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SHORT COMMUNICATION

Effect of Reduced Glutathione and Oxidised Glutathione on the Appearance of Multiple Forms of Glutathione S-transferases in Hevea brasiliensis *Latex*

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Glutathione S-transferase in latex of Hevea brasiliensis *appears to be a single protein containing presumably several labile disulphide bonds. Partial cleavage of these bonds occurs when the enzyme is eluted through a DEAE-Sephadex column in the presence of a sodium chloride gradient thus resulting in multiple forms. Glutathione but not oxidised glutathione (GSSG) in the eluting medium enhances the reduction process during ion-exchange chromatography. Reduced forms of the enzyme when dialysed free of sodium or sodium chloride plus the reducing agent after ion-exchange chromatography result in the formation of a major oxidised form with an isoelectric point similar to that of the crude enzyme.*

The glutathione (GSH) S-transferases [E.C. 2.5.1.18] are a group of enzymes that catalyse the conjugation of GSH on to a variety of substrates with suitable electrophilic centres in them[1]. These enzymes have been extensively studied in rat liver supernatant where at least six forms have been separated on CM-cellulose columns which were labelled in the inverse order of their elution as transferases *AA, A, B, C, D* and *E*. Each of these enzymes has an approximate molecular weight of 50 000 daltons and is made up of two sub-units[2–5]. At least four different types of sub-units can be identified among the transferases. They are referred to as *Ya, Yc, Yb* and *Yb'* of molecular weights 22 000, 22 500, 22 500 and 23 500 respectively. Although GSH S-transferases *A, AA, B* and *C* are known to correspond to protein dimers of *YbYb, YcYc, YaYc* and *YbYb'* respectively, the nature of transerases *D* and *E* are still not clear[6–13].

Recently an unusual form of GSH S-transferase that appeared homogenous on gel filtration and on isoelectric focusing columns but resolved into multiple forms when eluted through a DEAE-Sephadex anion-exchange column was reported[16]. Balabaskaran and Muniandy[16] had speculated that multiplicity in this case was probably due to partial transformation of the disulphide form of the enzyme to several thiol forms. Evidence in support of this theory is presented in this paper.

MATERIALS AND METHODS

Preparation of Crude Enzyme

The crude enzyme was prepared as described previously[16] except that the lyophilisation step was omitted. The dialysate obtained was used directly for chromatography.

Enzyme Assay

GSH S-transferase activity was assayed spectrophotometrically as described before[16].

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Ion-exchange Chromatography

A column of DEAE-Sephadex A-50 (2.5 × 12.5 cm) was equilibrated with 20 mM tris-HCl buffer, pH 6.0. A sample of the enzyme previously dialysed against this buffer was loaded and eluted first with it. Bound enzyme was eluted with 500 ml of a 0 – 0.5 M sodium chloride gradient in the same buffer. In experiments where the column was eluted with either reduced GSH or oxidised glutathione (GSSG), these were always present in the eluting buffer at 1 mM concentrations. Enzyme activity and protein content in the eluted fractions were estimated as described before[16].

Isoelectric Focusing

These studies were carried out as described previously[16] in a sucrose density gradient (0% – 50% weight/volume) super-imposed with an ampholine pH gradient (1% weight/volume) ranging from pH 3.5 to pH 10 at a constant voltage of 300 V for 48 h at 5°C.

