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REVIEW
Disulfide Proteomics: Current Status in Thioredoxin Biochemistry and Industrial Research

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Abstract
Redox regulation is a central control element across a broad spectrum of biology. Thioredoxin (Trx), a widely distributed disulfide enzyme, participates in the control of numerous target proteins, thus playing a key role in regulatory processes. Identification of Trx's targets will therefore aid the elucidation of redox biology. Two recently developed procedures, one based on thiol-specific probes and the other on affinity trapping, have facilitated the labeling or isolation of potential Trx targets that were later identified with proteomic approaches. Accordingly, the number of identified targets in plants has increased to 400. This review describes recent advances in proteomic strategies that are effectively overcoming challenging problems such as the evaluation of target authenticity. Also presented are two industrial research areas, food processing and medical investigation, in which disulfide-related proteomic studies beyond plant biology are underway.

Discipline: Genetic resources
Additional key words: biomarker, redox regulation

Introduction

Post-translational modification (PTM) functions in a broad spectrum of biology. Among the regulatory mechanisms involved, reversible protein phosphorylation has been most widely studied and developing the phosphoproteomics technique has enhanced our understanding of this mode of regulation. Meanwhile, a growing body of evidence suggests that reversible reduction/oxidation (redox) of protein disulfide bonds regulates critical processes in all cell types[12]. Thioredoxins (Trxs) are small (10–14 kDa), widely distributed redox proteins involved in the regulation of numerous target proteins via thiol/disulfide exchanges, and thus play key roles in maintaining cellular redox homeostasis[34,44]. Thus, a better understanding of redox biology could be achieved if more were known about their target proteins. Moreover, the knowledge obtained will be useful in developing new variety of plants/crops with improved yield, disease-resistance and cultivation suitability which should benefit developing countries. In medical studies, this knowledge may also reveal important clues to understand the mechanisms of various human diseases and, more practically, to identify potential drug targets and biomarkers.

In 2001, the concept of proteomics has been incorporated into Trx-linked studies. Yano et al.[57] and Motohashi et al.[37] independently developed major proteomic approaches based on discrete principles. The affinity column strategy[37] allows the concentration of the targets, while the fluorescent gel approach[57] is applicable to both *in vitro* and *in vivo* analyses. These two methods, with their improved versions, have since been applied to elucidate Trx-linked biochemistry[26], and the number of putative targets has reached 400[35]. Following a brief introduction of these two basic methods, the present short review focuses on the recent advancement of a technique that can effectively tackle problems such as target authenticity. The review also introduces two industrial research areas, food processing and medical investigation, in which proteomic studies are underway.

Proteomic approaches to identify Trx targets

In this section, basic proteomic methods are briefly introduced, while application of the techniques to plant

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seed biochemistry[54] as well as food allergens[53] has been summarized in recent reviews. A comprehensive list of putative targets, as of 2009, is also available[35].

1. Affinity column procedure

The affinity column procedure is based on the two-step reaction mechanism (Fig. 1A). First, the most reactive, N-terminal Cys residue in the active site (-WCGPC-) of Trx forms an unstable intermediate with the oxidized target protein. Subsequently, the second Cys residue (-WCGPC-) attacks the intermediate, releasing the reduced target and the oxidized Trx[20]. One approach to identifying target proteins involves isolating the Trx-target complex. However, in the case of Trx, the intermediate occurs only transiently. Verdoucq et al.[47] found that a mutant Trx, in which the second Cys in the active site is substituted by Ser, forms a stable intermediate with its target (Fig. 1B). Motohashi et al.[37] immobilized the monocysteinic Trx on a column resin. Incubation of the cellular extract with the resin made the targets link covalently with the immobilized Trx, and they were recovered by subsequent dithiothreitol (DTT) treatment.

