodies raised against

A. Rat liver affinity purified GSTs  B. Rat liver GST Yc subunit
C. Rat liver GST Yb subunit  D. Rat liver GST Ya subunit

Blot A to D
Lane 1. 1 μg rat liver affinity purified GSTs
Lane 2. 20 μg -E+Se rat liver cytosolic proteins
Lane 3. 20 μg +E-Se rat liver cytosolic proteins
Lane 4. 20 ng -E-Se rat liver cytosolic proteins
Lane 5. 20 μg +E+Se rat liver cytosolic proteins
Lane 6. 2 μg rat liver affinity purified GSTs
Figure 28A shows a clear induction of Yc and Ya subunits in -E+Se, +E-Se and -E-Se animals when cytosolic proteins separated on SDS-PAGE were probed with anti-affinity purified GST antibodies (compare lane 2,3,4 to lane 5). Similar results were observed when the proteins were probed with GST Yc antibody (Fig. 28B). Western blot analysis with GST Ya antibodies further showed the induction of Ya subunit in -E+Se, +E-Se and -E-Se animals, more so in -E-Se animals when compared to that of +E+Se animals (Fig. 28D). No significant changes were observed in Yb subunit (Fig. 28A&C).

4.2.1.3. SDS-PAGE analysis of affinity purified GSTs

In order to further characterize the GSTs, equal amounts (3 µg each) of affinity purified GSTs from vit.E and/or Se supplemented and deficient animals were separated on SDS-PAGE (Fig. 29). The relative concentration of subunits were analyzed by measuring subunit concentration on UVP-gel documentation system and also from the total protein recovered at the final step of purification (Fig. 30). The relative concentration of Ya subunit was significantly higher in liver tissues of -E+Se (34%), +E-Se (61%) and -E-Se (164%) animals compared to that of +E+Se animals. Further Yc subunit was also induced in -E+Se (24%), +E-Se (57%) and -E-Se (62%) animals when compared to that of +E+Se animals (compare lanes 2,3&4 with 1 in Fig. 30).

4.2.1.4. Reverse-phase HPLC analysis of affinity purified GSTs

In order to further quantify the specific induction of GST subunits during vit.E and/or Se deficiency, equal amounts of affinity purified GSTs were separated on reverse-phase HPLC (Fig. 31A,B,C&D). Individual peaks obtained were identified based on their order of elution as per the profile given by Johnson et al (1992) and were confirmed by comparison of HPLC profiles of individual isozymes and SDS-PAGE analysis. When relative subunit concentrations were compared, Ya2 was the major Ya-sized subunit in the rat liver, which was significantly higher in -E+Se (1.43 fold), +E-Se (2.42 fold) and -E-Se (3 fold)
Fig. 29: **SDS-PAGE** of liver and lung affinity purified GSTs of rats fed on vitE and/or Se supplemented and deficient diets

Lane 1 to 4 corresponds to liver GST proteins (3 μg each) purified from 1. +E+Se 2. -E-Se 3. +E-Se 4. -E+Se animals

Lane 5 to 8 corresponds to lung GST proteins (3 μg each) purified from 5. +E+Se 6. -E-Se 7. +E-Se 8. -E+Se animals
Fig. 30: Relative subunit concentration of liver affinity purified GSTs of rats fed on vit.E and/or Se supplemented and deficient diets separated on SDS-PAGE

Values represent mean + SD of five individual experiments. Data was analyzed with a one way ANOVA followed by SNK test Bars labelled with same letter are not significantly different from one another (p < 0.05).
Fig. 31: RP-HPLC analysis of affinity purified liver GSTs of (A)-E+Se (B)+E-Se (C)-E-Se (D)+E+Se rats

Equal protein (100 \( \mu g \)) was loaded to RP-HPLC

Column: ODS (Waters)
Solvent: A. 35% Acetonitrile + 0.1% TFA
   B. 85% Acetonitrile + 0.1% TFA
Flow rate: 1 mL/min
Detection: 214 nm
Fig. 32: Relative subunit concentration of liver affinity purified
GSTs of rats fed on vit.E and/or Se supplemented and
deficient diets separated on RP-HPLC

Bars represent mean ± SD of five individual experiments. Data was analyzed with a one way ANOVA
followed by SNK test. Bars labelled with same letter are not significantly different from one another (p ≤ 0.05).
animals (Fig. 32). Similarly \( Y_c \) was also induced in all deficient animals by 1.2 fold in \(-E+Se\), 1.63 fold in \(+E-Se\), and 1.82 fold in \(-E-Se\) animals compared to that of \(+E+Se\) animals. \( Y_{a1} \) subunit was induced only in \(-E-Se\) (1.78 fold) animals (Fig. 32C).

