Running title: Thioredoxin *h2* with both dehydroascorbate reductase and monodehydroascorbate reductase activities

Title of article:

Sweet potato storage root thioredoxin *h* with both dehydroascorbate reductase and monodehydroascorbate reductase activities

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Abstract

Recombinant thioredoxin *h* (Trx *h*2) overproduced in *E. coli* (M15) was purified by Ni^{2+}-chelated affinity chromatography as previously reported (Huang et al., 2004a). The molecular mass of Trx *h*2 is ca. 1.4 kDa determined by SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis). Trx *h*2 had antioxidant activity (Huang et al., 2004b). Trx *h*2 reduced dehydroascorbate (DHA) in the presence of glutathione to regenerate ascorbate (AsA). However, without glutathione, Trx *h*2 has very low DHA reductase activity. AsA was oxidized by AsA oxidase to generate monodehydroascorbate (MDA) free radicals. MDA was also reduced by Trx *h*2 to AsA in the presence of NADH mimicking the MDA reductase catalyzed reaction. These data suggest that Trx *h*2 have both DHA reductase and MDA reductase activities.

Keywords: Sweet potato storage roots; Thioredoxin *h*; Dehydroascorbate reductase; Monodehydroascorbate reductase;

INTRODUCTION

Ascorbic acid (AsA) plays an important role in protecting plant cells against the action of reactive oxygen species (Dalton et al., 1986; Kobayashi et al., 1995). In plants, peroxide-scavenging was accomplished through the AsA–glutathione pathway, a coupled series of redox reactions involving four enzymes: AsA-specific peroxidase (EC 1.11.1.11), monodehydroascorbate (MDA) reductase (EC 1.6.5.4), dehydroascorbate (DHA) reductase (EC 1.8.5.1), and glutathione reductase (EC 1.6.4.2) (Dalton et al., 1993; Leonardis et al., 1995). This pathway has been studied mainly in chloroplasts, in which the possible reactive oxygen species produced by PS I during photosynthesis might cause serious damage. However, the AsA–glutathione pathway has also been found in cytosol (Borraccino et al., 1986; Elia et al., 1992), mitochondria (Lunde et al., 2006), and peroxisomes (Jimenez et al., 1997). When AsA functions as an antioxidant in cells, it is oxidized to MDA free radical, and MDA reductase catalyzes the reduction of MDA back to AsA with NAD(P)H (Hossain et al., 1984). MDA was a sensitive endogenous index of oxidative stress in leaf tissues (Heber et al., 1996).

Thioredoxins, the ubiquitous small proteins with a redox active disulfide bridge, are important regulatory elements in a number of cellular processes (Buchanan, 1991; Vianey-Liaud et al., 1994). They all contain a distinct active site, WCGPC, which is able to reduce disulfide bridges of target proteins. Initially described as hydrogen carriers in ribonucleotide reduction in *E. coli*, they were found to serve as electron donors in a variety of cellular redox reaction (Holmgren, 1985). From genome sequencing data, a significant diversity of thioredoxin genes containing five different multigenic families (f, m, x, o and h) was observed (Mestres-Ortega and Meyer, 1999; Meyer et al., 2002; Balmer and Buchanan, 2002). The ferredoxin-thioredoxin system (thioredoxins f and m) has been proved to regulate several enzymatic activities associated with photosynthetic CO_2 assimilation in chloroplasts. Thioredoxin x contains a transit peptide similar to those required for chloroplast and mitochondria targeting; however, its function is not clearly defined (Mestres-Ortega and Meyer, 1999). A new type of plant mitochondrial thioredoxin o was also shown to regulate the activities of several mitochondrial proteins by disulfide bond reduction (Laloi et al., 2001).

Thioredoxin *h* is generally assumed to be cytosolic, which was supported by the absence of a transit peptide in the genes cloned for the isoforms from tobacco (Marty and Meyer, 2001; Brugidou et al., 1993), *Arabidopsis* (Rivera-Madrid et al., 1993;

1995), *Triticum aestivum* (Gautier et al., 1998), germinating wheat seeds (Serrato etal., 2001) and barley seed proteome (Kenji et al., 2003). Moreover, the existence of several forms of thioredoxin *h* detected in spinach leaves (Florencio et al., 1988), and wheat flour (Johnson et al., 1987) supports the view that higher plants possess multiple and divergent thioredoxin genes (Rivera-Madrid et al., 1995). In this study, we present evidence to show that the recombination protein, thioredoxin *h*2 exhibit both DHA reductase and MDA reductase activities.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, dehydroascorbic acid, electrophoresis grade acrylamide and Bis (*N*,*N*′-methylenediacrylamide), TEMED (*N*,*N*,*N*′,*N*′-tetramethylenediamine) and APS (ammonium persulfate) were from E. Merck Inc. (Germany). Other chemicals and solvents were purchased from Sigma Chemical Company (St. Louis, MO). The low molecular weight kits for electrophoresis were obtained from Pharmacia (Uppsala, Sweden).