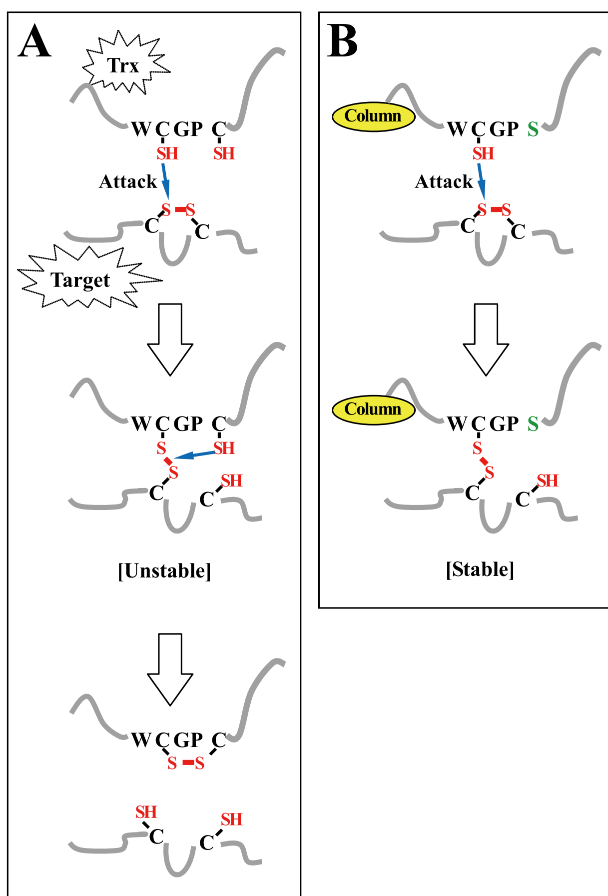


Fig. 1. Reduction mechanism of Trx (A) and stabilization of the Trx-target complex (B)

2. Fluorescent gel approach

Meanwhile, Yano et al.[57] used a specific fluorescent probe, monobromobimane (mBBr), to label free sulfhydryl groups of cellular proteins (Fig. 2A) followed by two-dimensional electrophoresis (2-DE) analysis (Fig. 2B). First, tissue extracts are treated with active Trx. Only the disulfide bonds of target proteins are reduced and expose the free SH group. Subsequently, the extracts are treated with mBBr, resulting in the selective fluorescent labeling of target proteins (Step 1). Next, the extracts are treated with DTT and all other disulfide bonds are re-

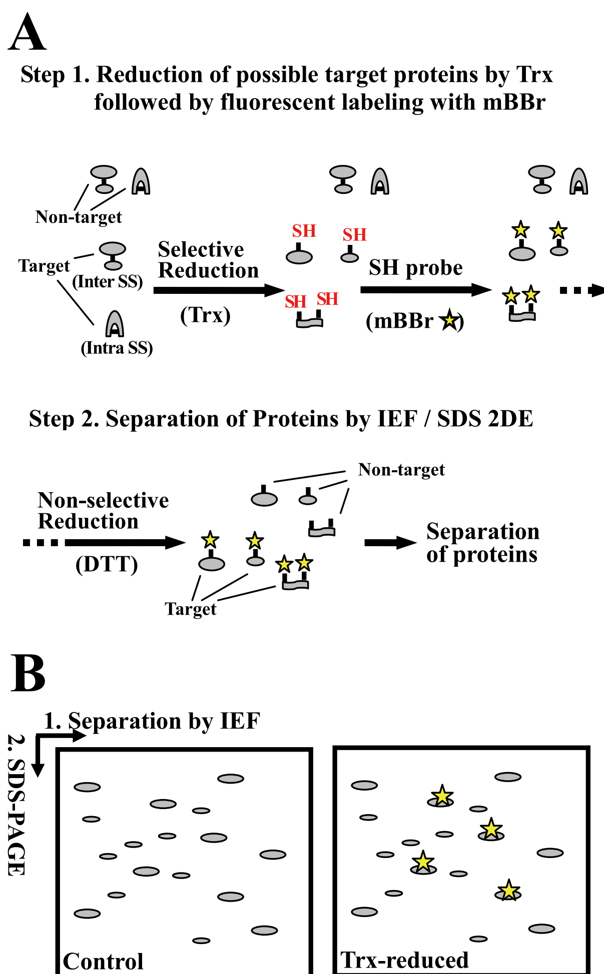


Fig. 2. Fluorescent labeling of target proteins with mBBr (A), followed by separation on the two-dimensional electrophoresis (2-DE) (B)

