Running title: thioredoxin *h2* with angiotensin converting enzyme inhibitory activity

Title of article:

Sweet potato storage root thioredoxin *h2* and their peptic hydrolysates exhibited angiotensin converting enzyme inhibitory activity in *vitro*

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Abstract:

Recombinant thioredoxin *h* (Trx *h*2) overproduced in *E. coli* (M15) was purified by Ni^{2+}-chelate affinity chromatography as previously reported (Huang et al., 2004a). The molecular mass of Trx *h*2 is ca. 14 kDa determined by SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis). Trx *h*2 had antioxidant (Huang et al., 2004b), dehydroascorbate reductase and monodehydroascorbate reductase activity (Huang et al., 2008). Trx *h*2 was shown by spectrophotometric methods to inhibit angiotensin converting enzyme (ACE) in a dose-dependent manner (50-200 μg/mL, with 31.9 ~ 65.9% inhibition) using *N*-[3-(2-*f*uryl) acryloyl]-Phe-*G*ly-*G*ly (FAPGG) as a substrate. The 50% inhibition (IC_{50}) of ACE activity required 151.75 μg/mL Trx *h*2 compared to that of 10 nM (868 ng/mL) Captopril. The use of TLC also showed Trx *h*2 as ACE inhibitor. Trx *h*2 acted as a mixed type inhibitor against ACE using FAPGG as a substrate. When 200 μg/mL Trx *h*2 were added, Vmax and Km were, respectively, 0.010 △A/min and 0.125 mM; while without Trx *h*2 they were 0.0096 △A/min and 0.495 mM. Pepsin was used for Trx *h*2 hydrolysis for different times. It was found that the ACE inhibitory activity increased from 52% to about 72% after 16 h hydrolysis. The results suggested that when small peptides increased by pepsin hydrolysis of the Trx *h*2 ACE inhibitory capacity also increased up to 16 hr, then decreased may be due to disappearance of some conformational

requirements. Four peptides, namely EVPK, VVGAK, FTDVDFIK and MMEPMVK, were synthesized based on the simulated pepsin digest of Trx *h*2, then tested for ACE inhibitory activity. IC_{50} values of individual peptides were 1.73 ± 0.24, 1.136 ± 0.13, 0.416 ± 0.02 and 1.028 ± 0.58 mM, suggesting that FTDVDFIK might represent the main active site for the ACE inhibition. Trx *h*2 and its hydrolysates might be good for hypertension and other disease control when people consume sweet potato tuberous roots.

Keywords: Angiotensin converting enzyme (ACE); thioredoxin *h*2; pepsin; sweet potato.

INTRODUCTION

Many bioactive peptides have common structural properties that include a relatively short peptide residue length (e.g. 2-9 amino acids), possessing hydrophobic amino acid residues in addition to proline, lysine or arginine groups. Bioactive peptides are among the many functional components identified in foods. These are small protein fragments that have biological effects once they are released during gastrointestinal digestion in the organism or by previous in vitro protein hydrolysis. Bioactive peptides with immunostimulating (Parker et al., 1984; Fiat et al., 1993), antithrombotic (Scarborough, 1991), caseino-phosphopeptic (Maubois, & Leonil, 1989), bactericidal (Bellamy et al., 1993), antioxidant or angiotensin-converting enzyme inhibitor (Ehlers, & Riordan, 1989) functions have been the research focus in recent years.

ACE (peptidyldipeptide hydrolyase EC 3.4.15.1) is a glycoprotein and a dipeptide-liberating exopeptidase classically associated with the renin-angiotensin system regulating peripheral blood pressure (Mullally et al., 1996). ACE removes a dipeptide from the C-terminus of angiotensin I to form angiotensin II, a very hypertensive compound. Several endogenous peptides, such as enkephalins, β-endorphin, and substance P, were reported to be competitive substrates and inhibitors of ACE. Several food-derived peptides from α-lactoalbumin,

β-lactoglobulin (Pihlanto-Leppälä et al., 1998), casein (Maruyama et al., 1987), zein, mucilage (Huang et al., 2006) and azein (Yano et al., 1996) also inhibited ACE. Several antioxidant peptides (reduced glutathione and carnosine-related peptides) (Hou et al., 2003) and synthetic peptides also exhibited ACE inhibitor activities (Chen et al., 2003).