Expression of thioredoxin *h2* in *E. coli*

Thioredoxin *h*2 (Gene Bank accession number: AY344228; Trx *h*2) was expressed in *E. coli*. The coding sequence was amplified from Trx *h*2 cDNA using an oligonucleotide (5´-GAG AGG ATC CAA TGG GAG GGG CT-3´), with a *BamH* I site (underlined) at the putative initial Met redisue, and an oligonucleotide (5´- ATT TGA AGC TTG ATT GAT GCT -3´), with a *Hind* III site at the 3´ end. The PCR fragment was subcloned in pGEM T-easy vector. And the plasmid was then digested with *BamH* I and *Hind* III and subcloned in pQE32 expression vector (QIAexpress expression system, Qiagen). The resulting plasmid, termed pQE-Trx *h*2, was introduced into *E. coli* (M15). Cultures of the transformed *E. coli* (M15) overexpressed a protein of the expected molecular mass, which was purified by affinity chromatography in Ni-NTA columns (Qiagen), according to the manufacturer´s instructions.

DHA reductase activity assay

The DHA reductase activity of Trx *h*2 was assayed according to the method of Trümper et al. (Tru¨mper et al., 1994) with some modifications. Ten milligrams DHA were dissolved in 5.0 ml of 100 mM phosphate buffer with two pH values (pH 6.0 and 7.0). The reaction was carried out at 30℃ by adding 100 μL Trx *h*2 solution (100 μg protein) to 0.9 ml DHA solution with or without 4 mM glutathione. The increase of

absorbance at 265 nm was recorded for 5 min. Non-enzymatic reduction of DHA in phosphate buffer was measured in a separate cuvette at the same time. A standard curve was plotted using 0.1– 50 nmol AsA.

MDA reductase activity assay

The MDA reductase activity of Trx *h*2 was assayed according to Hossain et al. (Hossain et al., 1984) by following the decrease in absorbance at 340 nm due to NADH oxidation. MDA free radicals were generated by AsA oxidase (EC 1.10.3.3) in the assay system (Yamazaki and Pette, 1961). The reaction mixtures contained 50 mM phosphate buffer (pH 6.0 and 7.0, respectively), 0.33 mM NADH, 3 mM AsA, AsA oxidase (0.9 U), and 200 μL Trx *h*2 solution (200 μg protein) in a final volume of 1 ml. Trx *h*2 solution was replaced with glutathione for controls.

Protein stainings of thioredoxin *h2* in 15% SDS–PAGE gels

Trx *h*2 were examined by protein staining in 15% SDS–PAGE (sodium dodecylsulfate–polyacrylamide gel electrophoresis) gels (Huang et al., 2004c). Twenty microliter samples were mixed with 25 μL sample buffer containing 60 mM

Tris buffer (pH 6.8), 2% SDS, 25% glycerol and 0.1% bromothymol blue, with 2-mercaptoethanol (2-ME) in a final concentration of 14.4 mM, and heated at 100℃ for 5 min for protein staining. Coomassie brilliant blue G-250 was used for protein staining

MDA reductase activity staining in 15% SDS–PAGE gels

Trx *h*2 were examined for MDA reductase by activity stainings in 15% SDS–PAGE gels. Diaphorase activity staining for MDA reductase activity of Trx *h*2 was according to the methods of Kaplan and Beutler (Kaplan and Beutler, 1967) in a 15% SDS–PAGE gel. After electrophoresis, the gel was washed with 25% isopropanol in 10 mM Tris buffer (pH 7.9) twice to remove SDS before activity staining.

Statistical Analysis. Means of triplicate were calculated. Student's *t* test was used for comparison between two treatments. A difference was considered to be statistically significant when $p < 0.05$.

RESULTS

Effect of pH (6.0 and 7.0) on dehydroascorbate reductase activity of thioredoxin *h2*.