A, In Step 1, only the disulfide bonds of the target proteins are labeled with fluorescence following their reduction by Trx. In Step 2, all other disulfide bonds are reduced. B, On the 2-DE gel, target proteins are observed as fluorescent spots (marked with ☆) under UV light. Inter SS, a protein with intermolecular disulfide bond(s); Intra SS, a protein with intramolecular disulfide bond(s); Trx, thioredoxin. From ref. 57 with modification. Copyright (2001) National Academy of Sciences, U.S.A.

duced (Step 2). Finally, the proteins in the extracts are separated by 2-DE (Fig. 2B). In the separation system, first, proteins are separated by isoelectric focusing (IEF) depending on their respective isoelectric points. Subsequently, proteins are treated with sodium dodecyl sulfate (SDS) followed by a separation according to their respective molecular sizes. Comparison of the gel profiles between the Trx-treated sample and control (non-treated) sample under UV light allows identification of the target spots (☆) because of their increased fluorescence. This chemical modification/2-DE based technique is applicable to both *in vitro* and *in vivo* analyses[55,56]. To allow high-sensitivity detection, mBBr (5,000 $M^{-1}cm^{-1}$) was replaced with Cy5 maleimide (250,000 $M^{-1}cm^{-1}$)[31] or ^{14}C-labeled iodoacetamide[33].

3. The two methods are complementary

Comparative studies of the two procedures were reported in some studies[1,32,49]. In each case, the two procedures were found to be complementary. Approximately one-third were detected by both methods, and the remaining one-third was unique to each[49]. Both Wong et al.[49] and Marchand et al.[32] discuss how each method has its own shortcomings and biases. With the fluorescent gel approach, the same spot could contain quantitatively-scarce protein which could be hidden by an abundant variety. In contrast, the affinity column procedure facilitates the identification of quantitatively-minor proteins, but may allow the detection of co-purified ones as false positives. Further studies may elucidate any bias and/or difference between the putative target groups identified by the respective methods. Overall, a combination of the two approaches should be useful for obtaining a comprehensive overview of potential targets.

Current challenges

These proteomic approaches have been utilized effectively in determining the putative Trx targets[26], and the number has reached approximately 400[35]. The data reveals the unexpected involvement of Trx-dependent redox regulation in almost all plant life processes[34]. Nevertheless, genetic methods of confirming the functions of cytosolic Trxs are limited due to the existence of identical phenotypes among single mutants, presumably attributed to functional redundancies among Trx and even glutaredoxin members[11,43]. Thus, we face a challenging problem: How can we identify specific targets at a proteomic scale with greater certainty?

As discussed in our previous review[54], molecular specificity and expression patterns are important determinants of Trx-target specificity. In brief, site-directed mutagenesis studies[36] suggested that electrostatic interactions between Trx and target induced the following formation of their non-covalent binary complex. The crystal structure of the Trx-target complex[30] identified the conserved hydrophobic motif of Trx as a key element in van der Waals contacts, while comparison of the Trx isoform structures[29] elucidated that charge complementarity near the binding site plays a key role in determining specificity. Thus, the challenge facing the disulfide proteome is how it can realize the specificity of the targets.

1. How to confirm specific targets?

(1) Quantitative procedure to evaluate authenticity

By site-directed mutagenesis, Mora-García et al.[36] modified *E. coli* Trx to make it more "*f*-type like", which improved the capacity to activate fructose-1,6-bisphosphatase (FBPase), a specific target of Trx *f*. The affinity for FBPase also increased from 4- to 11-fold. Thus, quantitative measurement of the Trx's reactivity toward individual targets would help in determining its authenticity. Hägglund et al.[15] developed a quantitative proteomics procedure based on thiol-specific differential labeling with iodoacetamide-based isotope-coded affinity tag (ICAT) reagents[14]. Quantification of the ratios of peptides labeled with "light" ^{12}C ($ICAT_L$) and "heavy" ^{13}C ($ICAT_H$) reagents makes it possible to estimate the extent to which individual targets are reduced. The authors successfully applied the method to determining targets in barley seed embryo[15] and aleurone layers[16].

