J. nat. Rubb. Res., 3(3), 184–189

Thiol: Disulphide Oxidoreductase in Latex of Hevea brasiliensis

BALABASKARAN SHANMUGAM* AND SEKARAN MUNIANDY*

Thiol: disulphide oxidoreductase is being described for the first time in the latex of Hevea brasiliensis. *The enzyme can be located in both 20 000 g and 100 000 g supernatants, and is stable at 25°C for up to 12 h at pH 8.3. The optimum pH for the enzyme is 8.2 and it is stable between pH 6.0 and 9.0 for up to 24 h. It appears as a single peak by gel filtration and isoelectric focusing studies and has a molecular weight around 100 000 daltons with an isoelectric point of 4.5.*

The enzyme thiol: disulphide oxidoreductase has the ability to transfer reducing equivalents from reduced glutathione on to the disulphide bonds in proteins and peptides[1] as well as on to cystine[2] and the oxidised glutathione so generated is subsequently reduced in the presence of $NADPH_2$ to reduced glutathione. Although both the steps are catalysed by the same enzyme, the latter step can also be carried out by glutathione reductase. The enzyme has been described in a variety of vertebrate tissues such as the liver, kidney, pancreas and pituitary[3] and is not only present as a membrane-bound enzyme[4] but also in the cytoplasm[2].

This paper describes for the first time the presence of a thiol: disulphide oxidoreductase in a plant and in particular in the latex of the rubber tree, *Hevea brasiliensis*.

MATERIALS AND METHODS

All chemicals and reagents were of analytical grade and used without further purification. Alcohol dehydrogenase, β-amylase, L-arginine-HCl, blue dextran, bovine serum albumin, carbonic anhydrase, cytochrome c, L-cysteine, L-cystine, glutathione (GSH) reduced, oxidised glutathione (GSSG), $NADPH_2$, sodium azide and tris-HCl were purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A.

Ampholine of pH range 3.5 to 10.0 was purchased from LKB Productor, Bromma, Sweden. Coomassie Brilliant Blue G-250 was purchased from Bio-Rad Laboratory, Richmond, California, U.S.A. while Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of Crude Enzyme from *Hevea* Latex

Freshly-tapped latex from a number of trees were pooled and immediately cooled to 5°C. Drops of glacial acetic acid were added with constant stirring to change the pH of fresh latex from 6.2 to 5.0. After standing the latex at pH 5.0 for 15 min, the viscous latex was centrifuged at 20 000 g for 1 h at 4°C. The rubber layer at the top of the centrifuge tube was removed with forceps, and the turbid supernatant below it was pooled and treated with cysteine to obtain a final concentration of 1.0 mM. The supernatant was dialysed against several changes of cold distilled water at 4°C and the clear supernatant was used as a source of the crude enzyme.

Protein Estimation

The protein content of column eluants was measured by the method of 280 nm absorbance

*Biochemistry Department, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

while the measurement of protein content of enzyme samples for specific activity measurements was carried out according to the method of Bradford[5] using BSA as the standard.

Sephadex Gel Filtration

A Sephadex G-200 column (80 × 2.5 cm) was equilibrated with 50 mM arginine-HCl buffer at a flow rate of 12 ml per hour at 5°C. A sample of the crude enzyme was loaded on to the column and 4.0 ml fractions were collected. Enzyme activity and protein content were estimated as described in the text. The column was also calibrated using two standard proteins and the molecular weight of the enzyme was estimated.

Enzyme Assays

Thiol: disulphide oxidoreductase was assayed essentially according to the method described previously[2] in a total reaction volume of 3.0 ml in 50 mM arginine-HCl buffer, pH 8.3 containing 1.0 mM GSH, 1.0 mM cystine, 0.1 mM $NADPH_2$ and an appropriate volume of enzyme. Glutathione reductase activity in the crude supernatant was assayed in the same buffer at pH 8.3 in a total reaction volume of 3.0 ml containing 3.3 mM oxidised glutathione (GSSG) and 0.1 mM $NADPH_2$ and 0.1 ml of the crude enzyme. With both assays the oxidation of $NADPH_2$ was measured at 340 nm using a Hitachi recording spectrophotometer.