To express sweet potato Trx *h*2 in *E. coli*, the coding sequence of Trx *h*2 was subcloned in a pQE-32 expression vector so that sweet potato thioredoxin *h* was produced with a 6x His-tag at the N-terminus. SDS-PAGE analysis of crude extracts from transformed *E. coli* (M15) showed a high level of a polypeptide with the expected molecular mass (ca. 14 kDa). The expressed protein was purified from crude extracts by Ni^{2+}-chelate affinity chromatography, which yielded highly purified His-tagged thiredoxin *h* (Huang et al., 2004b).

The purified Trx *h*2 samples were used to examine DHA reductase activity. Fig. 1 shows AsA regeneration (ΔA 265nm) from DHA at both pH 6.0 and 7.0 with (A) or without (B) glutathione. Fig. 1A shows that Trx *h*2 exhibited DHA reductase activity and could reduce DHA back to AsA. The specific activities of DHA reductase for Trx *h*2 in the presence of glutathione were 7.17 and 35.91 nmol AsA produced/min/mg protein at pH 6.0 and 7.0, respectively. However, in the absence of glutathione, very low DHA reductase activities of Trx *h*2 were found (Fig. 1B): only 0.01 and 0.68 nmol AsA produced/min/mg protein at pH 6 and 7.0, respectively. Trx *h*2 acts as a

GSH-dependent DHA reductase (Fig. 2), and the rate of reduction was closely proportional to the concentration of GSH. There was either a significant increase in the DHA activity treated with 1, 2, 4 and 4 μM GSH ($p < 0.05$). It was reported that thioredoxin m and thioredoxin f from spinach chloroplast and thioredoxin from *Escherichia coli* exhibit very low DHA reductase activities without glutathione [29].

Effect of pH (6.0 and 7.0) on monodehydroascorbate reductase activity of thioredoxin *h*.

MDA was reduced to AsA in coupling with NADH oxidation (Δ A340nm) at pH 6.0 and 7.0 when Trx *h*2 were used as MDA reductase. Trx *h*2 exhibited MDA reductase activity at pH 6.0 and 7.0 (Fig. 3), with higher activity at pH 6.0 than pH 7.0 in our assay system. Trx *h*2 acts as a GSH-dependent MDA reductase (Fig. 3), and the rate of reduction was closely proportional to the concentration of GSH.

Protein and diaphorase activity stainings in 15% SDS–PAGE gels for detection of monodehydroascorbate reductase activity of thioredoxin *h*.

MDA reductase activity staining of Trx *h*2 was done for diaphorase activity (Kaplan and Beutler, 1967) on SDS-PAGE gels (Fig. 4). Comparing Fig 5 (A)

(protein staining) with 4(B) of Trx *h*2 one can see that the diaphorase activity staining for MDA reductase activity came from 14 kD Trx *h*2. MDA reductase and DHA reductase were shown to contain free thiol groups in their catalytic sites (Borraccino rt al., 1989). When AsA is the sole hydrogen donor, the AsA peroxidase, guaiacol peroxidase, and AsA oxidase can produce MDA (Kaplan and Beutler, 1967). Nonenzymatic oxidations of AsA also produce MDA when cells were under oxidative stress (Hossain et al., 1984). Dimerization of heat shock protein 25 via S–S bond formation can occur in cells in response to various oxidative stresses (Zavialov et al., 1998).

DISCUSSION

This is the first report showing that TRX *h*2 displays both DHA reductase and MDA reductase activities with some unique characteristics.

In many physiological studies DHA reductase is regarded as one of the chloroplast enzymes involved in the protection against oxidative stress. A specific DHA reductase is frequently demanded as part of the enzymatic equipment to avoid oxidative stress. In plant extracts a glutathione-dependent DHA reductase activity has been observed (Hossain etal., 1984) which will recycle DHA to ascorbate. An increase of DHA

reductase activity and an accumulation of DHA have been frequently implied as biochemical indicators of oxidative stress in plant metabolism (Wise, 1995) but a characterization of DHA reductase has remained elusive because of rapid loss of enzyme activity.

The thioredoxin system is vital for chloroplast metabolism because redox control of at least 12 different enzymes is achieved by the reductive cleavage of regulatory disulfide bridges in these target enzymes (Buchanan, 1991). Trx *h*2 thiol-disulfide interchanges were found during DHA reduction to regenerate AsA. Thionin was reported to have intermolecular disulfide linkages with other proteins (Pinerio et al., 1995). Thiol groups are central to most redox-sensitive processes in the cell, and their redox state controls cellular processes such as growth, differentiation, and apoptosis. The intracellular thiol homeostasis is maintained by the thioredoxin systems, which utilize reducing equivalents from NADPH to reduce both protein and low molecular weight disulfides.