4.2.2.0. Lung GSTs

4.2.2.1. Affinity chromatography

Like liver GSTs, lung GSTs were also purified from equal quantities (16 g) of pooled tissue from vit.E and/or Se deficient and supplemented animals by affinity chromatography for 3 times. The data was presented in tables 10, 11, 12 & 13. Comparison of specific activities during the first step of purification (crude extract of 10,000 X g supernatant) indicated increase in the specific activities in \(-E+Se\) (33%), \(+E-Se\) (64%) and \(-E-Se\) (76%) animals compared to the level observed in \(+E+Se\) animals. The purification fold was decreased by 16% in \(-E+Se\), 40.12% in \(+E-Se\) and 39.13% in \(-E-Se\) animals. Further total GST protein obtained after affinity purification in lung tissues of \(-E+Se\) (48.5%), \(+E-Se\) (93%), and \(-E-Se\) (98%) animals were significantly higher when compared to the protein levels obtained for \(+E+Se\) animals. All these observations demonstrate the induction of lung GSTs in vit.E and/or Se deficient animals; no significant differences were observed in specific activity (17 U/mg protein) and % yield (~90%) of affinity purified lung GSTs of vit.E and/or Se supplemented and deficient diets. Comparison of total activity, total protein and yield of affinity purified lung GSTs for the vit.E and/or Se supplemented and deficient diets was presented in table 14.

4.2.2.2. Western blot analysis of cytosolic extracts

The affinity purified GSTs were resolved into 4 subunits on SDS-PAGE with molecular weights of 24, 25, 27 and 28 kDa, which were designated as \( Y_p \), \( Y_k \), \( Y_b \) and \( Y_c \) respectively, based on their molecular weights (Fig. 33). The subunit induction was analyzed by separating equal amounts (40 \( \mu g \) each) of
TABLE 10: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON -E+Se DIET

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 X g</td>
<td>15.56</td>
<td>1765</td>
<td>0.008</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>105,000 Xg</td>
<td>15.10</td>
<td>1019</td>
<td>0.015</td>
<td>97.02</td>
<td>1.805</td>
</tr>
<tr>
<td>Affinity pooled</td>
<td>14.30</td>
<td>0.815</td>
<td>17.61</td>
<td>92.24</td>
<td>2147</td>
</tr>
</tbody>
</table>

Each value is the mean of three individual observations.
One unit is defined as one \( \mu \text{mole} \) of thioether formed/min.

TABLE 11: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON +E-Se DIET

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 X g</td>
<td>19.9</td>
<td>1803</td>
<td>0.011</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>105,000 X g</td>
<td>18.45</td>
<td>2025</td>
<td>0.014</td>
<td>92.68</td>
<td>1.27</td>
</tr>
<tr>
<td>Affinity Pooled</td>
<td>17.92</td>
<td>1.062</td>
<td>16.80</td>
<td>90.0</td>
<td>1527</td>
</tr>
</tbody>
</table>

Each value is the mean of three individual observations.
One unit is defined as one \( \mu \text{mole} \) of thioether formed/min.
TABLE 12: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON -E-Se DIET

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 X g</td>
<td>21.75</td>
<td>1834</td>
<td>0.012</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>105,000 Xg</td>
<td>20.43</td>
<td>1121</td>
<td>0.018</td>
<td>93.92</td>
<td>1.54</td>
</tr>
<tr>
<td>Affinity pooled</td>
<td>18.36</td>
<td>1.07</td>
<td>17.1</td>
<td>90.67</td>
<td>1554</td>
</tr>
</tbody>
</table>

Each value is the mean of three individual observations.
One unit is defined as one umole of thioether formed/min.