Isoelectric Focusing

Isoelectric focusing was carried out according to the method of Vesterberg and Svensson[6] using an LKB 8101 analytical column. A 1% (weight/volume) ampholine solution of pH range 3.5 to 10.0 was used in a sucrose density gradient of 0%–50% (weight/volume). To prevent the carrier ampholytes from anodic oxidation and cathodic reduction, the electrodes were covered with acid and base respectively. The sucrose gradient was prepared by hand by mixing different volumes of dense and less dense solution. The fractions were vortex mixed and pipetted into the column in succession starting with the most dense solution. The crude enzyme solution was used to make up the less dense solution. Electrofocusing was carried out for 48 h at a constant voltage of 300 V at 5°C. At the end of the experiment, fractions of 2.0 ml were collected by hand, vortex mixed and their pH determined immediately at 5°C. The enzyme activity and protein content were evaluated as described in the text.

RESULTS AND DISCUSSION

This is the first report of the presence of a thiol: disulphide oxidoreductase in a plant and also in the latex of the rubber tree, *Hevea brasiliensis*. Both 20 000 g as well as 100 000 g supernatants from fresh latex could reduce the disulphide bonds of cystine in the presence of reduced GSH thereby generating oxidised GSSG and cysteine as products of the reaction. The GSSG was then detected indirectly by coupling it to endogenous GSH reductase *(Table 1)*. Although no GSH reductase enzyme was added to the assay system, there was sufficient amount of GSH reductase activity in the latex supernatant that was being assayed for thiol: disulphide oxidoreductase activity. Therefore, it must be assumed that the reduction of oxidised GSSG could have been due partly to GSH reductase although this reaction is also known to be catalysed by thiol: disulphide oxidoreductase in vertebrates.

The enzyme is stable at 5°C for up to a week with less than 10% loss of activity while at 25°C it retains almost 85% of activity at the end of 12 h. Generally, most proteins in their crude form are more stable than purified preparations. Carmichael *et al.*[4] found the enzyme from beef liver so stable that they used heat stability of the thiol: disulphide oxidoreductase as a means of enzyme purification. However, their purified enzyme was moderately heat stable, retaining only about 50% of the initial activity following a 30 min incubation at 60°C.

The optimum pH of this enzyme in latex supernatant was found to be around 8.2 in 50 mM arginine-HCl buffer *(Figure 1)*. It is interesting to note that a similar cytosolic enzyme isolated from porcine adenohypophysis had an optimum pH identical to that of

TABLE 1. SPECIFIC ACTIVITIES OF THIOL: DISULPHIDE OXIDOREDUCTASE AND GLUTATHIONE REDUCTASE

Enzyme	Substrate	Specific activity, 100 000 g supernatant	Specific activity, 20 000 g supernatant
Thiol: disulphide oxidoreductase	Cystine	10.10	0.12
	BSA	0.072	—
Glutathione reductase	GSSG	0.20	0.22

Fresh latex treated to pH 5.0 with glacial acetic acid was poured into two tubes and centrifuged at 20 000 g and 100 000 g respectively for 1 h at 4°C. The rubber layer from both the tubes were removed and their supernatants dialysed overnight in several changes of distilled water. The respective dialysates were again centrifuged at 20 000 g and 100 000 g and the clear supernatants decanted and assayed for thiol: disulphide oxidoreductase.