MDA reductase purified from potato was shown to contain thiol groups in their catalytic sites (Leonardis et al., 1995). Fernando et al., (1992) found that thioredoxin can act as a radical scavenger and facilitate the regeneration of oxidatively damaged proteins and TRX *h*2 might contribute to its antioxidant activities against hydroxyl and peroxyl radicals (Huang et al., 2004b). When AsA is the sole hydrogen donor, the

AsA oxidase can produce MDA (Yamazaki and Pette, 1961). Nonenzymatic oxidations of AsA also produce MDA when cells suffer from oxidative stress (Heber et al., 1996). Taking the above results into consideration, we construct a reduction scheme of both dehydroascorbate (DHA) and monodehydroascorbate (MDA) to ascorbate (AsA) catalyzed by Trx *h*2 of sweet potato roots. DHA and MDA can be reduced to regenerate AsA by Trx *h*2 in order to prevent oxidative damage to cytosols of sweet potato roots.

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甘藷塊根中硫氧化還原蛋白 *h* 具有去氫抗壞血酸還原酶和單去氫抗壞血酸還原酶的活性

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在大腸桿菌(M15)中大量表現重組蛋白質硫氧化還原蛋白 *h2* (Trx *h2*)，利用鎳離子螯合之親和性管柱純化。 Trx *h2* 經 SDS-PAGE 分析其分子量約為 1.4 kDa. 由於 Trx *h2* 具有抗氧化活性。Trx *h2* 在含有穀胱甘肽時，去氫抗壞血酸(dehydroascorbate, DHA)含量會降低而生成抗壞血酸(ascorbate, AsA)。但是，在不含有穀胱甘肽時，Trx *h2* 只有非常低 DHA reductase 活性。AsA 經由 AsA 氧化酶氧化生成單去氫抗壞血酸(monodehydroascorbate, MDA) 自由基。MDA 也可經由 Trx *h2* 而降低了 AsA 生成， 在 NADH 存在時模仿 MDA reductase 催化反應。這結果建議, Trx *h2* 同時具有去氫抗壞血酸還原酶和單去氫抗壞血酸還原酶的活性。

關鍵詞: 甘藷塊根; 硫氧化還原蛋白 *h*; 去氫抗壞血酸還原酶; 單去氫抗壞血酸還原酶;

Figure Legends

Figure. 1. Effect of pH (6.0 and 7.0) on dehydroascorbate reductase activity. Purified recombinant protein of thioredoxin *h*2 was with (A) or without (B) 4 mM glutathione in the reaction mixtures. The reaction was carried out at 30℃ by adding 100 μL thioredoxin *h*2 solution (100 μg protein, 100 mM phosphate buffer, pH 7.0 and 6.0) to 0.9 ml DHA solution with or without 4 mM glutathione. Glutathione was used to be a control. The increase of absorbance at 265 nm was recorded for 5 min.

Figure. 2. Dependence of dehydroascorbate reductase activity of thioredoxin *h*2 on GSH concentration. The reaction was carried out at 30°C by adding 100 μL thioredoxin *h*2 solution (100 μg protein, 100 mM phosphate buffer, pH 7.0) to 0.9 ml DHA solution with different concentrations of glutathione. The increase of absorbance at 265 nm was recorded for 5 min.

Figure. 3. Effect of pH (6.0 and 7.0) on monodehydroascorbate reductase activity of thioredoxin *h*2. The reaction mixtures contained 50 mM phosphate buffer (pH 6.0 and 7.0), 0.33 mM NADH, 3 mM AsA, AsA oxidase (0.9 U), and 200 μL thioredoxin *h*2 solution (200 μg protein) in a final volume of 1 ml.

Thioredoxin *h*2 solution was replaced with gluthioione for controls.

Figure. 4. Protein (A) and diaphorase activity (B) stainings in 15% SDS–PAGE gels for detection of monodehydroascorbate reductase activity of thioredoxin *h*2. The experiments were done twice and a representative one is shown. ‘M’ represents the molecular weight marker and 10 μg protein was loaded in each well.

Figure. 1.