TABLE 13: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON +E+Se DIET

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 X g</td>
<td>11.25</td>
<td>1677</td>
<td>0.007</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>105,000 Xg</td>
<td>10.35</td>
<td>1030</td>
<td>0.01</td>
<td>92.03</td>
<td>1.49</td>
</tr>
<tr>
<td>Affinity pooled</td>
<td>9.39</td>
<td>0.549</td>
<td>17.11</td>
<td>88.45</td>
<td>2553</td>
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</tbody>
</table>

Each value is the mean of three individual observations.
One unit is defined as one umole of thioether formed/min.
TABLE 14: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON vit.E AND/OR Se SUPPLEMENTED AND DEFICIENT DIETS

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity units (U)</th>
<th>Total protein (mg)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>-E+Se</td>
<td>14.3± 1.6*</td>
<td>0.82 ± 0.08*</td>
<td>17.6 ± 1.6*</td>
</tr>
<tr>
<td>+E-Se</td>
<td>17.92 ± 2.0*</td>
<td>1.06 ± 0.12*</td>
<td>16.8 ±1.9*</td>
</tr>
<tr>
<td>-E-Se</td>
<td>18.36 ± 2.9*</td>
<td>1.07 ± 0.11*</td>
<td>17.1 ± 2.0*</td>
</tr>
<tr>
<td>+E+Se</td>
<td>9.39 ± 1.3b</td>
<td>0.55 ± 0.07b</td>
<td>17.1 ± 1.8*</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD of three individual observations. One unit is defined as one umole of thioether formed/min. The same alphabet denotes no significant difference between the two groups in each column. Significance was set at p < 0.05.
cytosolic proteins from -E+Se, +E-Se and -E-Se and +E-Se animals on SDS-PAGE (Fig. 33) and probing them with polyclonal antibodies raised against affinity purified lung GSTs (Fig. 34A), lung Yc (Fig. 34B), lung Yb (Fig. 34C), lung Yk (Fig. 34D) and lung Yp subunits (Fig. 34E). Comparison of lanes 2,3&4 with that in lane 5 of Fig. 34A&B clearly showed an induction of Yc subunit in vit.E and/or Se deficient animals. No significant changes, however, were observed in Yb, Yk and Yp subunits in vit.E and/or Se deficient animal tissues compared to that of supplemented animals (Fig. 34A,C,D&E).

4.2.2.3. SDS-PAGE and RP-HPLC analysis of affinity purified GSTs

Equal amounts (3 µg) of affinity purified lung GSTs from vit.E and/or Se deficient and supplemented animals were separated on SDS-PAGE (Fig. 29) and relative subunit concentrations were analyzed based on UVP-gel documentation data and from the total protein recovered at the final step of affinity purification (Fig. 35). A significant induction of Yc subunit was observed in -E+Se (2.84 fold), +E-Se (4.82 fold) and -E-Se (7.48 fold) animals in comparison with +E+Se animals (compare lanes 6,7&8 with 5 in Fig. 35). No significant changes, however, were observed in Yb, Yk and Yp subunits.

In order to verify the specific induction of subunits in vit.E and/or Se deficient tissues, equal amounts (100 µg) of affinity purified GSTs were separated on RP-HPLC using Waters C18 analytical column (Fig. 36 A,B,C&D). When relative subunit concentrations were analyzed, a significant induction of Yc subunit was observed in -E+Se (2.9 fold), +E-Se (4.86 fold) and -E-Se (7.76 fold) animals when compared to that of +E+Se animals (Fig. 37).

Thus SDS-PAGE, immunoblots and RP-HPLC analysis of affinity purified GSTs have clearly demonstrated the induction of Yc2 and Yc subunits in liver and Yc only in lung tissue of vit.E and/or Se deficient animals when compared to those in +E+Se animals.
Fig. 33: **SDS-PAGE** of lung cytosolic proteins of rats fed on vit.E and/or Se supplemented and deficient diets

**Lane:**
1. 1 μg lung affinity purified GSTs
2. 40 μg -E+Se lung cytosolic proteins
3. 40 μg +E-Se lung cytosolic proteins
4. 40 μg -E-Se lung cytosolic proteins
5. 40 μg +E+Se lung cytosolic proteins
6. 3 μg liver affinity purified GSTs
Fig. 34: **Immunoblots** of lung cytosol and **affinity** purified GSTs of rats fed on vit.E and/or Se supplemented and deficient diets separated on SDS-PAGE and probed with antibodies raised against

A. **Rat** lung affinity purified GSTs  
B. **Rat lung** GST **Yc subunit**  
C. **Rat lung** GST **Yb subunit**  
D. **Rat lung** GST **Yk subunit**  
E. **Rat lung** GST **Yp subunit**