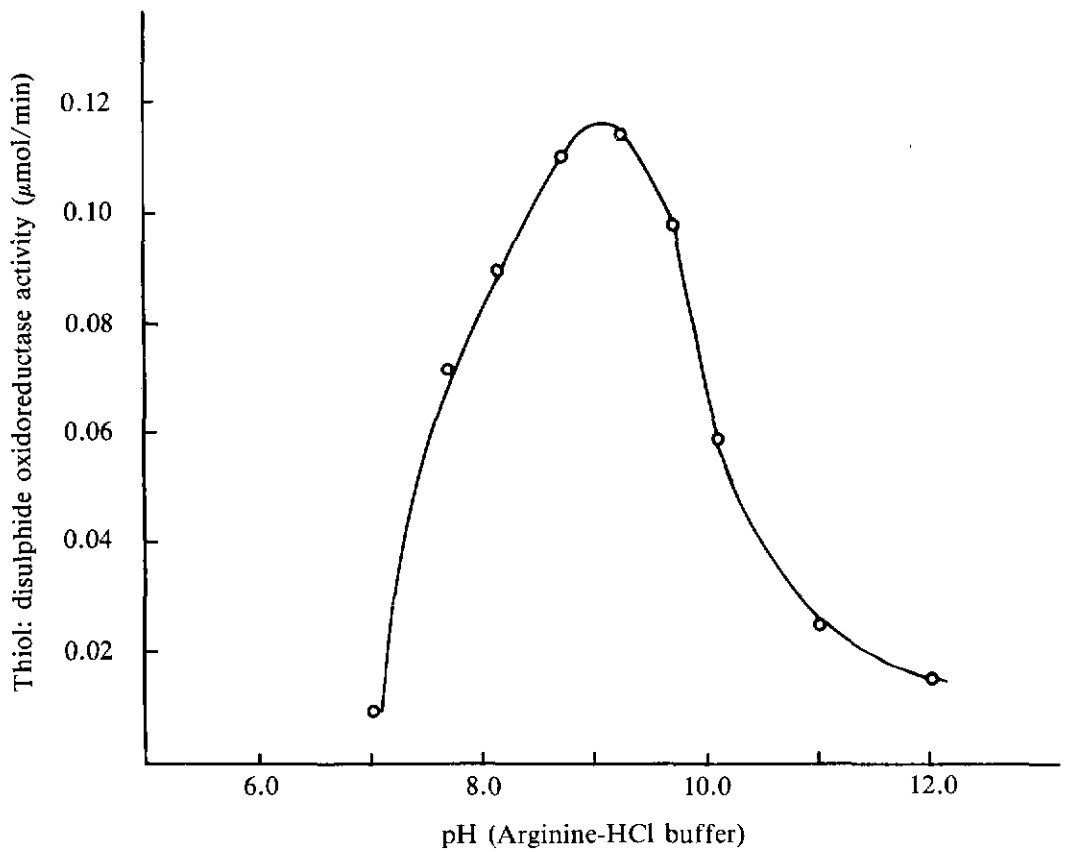

Figure 1. A sample of the crude enzyme was assayed for thiol: disulphide oxidoreductase at 28°C using 50 mM tris-HCl buffer between pH values of 7.0 and 12.0. The points represented in the graph are average values from duplicate determinations.

the latex enzyme[2]. Although the membrane-bound enzyme from beef pancreas had a broad optimum pH curve, its optimum pH was in the region of 8.2[7] unlike the rat liver microsomal enzyme which had a pH optimum around 7.3[8,9]. The cytosolic bovine liver thiol transferase had an optimum pH of 8.5[10]. Thus, it appears that the optimum pH of thiol: disulphide oxidoreductase from a variety of species ranges from about 7.3 to 8.5 and these values are certainly unrelated to the intracellular origin of this enzyme. The enzyme from latex supernatant was found to be stable between pH 6.0 and 9.0 for up to 24 h with less than 10% loss in activity while at pH 4, 5, and 10.0, the enzyme lost between 10% and 20% of its activity.