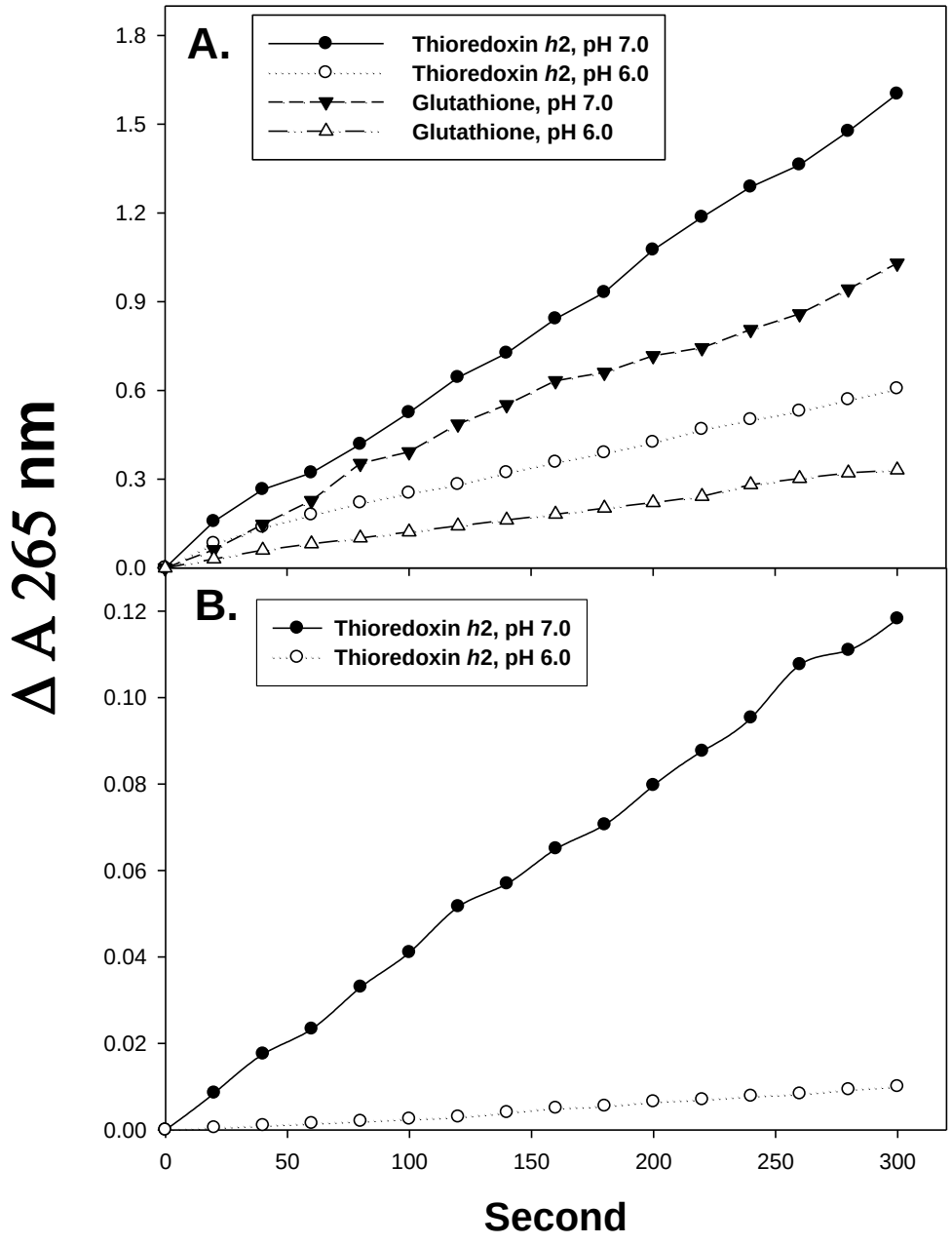
A.
Thioredoxin h2, pH 7.0
Thioredoxin h2, pH 6.0
Glutathione, pH 7.0
Glutathione, pH 6.0
B.
Thioredoxin h2, pH 7.0
Thioredoxin h2, pH 6.0
Δ A 265 nm
Second
0.0
0.3
0.6
0.9
1.2
1.5
1.8
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0
50
100
150
200
250
300

Figure. 2.