**Blot A**

Lane 1. 2 ug rat lung affinity purified GSTs  
Lane 2. 40 ug -E+Se rat lung cytosolic proteins  
Lane 3. 40 μg +E-Se rat lung cytosolic proteins  
Lane 4. 40 μg -E-Se rat lung cytosolic proteins  
Lane 5. 40 ug +E+Se rat lung cytosolic proteins  
Lane 6. 1 ug rat liver affinity purified GSTs

**Blot B to D**

Lane 1. 2 μg rat lung affinity purified GSTs  
Lane 2. 40 ug -E+Se rat lung cytosolic proteins  
Lane 3. 40 μg +E-Se rat lung cytosolic proteins  
Lane 4. 40 ug -E-Se rat lung cytosolic proteins  
Lane 5. 40 μg +E+Se rat lung cytosolic proteins  
Lane 6. 2 μg rat lung affinity purified GSTs
Fig. 35: Relative subunit concentration of lung affinity purified GSTs of rats fed on vit.E and/or Se supplemented and deficient diets separated on SDS-PAGE

Bars represent mean ± SD of three individual experiments. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p ≤ 0.05).
Fig. 36: RP-HPLC analysis of affinity purified lung GSTs of (A)-E+Se (B)+E-Se (C)-E-Se (D)+E+Se rats

Equal protein (100 μg) was loaded to RP-HPLC

Column: ODS (Waters)
Solvent: A. 35% Acetonitrile + 0.1% TFA  
B. 85% Acetonitrile + 0.1% TFA
Flow rate: 1 ml/min
Detection: 214 nm
Fig. 37: Relative subunit concentration of lung affinity purified GSTs of rats fed on vit. E and/or Se supplemented and deficient diets separated on RP-HPLC

Bars represent mean ± SD of five individual experiments. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p ≤ 0.05).
4.3.0.0. DISCUSSION

Vitamin E and Se deficiency is known to induce oxidative stress and simultaneously induce alternative antioxidant defenses, specifically glutathione-dependent defenses including GSTs (Mehlert and Diplock, 1985; Kim and Comb, 1993). In the present study also, induction of GSTs was observed in response to vit.E and/or Se deficiency (Chapter II, Fig. 25). However, it is not clear whether the increase in GST activity is due to an increased amount of GST protein or the activation of the enzyme systems. To unravel this, GSTs were purified by affinity chromatography and individual subunits were separated on SDS-PAGE and RP-HPLC.

GST activity is increased in many organisms following exposure to foreign compounds. From studies of rodents, the adaptive response to chemical stress is clearly pleiotropic in character and involves the induction of many drug-metabolizing enzymes (Spencer et al., 1991; Hayes et al., 1993; Borroz et al., 1994). Collectively, these detoxification enzymes provide protection against a diverse spectrum of harmful compounds. Evidences suggest that, besides providing protection against chemicals of foreign origin, GSTs are involved in the protection against oxidative stress. It has been reported that in Selenium- and Copper deficient rats, which are chronically exposed to increased intracellular levels of hydrogen peroxide due to lack of Se-GSH Px, marked over-expression of hepatic GST isozymes is observed (Arthur et al., 1987).

Rat GST subunits are preferentially induced by various drugs, including carcinogens like 3’-methyldiaminobenzene, 2-acetylaminofluorene (Kitahara et al., 1984) and trans-stilbene oxide (Tahir et al., 1989) and anticarcinogenic agents such as BHA (Kitahara et al., 1984) and ethoxyquin (Kensler et al., 1986).

4.3.1.0. Structural characterization of liver GSTs

In the present study oxidative stress induced by vit.E and Se deficiency resulted in the induction of hepatic GSTs, particularly Ya and Yc subunits.
containing GSTs (Fig. 28). Therefore, increased GST protein appears to be responsible for the total elevation in GST activity with the classical substrate, CDNB. Similar induction of hepatic GSTs in Se deficiency was reported by Chang et al (1990) and Christensen et al (1994). The transcriptional activation of the GST genes may account for this increase in GST subunit levels. Chang et al (1990) proposed that an accumulation of peroxides during Se-deficiency might possibly bind to the corresponding receptor and the receptor-ligand complex would presumably interact with chromatin to activate the corresponding genes.