RESULTS AND DISCUSSION

It was reported earlier[16] that GSH S-transferase from the latex of *Hevea brasiliensis* behaved anomalously in that although the enzyme appeared homogenous on isoelectric focusing as well as on gel filtration, it appeared in multiple forms when eluted through DEAE-Sephadex anion-exchange chromatography. This phenomenon was not only true with a solution of the lyophilised powder but also with a 50% – 80% ammonium sulphate fractionated enzyme prepared from a solution of the lyophilised powder. To ensure that lyophilisation

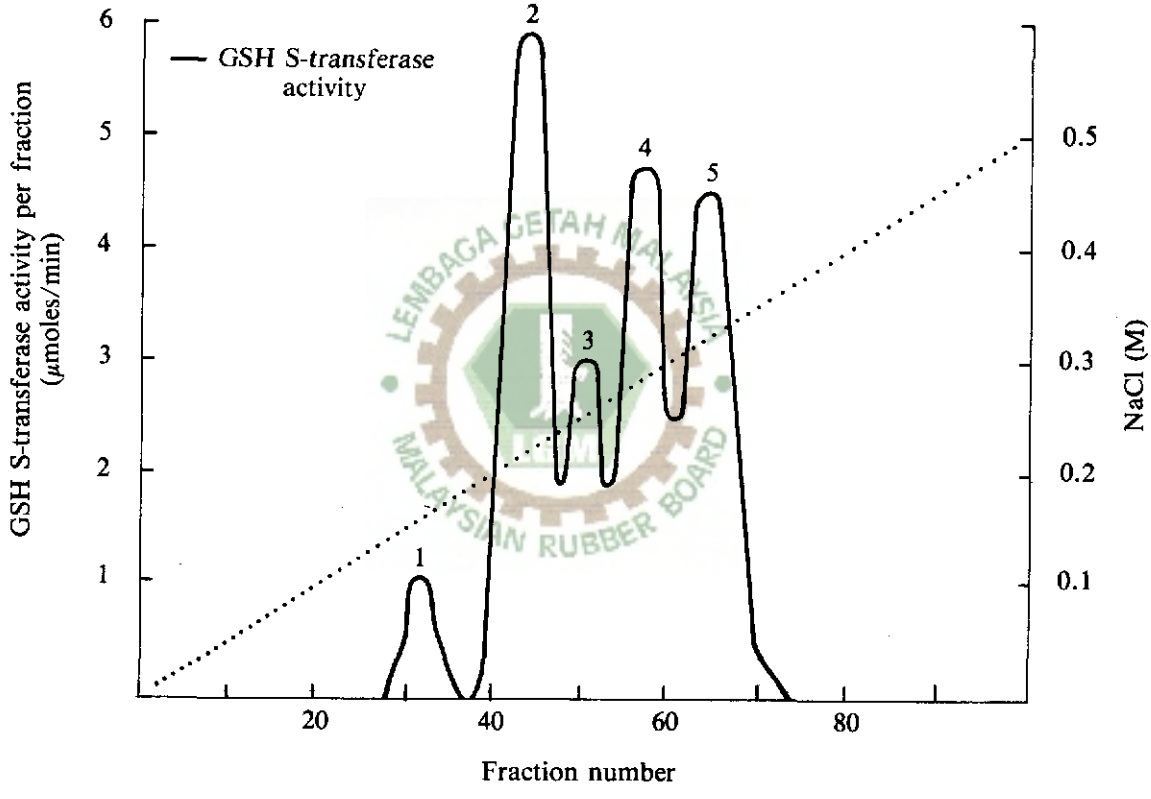

Figure 1. Elution profile of the enzyme (82 mg protein, 65 μmoles/min of activity) eluted with a sodium chloride gradient of 0–0.5 M.

was not a contributory factor to the presence of multiple forms, the lyophilisation step was omitted and instead the crude supernatant was used immediately for chromatography. When such crude supernatants were eluted through a DEAE-Sephadex column using a 0 - 0.5 M sodium chloride gradient, five peaks were again eluted at approximately similar sodium chloride concentrations *(Figure 1)*. Since these results were consistent and reproducible they are indicative of the presence of different negatively-charged forms of the enzyme. These would be eluted in the order of their net negative charge with the most negatively-charged form being eluted last.