When a sample of the crude enzyme was eluted through a Sephadex G-200 column, a single peak of activity was detected appearing between bovine serum albumin (molecular weight 66 000 daltons) and alcohol dehydrogenase (molecular weight 150 000 daltons). This could imply that the molecular weight of the enzyme could be in the region of 100 000 daltons *(Figure 2)*. In a number of vertebrates studied so far, more than a single form of thiol: disulphide oxidoreductase has been detected. In rat liver, the microsomal membrane-bound enzyme appeared with molecular weights of 60 000 and 121 000 daltons by sodium dodecyl sulphate polyacrylamide gel electrophoresis while a value of 50 000 – 55 000 daltons was obtained by molecular sieve chromatography[11]. In bovine liver, a value of 92 000 daltons was obtained using molecular sieve chromatography while a value of 60 000 daltons was estimated using sedimentation equilibrium centrifugation as well as by sodium dodecyl sulphate polyacrylamide gel electrophoresis. In another study using the same enzyme source, two forms with molecular weights of 57 000 and 65 000 daltons were reported[11]. However, it is important to

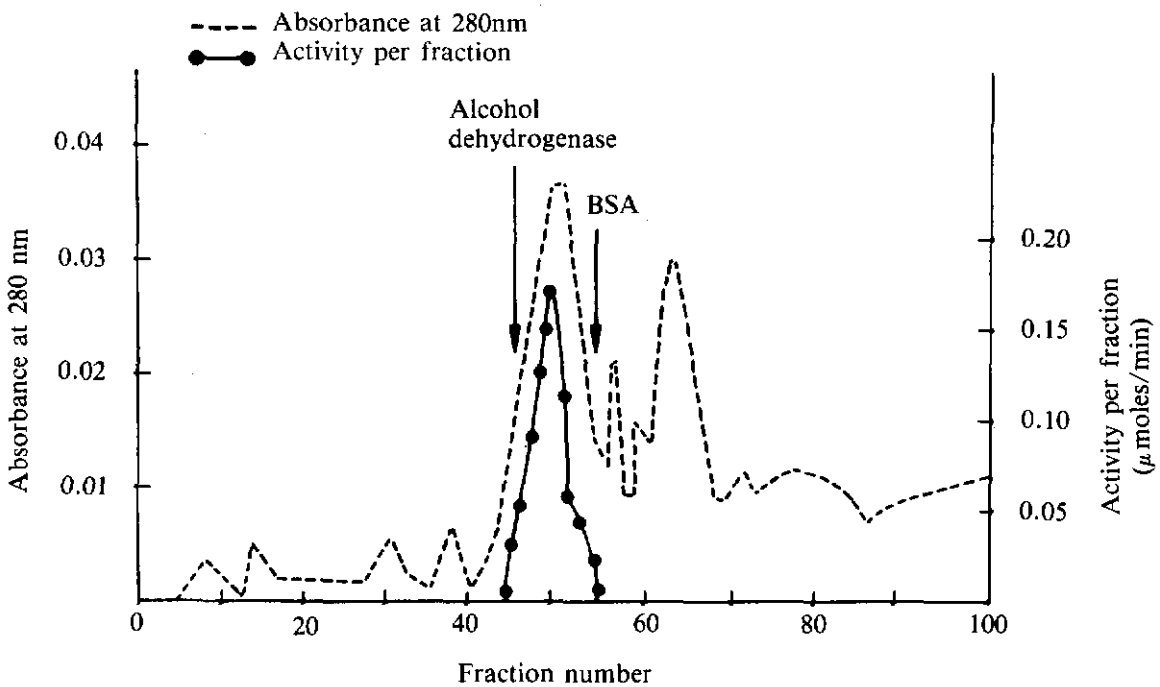

Figure 2. A sample of the crude enzyme was chromatographed on a Sephadex G-200 column (2.5 × 80 cm) with 50 mM tris-HCl buffer at pH 8.3 at a flow rate of 12 ml per hour. Fractions of 4 ml were collected and assayed for thiol: disulphide oxidoreductase activity and protein content.

note that the cytosolic thiol: disulphide oxidoreductase isolated from bovine liver[10] and rat liver[12] is made up of one subunit of 11 000 daltons[14] and in the latter case the enzyme contains 8.6% carbohydrates. Presently, the subunit nature of the membrane-bound forms of vertebrate liver thiol: disulphide oxidoreductases is still not clear.