The liver Ya subunit was resolved into 2 different peaks, Ya1 and Ya2 on reverse-phase HPLC. In the present study Ya2 is the major form of GSTs observed in the hepatic tissue of +E+Se animals (Fig. 31D). This observation is quite contradictory to the studies reported earlier for albino rats (Hayes et al., 1990), where Ya1 was shown to be the major form of Ya GSTs. It is not clear why Ya2 was the more abundant form in the present study. One difference between the present work and the previous reports is the use of female rats instead of male rats. These subunits seem to have different inducibility. Ya2 is much more inducible than Ya1 in liver tissue of vit.E and Se deficient animals (Fig. 31A,B&C).

Lai et al (1984) for the first time isolated a full length clone of Ya, pGTR 261, which encompasses the cDNA described by Kalinyak and Taylor (1982). Further Ya subunit which was deduced from the clone cDNA pGTB 38 was described by Pickett et al (1984). The subunits encoded by pGTB 38 and pGTR 261 show considerable sequence homology and over their 222-amino-acid length show residue differences in only eight positions. At these positions (residues 31, 34, 96, 107, 117, 206, 207 and 219) pGTB 38 encodes glutamic acid, leucine, serine, isoleucine, arginine, proline, alanine and valine respectively, while pGTR 261 encodes aspartic acid, phenylalanine, threonine, methionine, lysine, leucine, proline and isoleucine at the respective positions. Hayes et al (1990) showed sequence data for Ya that include five of the eight 'difference residues' (namely 31, 34, 96, 107 and 117) and at each of these positions Ya1 shows sequence
identity with pGTR 261. Ya\textsubscript{2} also includes five of the eight different residues (namely 31, 34, 107, 117 and 219), and at all of these positions Ya\textsubscript{2} contains the amino acids that would be predicted by pGTB 38. This provides the proof for the existence of multiple Ya-type subunits at the protein level. Hayes \textit{et al} (1990) also reported that Ya\textsubscript{2} subunit was over-expressed in liver bearing aflatoxin-induced preneoplastic nodule, which was appear to be an adaptive response to aflatoxin exposure.

Waxman \textit{et al} (1992) used gene specific oligonucleotide probes to monitor the expression of individual GST mRNA in liver and kidney of adult rats treated with different drugs. The study provided an unambiguous discrimination between the closely related GST Ya\textsubscript{1} and Ya\textsubscript{2} mRNA and have shown differential induction of these messages by different agents. They can not be distinguished at the RNA level using conventional cDNA probes (Lai \textit{et al}., 1984; Pickett \textit{et al}., 1984) or by immunoprecipitation analysis (Pemble \textit{et al}., 1986). GST Ya\textsubscript{1} is the major Ya form expressed in kidney while Ya\textsubscript{2} is induced in response to dexamethasone or phenobarbital treatment without any changes in Ya\textsubscript{1}. Important differences were also observed between the responses of these two GST class a mRNA to cisplatin, which suppressed Ya\textsubscript{1} without any effect on Ya\textsubscript{2} levels. Our previous studies (Veera Reddy \textit{et al}., 1995) have revealed that Ya\textsubscript{1} subunit is induced in response to exercise training in female rats.

Regulation of GST expression is a very complex process. They are subjected to developmental control, with tissue and sex specific expression. They are responsive to physiological stress and are inducible by many drugs and chemicals. The GSTA1 (Ya\textsubscript{1}) and GSTA2 (Ya\textsubscript{2}) subunits in rodent liver are markedly inducible by drugs. The rat GSTA2 gene appears to contain at least four cis-acting elements in the 5’-flanking region that respond to xenobiotics. One of these elements is responsible for induction by PAH and is identical to the xenobiotic-responsive-element (XRE) found in the rat CYPIA1 gene (Rushmore \textit{et al}., 1990). A second element on the rat GSTA2 gene has been designated as the antioxidant-responsive element (ARE) because it mediates responsiveness to
phenolic antioxidants (Rushmore et al., 1991). The third element identified in this gene is identical to the glucocorticoid-responsive element (GRE) and may render expression of GSTA2 responsive to dexamethasone (Rushmore et al., 1993). A fourth element exists in rat GSTA2 which is responsible for the induction of this gene by barbiturates. The activation of transcriptional Ya gene through ARE by H$_2$O$_2$ was clearly shown by Rushmore et al (1990). β-Naphthoflavone, a planar aromatic compound activates the gene through either XRE or ARE, but the presence of Ah receptors and metabolism of β-naphthoflavone by cytochrome P-450 IA are required for its transcriptional activation. On the other hand, t-butyl hydroquinone, a phenolic antioxidant, activates through ARE, independently of Ah receptors or cytochrome P-450 IA1. In the presence of Ah receptors, XRE reacts with 2,3,7,8-tetrachlorodibenzo-p-dioxin, but ARE does not. These results support the proposal by Spencer et al (1991) that phase II drug metabolizing enzymes, including GSTs are induced by monofunctional and bifunctional inducers by different mechanisms and that induction by mono functional inducers such as t-butyl hydroxyquinone is mediated by an electrophilic signal independently of Ah receptor. The difference between ARE and XRE is their capacity to induce phase I (cytochrome P450) gene expression. The XRE mediates induction of both phase I and phase II enzymes while ARE mediates induction of only phase II enzymes (Belinsky and Jaiswal, 1993). The induction of phase I enzymes by XRE element in response to xenobiotics may cause increased risk of mutagenecity and carcinogenecity as the products of xenobiotics activated by phase I enzymes increase the oxidative damage to DNA and membranes. The ARE-mediated phase II enzymes, on the other hand, detoxify the XRE-activated xenobiotics. Therefore, the ARE mediated increase in phase II enzymes is the safety way to stimulate the chemoprotective power of the cells.