(2) "Stringent" column procedure to raise the threshold

Traverso et al.[45] sought to identify the respective targets of pea Trx isoforms, PsTrxh1 and PsTrxh2, which behaved differently during *in vitro* and *in vivo* assays. They considered that the success of an affinity column procedure was highly dependent on the exposure of the interaction site as well as discrimination of nonspecific interactions. Accordingly, the N-terminus of each Trx was fused with a His-Tag on the opposite side to the active site, and thus did not interfere with the measurements. They also produced the protein in the absence of reductants to promote the formation of disulfide bonds, thus avoiding any reaction of the Cys residues with the resin. Discrimination of the nonspecific interaction was approached by loading the sample or washing the column at moderate ionic strength, even at the risk of being too strict. In addition, they used non-mutant versions of the PsTrxs (–WCGPC–) because the mutation of Cys to Ser modifies the p*Ka* of the active center and varies the physiological conditions of the interaction[3]. As a result, the two Trx isoforms captured different proteins. PsTrxh1 interacted with a transcription factor, embryonic proteins, transposases and a photolyase, while PsTrxh2 showed af-

finity for classical antioxidant proteins. Such refinement would also be useful to define the targets of the respective Trx isoforms.

(3) Comparison between *in vivo* and *in vitro* data

As stated above, the mBBr-labeling procedure is applicable to both *in vitro* and *in vivo* analyses. It is therefore possible to confirm whether the putative targets of Trx identified by the *in vitro* reaction are reduced *in vivo*. In a study with the legume, *Medicago truncatula*, more than half the 43 potential Trx targets identified by the *in vitro* mBBr-labeling reaction were also labeled *in vivo* during germination[1]. In addition, Yano & Kuroda[55] reported that rice aleurone ESP2, identified as putative targets of Trx, were degraded both *in vivo* and *in vitro* by a cysteine protease also activated by Trx. They also confirmed that disulfide bonds of ESP2 were reduced *in vivo* in the presence of a cysteine protease inhibitor.

Such technological refinements will improve the authenticity of the putative targets, and eventually promote a more accurate understanding of the redox biology.

Possible breakthroughs by disulfide proteomics

In this section, we present examples of the potential application of the proteomic strategy to several contemporary food and medical issues.

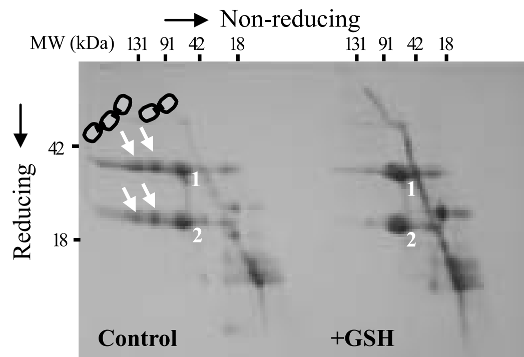
1. Application to food chemistry

Disulfide bond formation affects the physical properties and texture of food. A typical example is wheat gluten. Gliadin and glutenin are the major wheat proteins. Kneading wheat dough promotes the formation of intermolecular disulfide bonds among them, which produces gluten. Gluten demonstrates a unique property to retain the carbon dioxide produced during yeast-fermentation, making wheat flour the representative ingredient of bread. Conversely, peptides released from wheat gluten during digestion are responsible for celiac disease[13] as well as wheat allergy[6]. Some food chemists are therefore trying to develop a wheat alternative that works as gluten. Rice is considered a suitable substitute for wheat, as it is available worldwide and is less allergenic. Moreover, as rice is grown in developing countries, the development of rice-based food products, such as bread, will sustain their economic growth. However, rice proteins lack the viscoelastic properties typically found in gluten. With this in mind, we sought to increase the bread-making property of rice proteins by changing their disulfide bonding structure. In this study we used glutathione, a bioactive small peptide, which is capable of cleaving the disulfide bond of protein as well as Trx. It is also inexpensive and usable as a foodstuff in Southeast Asian countries as well as the USA. We[51,52] found that the addition of glutathione improved the bread-making quality of rice batter (Fig. 3). Also, the non-reducing/reducing 2-DE demonstrated that glutelin polymers, which are usually observed in rice batter, were decomposed to monomers in the case of glutathione-added rice bread (Fig. 4A). It has been reported that starch granules are surrounded by disulfide-linked macromolecular proteins natively present in the rice endosperm or formed/strengthened during cooking[9] (Fig. 4B). They function as a “barrier” and restrict the heat-induced water adsorption or swelling of rice starch. Glutathione seems to hinder the formation of the macromolecules by cleaving or preventing the intermolecular disulfide crosslinking among glutelins. The microstructure of the bread (Fig. 5) shows that both the glutathione-added and wheat breads had perforated structures (× 30 resolution), demonstrating their gas-retaining capacity in the fermentation process. Conversely, further magnification (× 1,000) shows that the glutathione-treated bread had a smooth-looking surface without visible traces of starch granules, in contrast to the control rice/wheat breads, which appeared to have rougher surfaces. Thus, changing the disulfide bond of food protein affected both the chemical and physical properties of the food component and ultimately had a drastic impact on its quality.