When a sample of the crude enzyme was subjected to isoelectric focusing, a single peak of activity was detected with an isoelectric point of 4.5 *(Figure 3)*. With all the vertebrate membrane-bound enzymes, the pI values have been found to be in the range of 4.1 to 4.8[4,11]. However, the vertebrate cytosolic enzymes from rat liver as well as bovine liver have alkaline pI values. The former has a pI of 9.6[12] while the latter has a pI of 8.1[10]. Although the latex enzyme resembles the membrane-bound vertebrate enzyme with respect to its isoelectric point it differs from the soluble vertebrate enzymes markedly in this regard.

No physiological role can be attributed to this enzyme in *Hevea brasiliensis* as yet. However, we had recently shown that multiple forms of GSH S-transferase detected on DEAE-Sephadex columns could be reduced to a strongly anionic form in the presence of

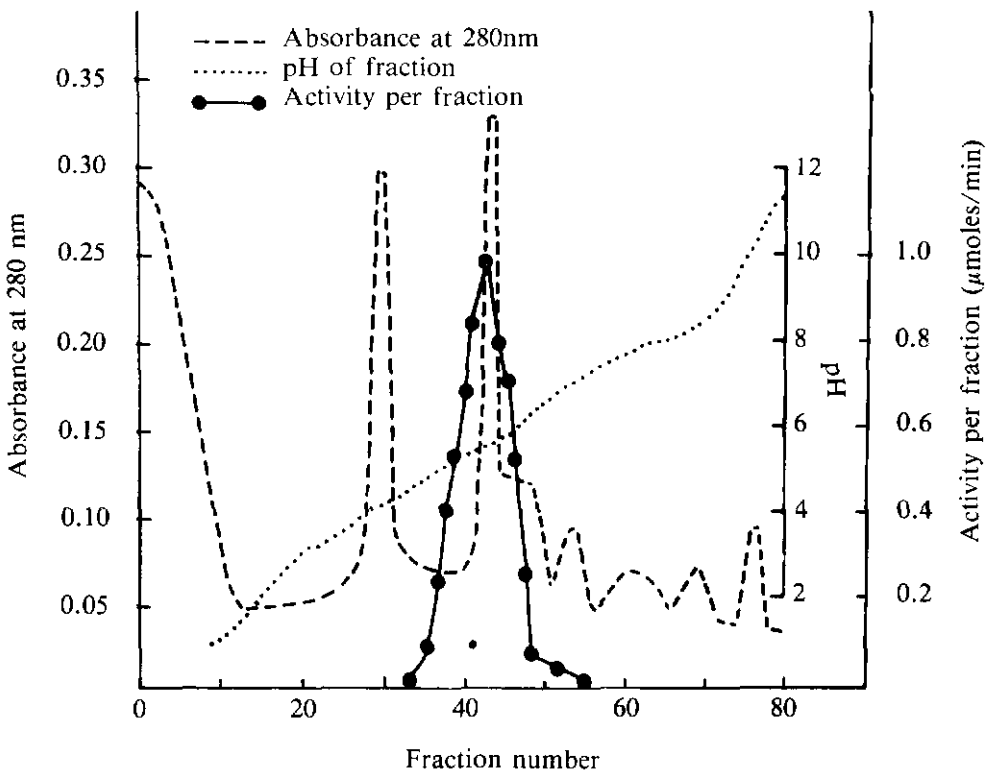

Figure 3. A sample of the crude enzyme (80.85 mg protein) was electrofocused for 48 h as described previously. Fractions of 2 ml were eluted manually and their pH determined at 5°C. Thiol: disulphide oxidoreductase activity and the protein content of each fraction were determined.

reduced GSH and not by oxidised glutathione (GSSG)[13]. We speculated that if the native GSH S-transferase had labile disulphide bonds then the presence of reduced GSH could help to reduce these multiple forms to a strongly anionic form. This step could further be enhanced if there was an enzyme to catalyse the thiol: disulphide oxidoreduction in the presence of reduced GSH. Such an enzyme can now be detected in latex supernatant although the substrates for the moment are cystine and bovine serum albumin rather than GSH S-transferase. Further work is now being carried out to investigate this possibility.