It is unlikely that these charged forms could have been present in the original crude supernatant as they would have resolved into multiple forms on isoelectric focusing experiments. The fact that only a single form with a pI of 4.3 was detected on isoelectric focusing studies of a crude supernatant *(Figure 2)* confirms the above assumption. Further when the five peaks resolved on the DEAE-Sephadex column were pooled, dialysed and subjected to isoelectric

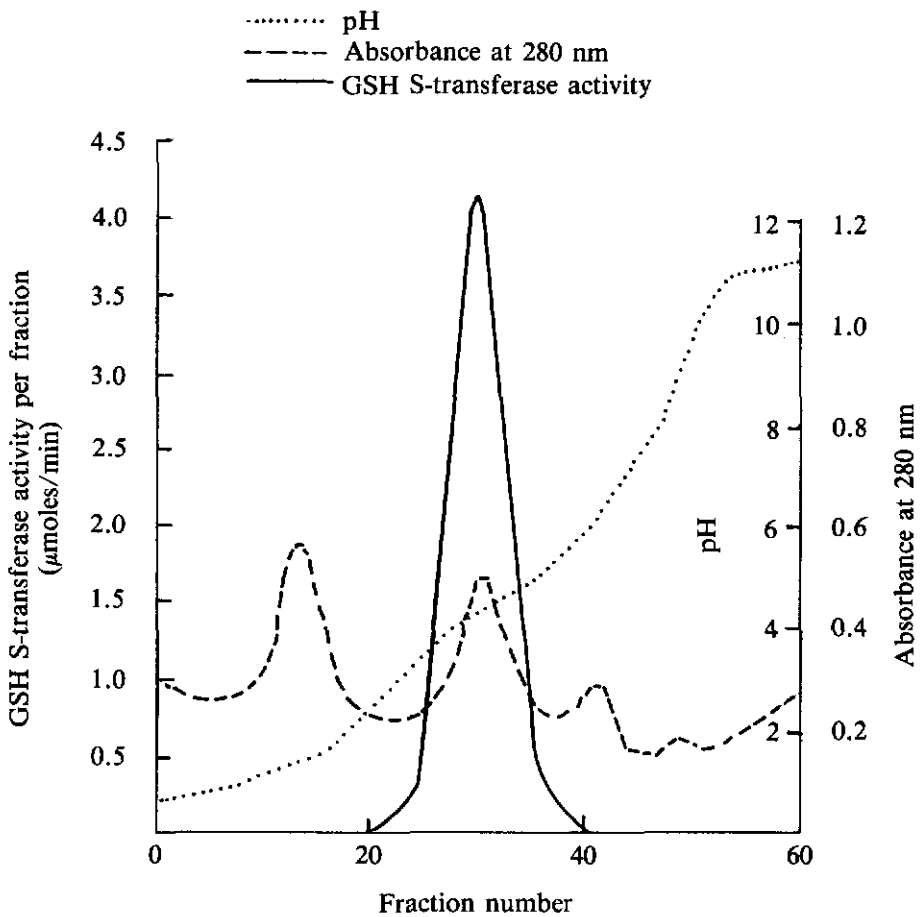

Figure 2. A solution of the crude enzyme containing 8.0 mg protein was electro-focused for 48 h. Fractions were eluted manually and the pH of each fraction was determined at 5°C. Protein contents were determined by the absorbance at 280 nm while GSH S-transferase activity was measured with 100 μlitre aliquots.

focusing, a single peak with a similar pI as reported above was again obtained (data not included). It is possible that the multiple forms could have been created on the DEAE column during the elution procedure by the sodium chloride gradient, resulting in various negatively-charged forms of the enzyme. This would also explain why a single form was obtained on isoelectric focusing studies when the five peaks obtained from the DEAE column were pooled, dialysed free of sodium chloride and electro-focused, as the removal of sodium chloride allowed the multiple forms to return to the native state.