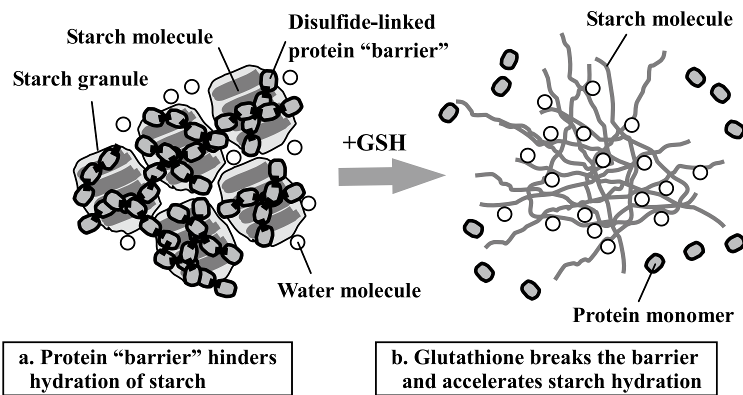
Control of inter- and intramolecular disulfide bonds of food proteins by Trx or glutathione will be useful for developing foods with new characteristic features. Also, if glutathione and Trx work on different sets of proteins in food, they should have different effects on its physical property, digestibility, allergenicity and so on. Moreover,

Fig. 3. Comparison of the control and glutathione-added rice breads[52]

First, in the bread pan of a home bakery, 280 g of water and 0.75 g of glutathione (GSH) were added to 280 g of rice powder, whereupon they were mixed and left overnight at room temperature. Subsequently, 4 g of dry yeast and 15 g of sugar were added to the mixture, followed by yeast fermentation and baking. The addition of glutathione made the gluten-free rice bread swell (+GSH), while it did not swell when glutathione was absent (control).



A. Cleavage of the disulfide bonds by glutathione



B. Illustrative drawing of the barrier theory

Fig. 4. A. Non-reducing/reducing two-dimensional electrophoresis of protein taken from the control and glutathione-added (+GSH) rice batters in the early stage of the baking process

Arrows, disulfide-linked dimer and trimer. From ref. 52, with modification. Copyright (2010) American Chemical Society. B. Illustrative drawing of the barrier theory. The addition of glutathione cleaves the intermolecular disulfide bonds that support the barrier, improving starch hydration.