REFERENCES

1. MORIN, J.E., CARMICHAEL, D.F. AND DIXON, J.E. (1978) Characterization, Kinetic and Corporative Properties of Thiol: Protein Disulphide Oxidoreductase. *Arch. Biochem. Biophys.*, **189**, 354.
2. LORENSON, M.Y. AND JACOBS, L.S. (1981) Characterization and Partial Purification of a Cytoplasmic Glutathione: Disulphide Oxidoreductase (Thioltransferase) from Adenohypophysis. *Biochem. Biophys. Acta*, **661**, 63.
3. LORENSON, M.Y., LEE, Y.C. AND JACOBS, L.S. (1981) Regulation of Hormonal and Secretory Granule Membrane Disulphides by Adenohypophysial Glutathione: Disulphide Oxidoreductase. *Life Sci.*, **28**, 2309.
4. CARMICHAEL, D.F. MORIN, J.E. AND DIXON, J.E. (1977) Purification and Characterization of a Thiol: Protein Disulphide Oxidoreductase from Bovine Liver. *J. biol. Chem.*, **252**, 7163.
5. BRADFORD, M.M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilising the Principles of Protein-dye Binding. *Analyt. Biochem.*, **72**, 248.
6. VESTERBERG, O. AND SVENSSON, H. (1966) Isoelectric Fractionation, Analysis and Characterization of Ampholytes in Normal pH Gradients. *Acta Chem. Scand.*, **20**, 820.
7. VARANDANI, P.T. AND TOMIZAWA, H.H. (1966) Purification and Properties of Pancreatic Glutathione-Insulin Transhydrogenase. *Biochem. Biophys. Acta*, **113**, 498.
8. ANSORGE, S., BOHLEY, P. KIRSCHKE, H., LANGNER, J. WIEDERANDERS, B. AND HANSON, H. (1973) Metabolism of Insulin and Glucagon: Glutathione-Insulin Transhydrogenase from Microsomes of Rat Liver. *Eur. J. Bicohem.*, **32**, 27.
9. ANSORGE, S., BOHLEY, P. KIRSCHKE, H., LANGNER, J. AND HORST, H. (1971) Metabolism of Insulin and Glucagon: Breakdown of Radio-iodinated Insulin and Glucagon in Rat Liver Cell Fractions. *Eur. J. Biochem.*, **19**, 283.
10. HATAKEYAMA, M. TANIMOTO, Y. AND MIZOGUCHI, T. (1984) Purification and Some Properties of Bovine Liver Cytosol Thioltransferase. *J. Biochem.*, **95**, 1811.
11. HAWKINS, H.C. AND FREEDMAN, R.B. (1976) Thiol-Protein Disulphide Oxidoreductase: Differences between Protein Disulphide Isomerase and Glutathione-insulin Transhydrogenase Activities by Covalent Chromatography.
12. MANNERVIK, B., AXELSON, K. AND KERSTIN, L. (1981) Thioltransferase: Methods in Enzymology, **77**, 281.
13. BALABASKARAN, S. AND SEKARAN, M. (1987) Effect of Reduced Glutathione and Oxidised Glutathione on the Appearance of Multiple Forms of Glutathione S-transferase in *Hevea brasiliensis* latex. *J. nat. Rubb. Res.*, **3(2)**, 136.
14. MANNERVIK, B. AND AXELSSON, K. (1980) Role of Cytoplasmic Thioltransferase in Cellular Regulation by Thiol-disulphide Interchange. *Biochem. J.*, **190**, 125.