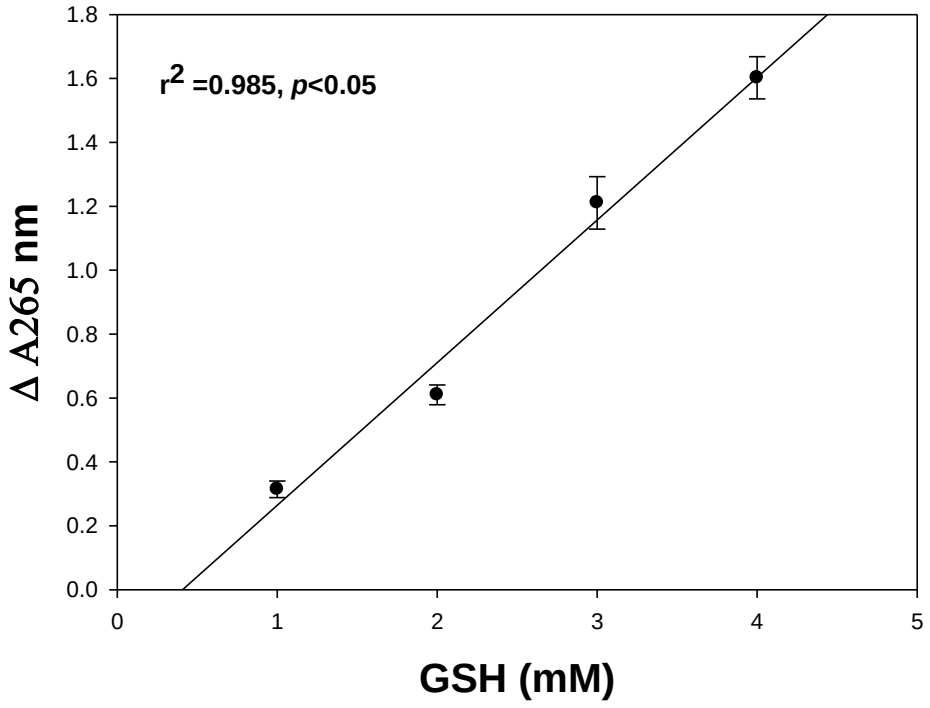

Figure. 3.

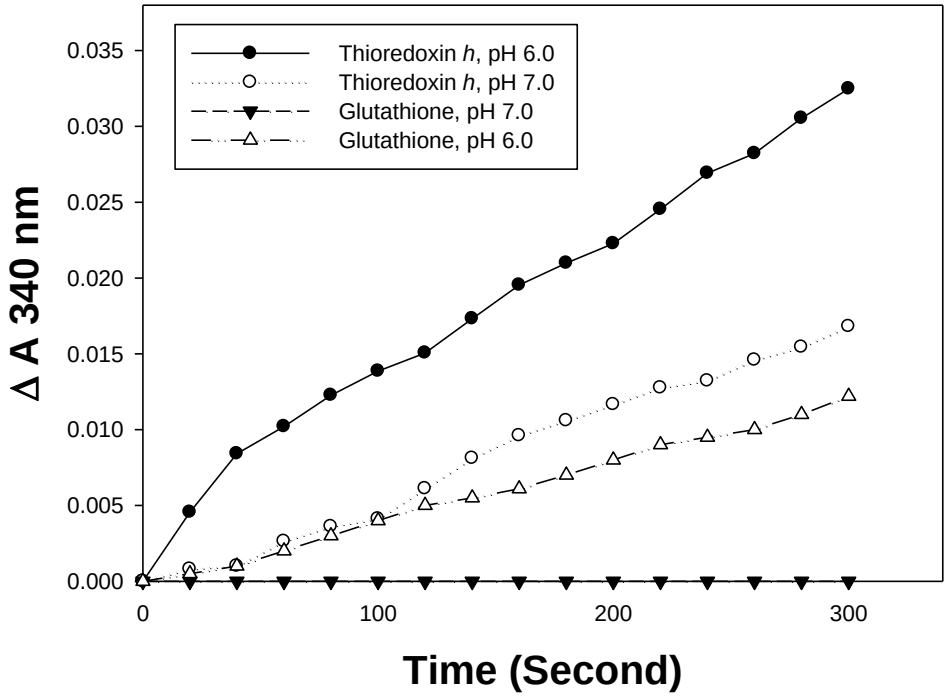
Thioredoxin h, pH 6.0
Thioredoxin h, pH 7.0
Glutathione, pH 7.0
Glutathione, pH 6.0
Δ A 340 nm
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0
100
200
300
Time (Second)

Figure. 4.