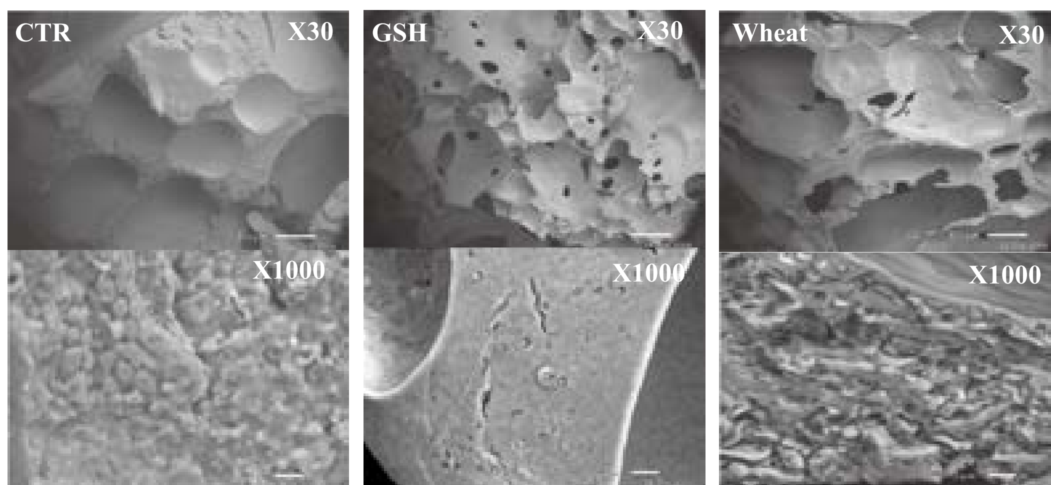


Fig. 5. Low-vacuum scanning microscopic analysis of control rice (CTR), glutathione-added rice bread (GSH) and wheat bread (Wheat)

From ref. 52. Copyright (2010) American Chemical Society.

we[51] have recently found that reduced glutathione (GSH) and oxidized glutathione (GSSG) work differently on rice proteins. A proteomic approach should be beneficial in clarifying the mechanism, and will provide information which should be valuable in developing foods with better properties and digestibility and reduced-allergenicity. To date, however, disulfide-linked large protein networks remain to be investigated, as the molecular size limit of the non-reducing/reducing 2-DE is less than 200 kDa, and such macromolecules are too large to enter the polyacrylamide gel matrix. Thus, if a proteomic technique is developed that identifies disulfide changes of macromolecular proteins, it will be utilized effectively in the food industry.

2. Application to medicine: Biomarker development

As mentioned above, the Trx system is related to a broad range of cellular processes, including DNA synthesis, defenses against oxidative stress, apoptosis, and redox signaling[18]. Accordingly, alteration of the cellular redox balance may result in significant pathological alterations and human diseases. Many research results have demonstrated that the quantity of proteins responsible for the mammalian Trx system varies in many diseases[18]. It is also well-known that tumors possess an altered redox status, lower pH, and nutrient supply limitations. Under these conditions, various malignant tissues/cells have an increased expression of Trx and Trx-reductase (TrxR), which may be associated with aggressive tumor growth and poor survival[19,42,48]. Moreover, higher levels of Trx1 and TrxR1, the isoforms of Trx and TrxR, in blood plasma have been used as molecular markers of inflammation, cancer, and human immunodeficiency virus (HIV) infection[39]. These findings may result from protection against apoptosis and the promotion of cell growth[2,38,46]. Meanwhile, inhibition assays *in vitro* and *in vivo* have suggested that TrxR is a novel target of antitumor therapy[46,50].

Cellular metabolic processes generate reactive oxygen and nitrogen oxide species (ROS/RNS) as byproducts. Meanwhile, cys-thiol modifications by ROS are associated with aging[17] and have been identified in various diseases with oxidative stress, including type II diabetes, cancer, neurodegenerative diseases such as Alzheimer's disease, and cardiovascular disease[8]. Thus, redox status in human diseases has a close relevance to proteome-wide alterations in related cells, tissues, and/or biofluids.

In clinical practice, there is a need for biomarkers to help improve treatment outcomes. The term "biomarker" refers to a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic intervention[5]. At the protein level, it is estimated that disease-related proteins would be closely correlated in quantity with the clinical characteristics, *e.g.* histopathological observations of the tissues[21], the onset and prognosis of the related disease[21,28], and the effects/side effects of clinical treatment[28,41]. To date, most biomarker investigations have targeted the intact protein molecules or the degradation-generated peptides involved in the specimens[24,27]. However, there is increasing evidence that PTM moieties can alter structurally or quantitatively in a single protein with relevance to the disease states[4,10]. Recent advances in proteomic technologies have enabled comprehensive and systematic identification of the candidate biomarkers based on altered thiol redox-/disulfide-PTMs[8]. For example, Parkinson disease protein 7 (protein DJ-1) in its oxidized form is a biomarker for cancer and neurodegenerative disease. Using the OxICAT method[25], based on differential thiol-labeling and trapping, Chiappetta et al.[7] have shown the selective oxidation at Cys106 of DJ-1 via disulfide linkage with glutathione or another protein.