Interestingly enough this multiplicity could be reduced to almost a single form when the DEAE column was eluted with a similar sodium chloride gradient but containing 1.0 mM of reduced GSH *(Figure 3)*. The sodium chloride concentration required to elute this peak was similar to that required to elute *Peak 5* in the normal run *(Figure 1)*. This would suggest that they both must have similar net negative charge. Since GSH like all other thiol reagents is known to reduce disulphide bonds in proteins, it appears that this reagent has further reduced the partially reduced forms generated during elution from the DEAE column to a major negatively-charged form. It is not known if this form represents a completely reduced form and neither can it be said that small amounts of the less negatively-charged forms seen in the normal run *(Figure 1)* are totally absent in this study. The effect of reduced GSH on the transformation of the multiple

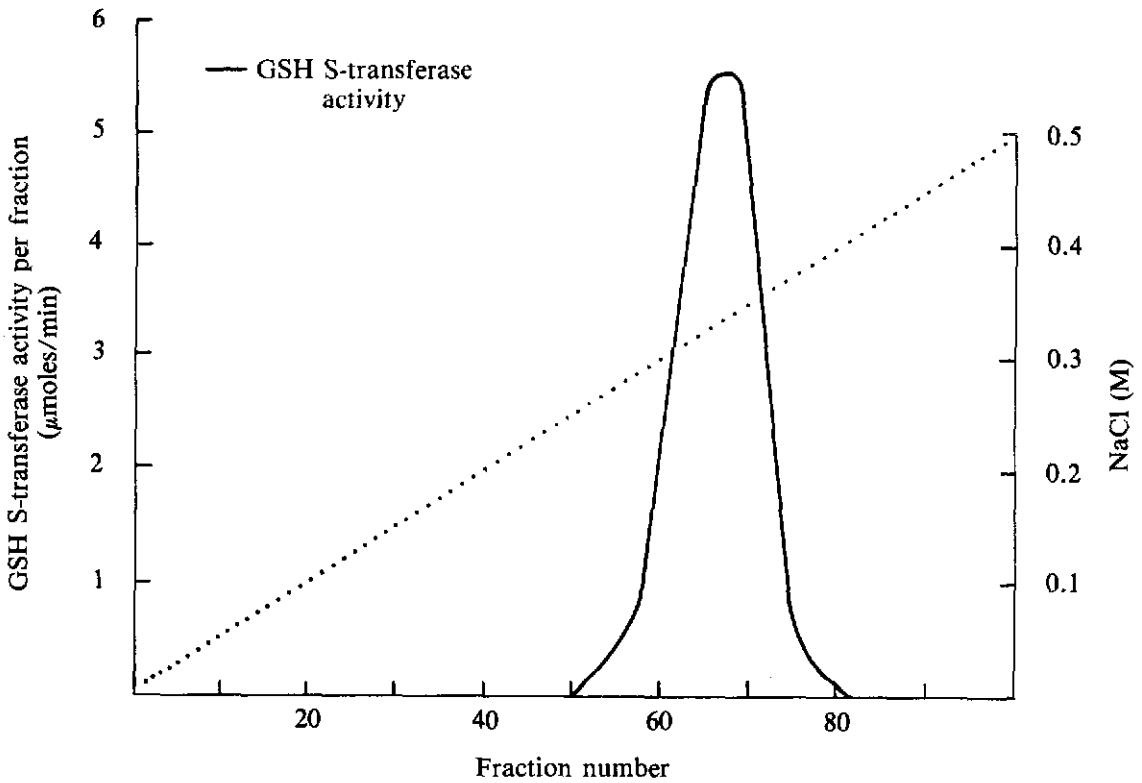

Figure 3. Elution profile of the enzyme (79 mg protein, 60 μmoles/min of activity) eluted with a sodium chloride gradient of 0–0.5 M. The eluting buffer as well as the gradient contained 1.0 mM GSH.