Thioredoxins, the ubiquitous small proteins with a redox active disulfide bridge, are important regulatory elements in a number of cellular processes (Buchanan, 1991;). 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; *Arabidopsis* (Rivera-Madrid et al., 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 our previous report, Trx *h*2 exhibited both dehydroascorbate reductase and monodehydroascorbate reductase activities. And Trx *h*2 exhibited antioxidant activities against different radicals (Huang et al., 2004). In this work we report for the first time that Trx *h*2 exhibited dose-dependent ACE inhibitory activity when Captopril was used as a positive control. Commercial bovine serum albumin (BSA), which was frequently found in the literature as the peptide resources of ACE inhibitors, was chosen for comparison. The K_i values of Trx *h*2 against ACE were calculated. We also used pepsin to hydrolyze Trx *h*2 for different times, and the

changes of ACE inhibitory activity were determined. IC_{50} of ACE inhibitory activities by synthetic peptides were also determined.

MATERIALS and METHODS

Materials.

Tris, electrophoretic reagents, and silica gel 60 F254 were purchased from E. Merck Inc. (Darmstadt, Germany); Captopril was purchased from Calbiochem Co. (CA, USA); Seeblue prestained markers for SDS-PAGE including myosin (250 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (30 kDa), and lysozyme (16 kDa) were from Invitrogen (Groningen, The Netherlands); FAPGG, ACE (1 unit, rabbit lung); coomassie brilliant blue G-250; peptide (GL Biochem, China), and other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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

143 TGA AGC TTG ATT GAT GCT -3´), with a *Hind*III site at the 3´ end. The PCR
144 fragment was subcloned in pGEM T-easy vector. And the plasmid was then digested
145 with *BamH*I and *Hind*III and subcloned in pQE32 expression vector (QIAexpress
146 expression system, Qiagen). The resulting plasmid, termed pQE-Trx *h*2, was
147 introduced into *E. coli* (M15). Cultures of the transformed *E. coli* (M15)
148 overexpressed a protein of the expected molecular mass, which was purified by
149 affinity chromatography in Ni-NTA columns (Qiagen), according to Huang et al
150 (Huang et al., 2007).
151
152 **Protein staining of thioredoxin *h*2 on 15% denaturing polyacrylamide gels.**
153 Samples were mixed with sample buffer, namely 60 mM Tris-HCl buffer (pH 6.8)
154 containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue with or without
155 2-mercaptoethanol. Coomassie brilliant blue G-250 was used for protein staining
156 (Huang et al., 2004).
157
158 **Determination of ACE inhibitory activity by spectrophotometry.**
159 The ACE inhibitory activity was measured according to the method of Holmquist
160 et al. (Holmquist et al., 1979) with some modifications. Four microliters (4 microunits)
161 of commercial ACE (1 unit, rabbit lung) was mixed with 50μ L of different amounts

of Trx *h*2 or BSA (50, 100, and 200 μg/mL), and then 200 μL of 0.5 mM *N*-[3-(2-*f*uryl) *acryloyl*]-*P*he-*G*ly-*G*ly [FAPGG, dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl] was added. The decreased absorbance at 345 nm (Δ*A* inhibitor) was recorded during 5 min at room temperature. Deionized water was used instead of sample solution for blank experiments (Δ*A* control). Captopril (molecular mass 217.3 Da) was used as a positive control for ACE inhibitor (1.25, 2.5, 5, 10, 20, 40, and 80 nM). The ACE activity was expressed as Δ*A* 345 nm, and the ACE percent inhibition was calculated as follows: [1 - (Δ*A* inhibitor / Δ*A* control)] x 100. Means of triplicates were determined. The 50% inhibition (IC_{50}) of ACE activity was defined as the concentrations of samples that inhibited 50% of ACE activity under experimental conditions.

Determination of ACE inhibitory activity by TLC.