Following the discovery phase, the proteomic candidate markers are subjected to validation: Usually, the same clinical specimens as those used for the discovery study as well as other sample sets from the larger population are investigated for their statistical significance, as indicated by proteome analysis. In this phase, conventionally immunological detection methods with specific antibodies have selected and narrowed these candidates towards routine assay in the clinical utility. A lack of antibodies recognizing oxidized Cys residues, however, hampers these strategies. Mass spectrometry (MS)-based assay is promising for measuring modifications in the redox moieties of proteins, due to its high detection sensitivity and molecule selectivity as well as the potential for high-throughput analysis. Niwa[40] has reported elevated glutathionylation levels of hemoglobin in patients with diabetes, hyperlipidemia, uremia, and Friedreich's ataxia, where liquid-chromatography (LC)/electrospray ionization (ESI)/MS were used. Recently, Yamada and coworkers[22,23] established an analytical system using oxidized human serum albumin. Serum albumin has Cys34 as the only free thiol group. Various human conditions, *e.g.* hepatic disease diabetes, renal disease, and aging, can cause several types of partial oxidation of this residue, including disulfide-binding with Cys[22]. LC/ESI/time-of-flight (TOF) MS of intact proteins has been used to monitor the ratio of oxidized forms of serum albumin in plasma or whole blood. Notably, the acidification of specimens stabilized the modifications of the thiol groups, enabling precise determination of the proportion of oxidized and reduced forms[7]. These examples contain useful informa-

tion for the preparation of post-proteomics thiol assay systems.

Redox status with Cys-thiol modification is a potential target for a molecular indicator of various diseases. Disulfide-related proteomics, meanwhile, may provide clues to early diagnostics and therapy. Establishment of the assay systems will lead to mass-screening, *i.e.* screening of larger populations, demonstrating the clinical utility of the candidate modifications. Bridging the gap between research findings and clinical practice, technical improvements to stably detect and quantify the redox alteration will facilitate biomarker development directed by disulfide-related proteomics.

Concluding remarks

The disulfide proteomics has improved our knowledge of redox biology. It will also be useful for applicable studies, e.g. in the food industry or medical research. As technology progresses, proteomic studies will overcome challenging problems and contribute significantly to basic as well as applicative studies (Fig. 6). This knowledge will eventually spawn the development of high-yield crops, rice breads and clinical biomarkers that should benefit developing countries and may also boost the global quality of life.

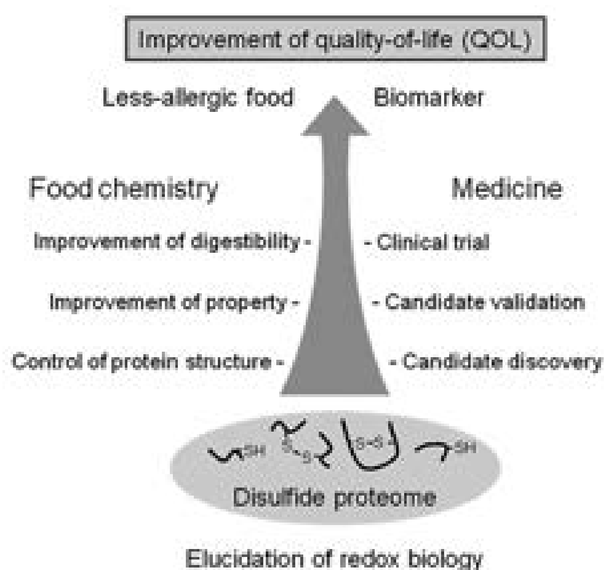


Fig. 6. Future direction of disulfide proteomics
Improvement of human quality-of-life (QOL) is a shared goal for disulfide proteomics applications. Based on knowledge of redox biology, industrial research works have identified various candidate redox PTMs potentially useful in practice. The following long-term improvement/validation processes will support the development of products such as less-allergenic food and clinical biomarkers.

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