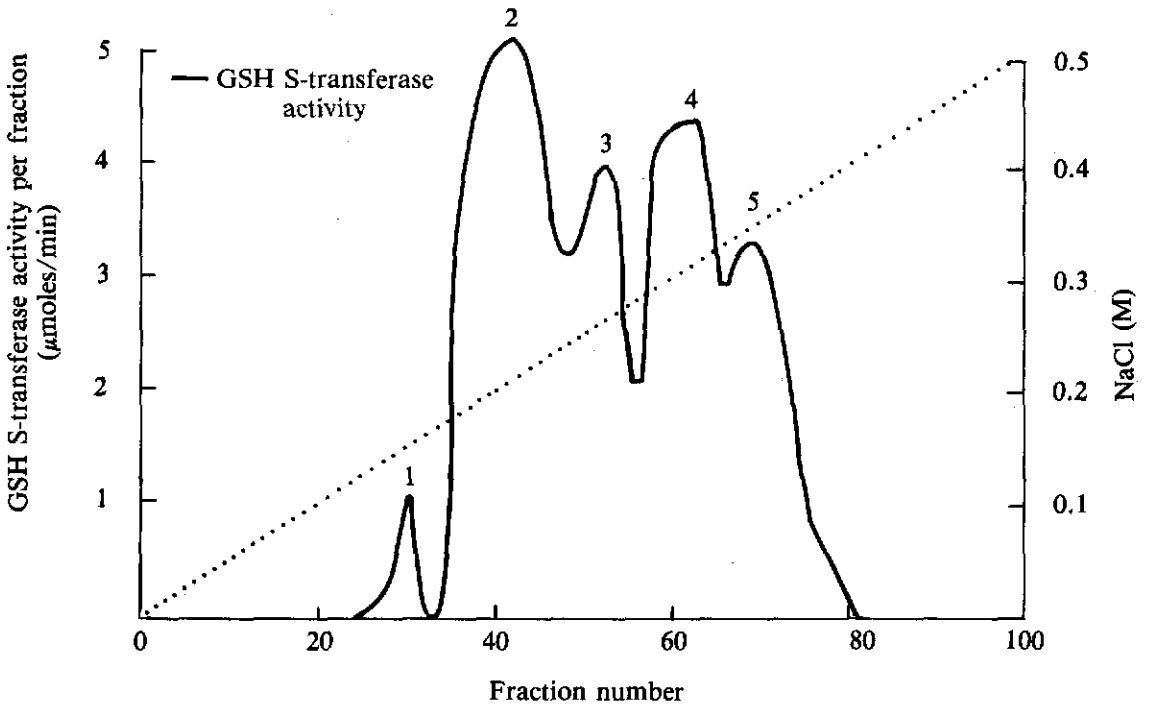

Figure 4. Elution profile of the enzyme (85 mg protein, 68 μmoles/min of activity) eluted with a sodium chloride gradient of 0–0.5 M. The eluting buffer as well as the gradient contained 1.0 mM GSSG.

forms to a major negatively-charged form could not be reproduced by GSSG *(Figure 4)* suggesting that the thiol group of reduced GSH is essential for this transformation. Further when the single peak of transferase activity obtained from the DEAE column that had been eluted with a NaCl gradient containing 1.0 mM GSH was dialysed overnight and eluted through a second DEAE column, again five peaks appeared at approximately the same sodium chloride concentrations (data not included). Thus the effect of sodium chloride and reduced GSH on GSH S-transferase from latex can be interpreted as follows. The enzyme in the crude supernatant is probably in the oxidised state containing multiple labile disulphide bonds. The enzyme when eluted through the DEAE column suffers a disruption of labile disulphide bonds to expose anionic groups to different extents creating multiple forms which require different concentrations of sodium chloride to elute each of them. The presence of reduced GSH in this environment further enhances the reduction process while GSSG is unable to do the same. Removal of both reduced GSH and sodium chloride from the environment of the enzyme by dialysis allows the free thiol groups on the enzyme to undergo auto-oxidation to form disulphide bonds thereby returning the enzyme to its native state.

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