The ACE inhibitory activity of Trx *h*2 was determined by TLC method (Holmquist et al., 1979). The reactions between Trx *h*2 and ACE or BSA and ACE were according to the method of Anzenbacherova et al. (Anzenbacherova et al., 2001) with some modifications. Each 100μL of Trx *h*2 and BSA (225μg/mL) was premixed with 15 microunits ACE for 1 min, and then 200 μL of 0.5 mM FAPGG was added and allowed to react at room temperature for 10 min. Then 800 μL of

methanol was added to stop the reaction. The blank experiment contained FAPGG only; in the control experiment, ACE reacted with FAPGG under the same conditions. Each was dried under reduced pressure and redissolved with 400 μ L of methanol, and 50 μ L was spotted on a silica gel 60 F254. The FAPGG and FAP (ACE hydrolyzed product) were separated by TLC in 1-butanol-acetic acid-water, 4:1:1 (v/v/v), and observed under UV light.

Determination of the kinetic properties of ACE inhibition by thioredoxin *h*2.

The kinetic properties of ACE (4 mU) without or with Trx *h*2 (200 μg/mL) in a total volume of 250 μL were determined using different concentrations of FAPGG as substrate (0.1 mM to 0.5 mM). The Km (without Trx *h*2) and Km´ (with Trx *h*2) were calculated from Lineweaver-Burk plots, where Km´ was the Michaelis constant in the presence of 200 μg/mL Trx *h*2.

Determination of the ACE Inhibitory Activity of Peptic Hydrolysates of Thioredoxin *h*2.

Six mg of Trx *h*2 were dissolved in 1 mL of 0.1 M KCl buffer (pH 2.0). Then 0.1 mL of 12 mg of pepsin was added at 37 °C for 8, 12, 24, and 32 h. After hydrolysis,

0.5 mL of 0.5 M Tris-HCl buffer (pH 8.3) was added, and the solution was heated at 100 °C for 5 min to stop enzyme reaction. The pepsin was heated before thioredoxin *h*2 hydrolysis for the 0 h reaction. Each of the 60 μL Trx *h*2 hydrolysates was used for determinations of ACE inhibition by spectrophotometry.

Chromatograms of Peptic Hydrolysates of thioredoxin *h*2 on a Sephadex G-50 Column.

The unhydrolyzed Trx *h*2 and peptic Trx *h*2 hydrolysates at 24 h were separated by Sephadex G-50 chromatography (1 x 60 cm). The column was eluted with 20 mM Tris-HCl buffer (pH 7.9). The flow rate was 30 mL/h, and each fraction contained 2 mL, the absorbance of which was determined at 280 nm.

Statistical Analysis. Means of triplicates 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 and DISCUSSION

Expression and Purification of Thioredoxin *h*2. To express sweet potato thioredoxin *h* in *E. coli*, the coding sequence of Trx2 was subcloned in a pQE-32 expression vector so that sweet potato Trx *h*2 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). This polypeptide was found as a soluble protein in the supernatant and was absent in protein extracts obtained from *E. coli* transformed with pQE-32 vector. The expressed protein was purified from crude extracts by Ni^{2+}-chelate affinity chromatography, which yielded highly purified His-tagged thiredoxin *h2* (Fig. 1) .

Determination of ACE inhibitory Activity of Thioredoxin *h*2 by Spectrophotometry.

The purified Trx *h*2 was used for determinations of ACE inhibitory activity. Figure 2 shows time course of the effect of the different amounts of Trx *h*2 (0, 50, 100, and 200 μg/mL) on the ACE activity (ΔA 345 nm). Compared with the ACE only (control), it was found that the higher the amount of Trx *h*2 added the lower the ΔA 345 nm found during 300 sec reaction period. Results of Figure 2 shows that purified

234 Trx *h*2 could inhibit ACE activity in a dose-dependent manner.

235

236 **Effects of Thioredoxin *h*2, BSA and Captopril on ACE Activity shown by**
237 **Spectrophotometry.**