The observed induction of Ya$_2$ subunit in the present study (Fig. 31A,B&C) like the induction of Ya GST in Se deficiency (Chang et al., 1990), may be the livers response to rising hydroperoxide levels or free radicals when antioxidant systems are impaired due to vit.E and Se deficiency. Thus from studies
on affinity purification, western blot of crude cytosolic fractions, subunit concentration on SDS-PAGE and RP-HPLC, it is concluded that \textbf{Ya}_2 and \textbf{Yc} subunits are induced in liver tissue in response to vit.E and Se deficiency. The induction of \textbf{Ya}_2 subunit in vit.E and Se deficiency states might be regulated by ARE.

\subsection*{4.3.2.0. Structural characterization of lung GSTs}

In comparison to liver, there is limited information on lung GSTs and their involvement in oxidative stress. In the present study lung GSTs resolved into four bands on SDS-PAGE, which were designated as \textbf{Yc}, \textbf{Yb}, \textbf{Yk} and \textbf{Yp} (Fig. 33). Further separation of affinity purified GSTs on RP-HPLC showed \textbf{Yb}_1, \textbf{Yb}_2, \textbf{Yp}, \textbf{Yc} and \textbf{Yk} among which \textbf{Yp} is the major subunit (Fig. 36). Both a- and u- class GSTs are found in low concentrations as reported by Cossar \textit{et al.} (1990) and Coursin \textit{et al.} (1992). Rat lung GST 7-7 is immunologically identical to the rat \textit{placental} form GST-P (Robertson \textit{et al.}, 1986).

Lung is the first and foremost tissue exposed to airborne toxicants and pollutants. Also it is more vulnerable to oxidative stress compared to other tissues. Lung is equally well equipped with antioxidant defenses, though not to the extent observed in liver. This is evident by an increase in lipid peroxides and GSSG levels in response to vit.E and Se deficiency (Fig. 18&21).

It is noteworthy that \textbf{Ya} which shows a high inducibility in liver is absent in lung. The lower recovery of liver vs lung GSTs (70% vs 90%) may be due to some of the isozymes specific to liver were not completely bound to affinity column or may be due to higher flow through loss (Table 8vs13). In the present study vit.E and Se deficiency significantly induced \textbf{Yc} subunit (7.76 fold) of a class in the lung tissue (Fig. 37). The increased GST activity might be aimed to compensate for the loss of Se-GSH Px during Se deficiency and to improve antioxidant defenses which were impaired due to vitamin E deficiency.
From these studies it is concluded that GSTs exhibit tissue-specific expression. After purification, western blot analysis of cytosolic proteins, subunit concentration on SDS-PAGE and RP-HPLC it is concluded that $\text{Ya}_2$ and $\text{Yc}$ subunits are induced significantly in liver and $\text{Yc}$ subunit in lung in response to oxidative stress induced by vit.E and/or Se deficiency. The results demonstrate that $\text{Ya}_1$ and $\text{Ya}_2$ have differential inducibility, $\text{Ya}_2$ being more inducible than $\text{Ya}_1$. However it is not clear whether the GSTs induced in liver and lung tissues in response to vit.E and/or Se deficiency are involved in any antioxidant protection. Further studies on their functional characterization will throw more light on the participation of GSTs in cellular antioxidant defenses.