238 It was interesting to know whether BSA also exhibited the ACE inhibitory activity.
239 Figure 3A shows the effects of Trx *h*2 (0, 50, 100, and 200 μg/mL), BSA (0, 50, 100,
240 and 200 μg/mL) or Captopril (Figure 3B; 0, 1.25, 2.5, 5, 10, 20, 40 and 80 nM;
241 corresponding to 0, 108.5, 217, 434, 868, 1,736, 3,472 and 6,844 ng/mL, respectively)
242 on ACE activity. It was found that BSA showed less ACE inhibitory activity (less
243 than 15% inhibition) and without dose-dependent inhibition patterns. However, Trx
244 *h*2 exhibited dose-dependent ACE inhibitory activity (50~200 μg/mL giving,
245 respectively, 31.9 ~ 65.9% inhibition). From calculations, the 50% inhibition (IC_{50}) of
246 Trx *h*2 against ACE activity was 151.75 μg/mL compared to that of 10 nM (868
247 ng/mL) for Captopril, which was similar to the report (7 nM) of Pihlanto-Leppäl¨a¨ et
248 al. (Pihlanto-Leppälä et al., 1998); while the IC_{50} of yam dioscorin was 250 μg/mL
249 (Hsu et al., 2002). Both BSA and purified Trx *h*2 were proteins, but only the purified
250 Trx *h*2 showed specific dose-dependent ACE inhibitory activity. In the literature, the
251 protein hydrolysates were used as sources to purify peptides as ACE inhibitors
252 (Mullally et al., 1996; Maruyama et al., 1987). From calculations, the IC_{50} of Trx *h*2

against ACE activity was 151.75 μg/mL, which was smaller than the synthetic peptide α-lactorphin (YGLF, 322.7 μg/mL). Several identified peptide fragments exhibited much lower IC_{50} values than our purified Trx *h*2; for example, Tyr-Pro of whey proteins, 8.1 μg/mL (Yamamoto et al., 1999) and HHL of soybean proteins, 2.2 μg/mL (Shin et al., 2001). On the opposite, several identified peptide fragments exhibited much higher IC_{50} values than our purified Trx *h*2; for example, hydrolysates of whey proteins (α-lactalbumin and β-lactoglobulin) were effective with IC_{50} values between 345-1,733 μg/mL (Pihlanto-Leppälä et al., 2000), LAHKAL of α-lactalbumin hydrolysates, 406 μg/mL; GLDIQK of β-lactoglobulin hydrolysates, 391 μg/mL; and VAGTWY of β-lactoglobulin hydrolysates, 1,171 μg/ mL. In our previous paper, the IC_{50} of ACE activity required 187.96 μg/mL trypsin inhibitor from sweet potato, which was higher IC_{50} values than our purified Trx *h*2 (Huang et al., 2007).

Determinations of ACE Inhibitory Activity of Thioredoxin *h*2 by TLC.

The FAPGG and FAP (product of ACE catalyzed hydrolysis reaction) were separated by TLC using water saturated 1-butanol: acetic acid: water, 4:1:1 (V/V/V) as developing solvents according to the method of Holmquist et al.(Holmquist et al., 1979) . Figure 4 shows the qualitative results of TLC chromatograms of a silica gel 60

F254 about the effects of 225μ g/mL of commercial BSA (lane 3) or Trx *h*2 (lane 4) on 15 microunits of ACE. Compared to the control test (lane 2), it was found that Trx *h*2 (lane 4) inhibited ACE reaction showing less amounts of FAP production observed under UV light. However, similar amounts of FAP were found between the control test (lane 2) and BSA (lane 3). These results demonstrated again that Trx *h*2 exhibited ACE inhibitory activity.

Determination of the Kinetic Properties of ACE Inhibition by Thioredoxin *h*2. The Lineweaver-Burk plots of ACE (4 mU) without or with purified Trx *h*2 (200 μg/mL) under different concentrations of FAPGG are shown in Figure 5. The results indicated that purified Trx *h*2 acted as a mixed type inhibitor against ACE using FAPGG as a substrate. When 200 μg/mL Trx *h*2 were added, Vmax and Km were, respectively, 0.010 △A/min and 0.125 mM; while without Trx *h*2 they were 0.0096 △A/min and 0.495 mM. In conclusion, Trx *h*2 exhibited dose-dependent ACE inhibitory activity and acted as a mixed type inhibitor with respect to the substrate (FAPGG). A similar work was reported with the calculated Km as 0.255 mM FAPGG for ACE and in the presence of purified dioscorin, the calculated Km´ was 0.3304 mM (Hsu et al., 2002).

290

291 **Determination of the ACE Inhibitory Activity of peptic Thioredoxin *h*2**
292 **Hydrolysates and their peptide Distributions.**

293 The pepsin was frequently used for protein hydrolysis to purify potential ACE
294 inhibitory peptides (Pihlanto-Leppälä et al., 2000). Therefore, we used pepsin to
295 hydrolyze Trx *h*2. Figure 6 shows the ACE inhibitory activity (ΔA 345 nm) of peptic
296 Trx *h*2 hydrolysates. Figure 6A shows the ACE inhibition (percent) of peptic Trx *h*2
297 hydrolysates collected at different pepsin hydrolysis times. From the results (Figure
298 6A), it was found that the ACE inhibitory activity increased from 52% (0 h) to about
299 72% (16 h). Figure 6B shows the chromatograms of unhydrolyzed Trx *h*2 and peptic
300 Trx *h*2 hydrolysates (16 h) on Sephadex G-50 chromatography. It was found that
301 smaller peptides increased with increasing pepsin hydrolytic time. The ACE inhibitor
302 activities of peptic Trx *h*2 hydrolysates decreased after 16 h hydrolysis (Fig. 6A)
303 suggesting that some proper conformational requirements got lost after 16 h
304 hydrolysis.

305 We used synthetic peptides to measure ACE inhibitor activity by Trx *h*2 genes
306 sequence. Kohmura et al (1989) synthesized some peptide fragments of human
307 β-casein and found that the length of those peptides had an influence on the ACE

308 inhibitory activity. Namely, peptides composed of 3-10 amino acids with proline on
309 the C-terminal were necessary for ACE inhibitors (Kohmura et al., 1990). Thus the
310 peptide Leu-Arg-Pro from food protein hydrolysates has been reported to be the most
311 potent natural ACE inhibitor, with an IC_{50} value of 0.27 or 1.0 μM. Byun et al. (1980)
312 studied the ACE inhibitory activity of a series of dipeptides, and indicated that
313 tryptophan, tyrosine, proline, or phenylalanine at the *C*-terminal and branched-chain
314 aliphatic amino acid at the *N*-terminal was suitable for a peptide binding to ACE
315 (Byun & Kim, 2002).

316 Synthetic peptides were designed by simulated pepsin cutting sites of *Trx h2* gene
317 (accession number: AY344228) products from sweet potato (pH >2,
318 http://expasy.nhri.org.tw/tools/peptidecutter/). Four new inhibitory peptides (Table I)
319 for ACE, that is, EVPK, VVGAK, FTDVDFIK and MMEPMVK, were synthesized
320 according to simulation. IC_{50} values of individual peptides were 1.73 ± 0.24, 1.136 ±
321 0.13, 0.416 ± 0.02 and 1.028 ± 0.58 mM, respectively. These results demonstrated that
322 simulated synthetic peptides from peptic Trx *h*2 hydrolysates exhibited ACE
323 inhibitory activities. Our work suggests that (1) FTDVDFIK might represent the main
324 active site for the ACE inhibition; (2) there are marked structural similarities for
325 peptides with antihypertensive, immunomodulatory and antioxidant activities and may

be used as criteria for selecting or designing multifunctional ingredients of functional foods to control cardiovascular diseases.

In summary, Trx *h*2 exhibited dose-dependent ACE inhibitory activity. Trx *h*2 acted as a mixed type inhibitor toward ACE with IC_{50} of 151.75 μg/mL. Its peptic hydrolysates also showed ACE inhibitory activities. Some peptides derived from food proteins were demonstrated to have antihypertensive activities against spontaneously hypertensive rats (Fujita et al., 2000; Yoshii et al., 2001). The potential for hypertension control when people consume sweet potato deserves further investigations.

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