

Notes & Tips

## General method for facile intramolecular disulfide formation in synthetic peptides

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There are several methods by which intramolecular disulfide bonds are formed in synthetic peptides and proteins. Most procedures are time consuming, requiring several concentration and purification steps to ensure the exclusive formation of intramolecular disulfides over intermolecular disulfide-bonded aggregates. Herein we describe a simple procedure to form intramolecular disulfide bonds in dicysteine peptides under the dilute, highly acidic, mixed solvent systems of preparatory reversed-phase (RP)<sup>1</sup> HPLC.

Oxidation procedures for forming single intramolecular disulfide bonds have been extensively reviewed [1]. A procedure common to many peptide labs involves cleaving the peptide from resin and subsequently purifying the reduced peptide by RP-HPLC. After lyophilization, solid peptide is dissolved in basic ammonium acetate (pH 8–9) under dilute conditions (10–100  $\mu$ M). Trace metals in the buffer catalyze the air oxidation over several hours to days, depending on amino acid sequence. Although reaction times can be greatly accelerated by use of exogenous oxidizing agents (iodine, dimethyl sulfoxide, glutathione, potassium ferricyanide, thallium trifluoroacetate, or silver triflate [1] and some metal complexes of Pt<sup>4+</sup> [2,3]), their use may result in the overoxidation of cysteine and undesirable side chain oxidations of tryptophan, tyrosine, and methionine. After oxidation, the resulting solution must be lyophilized a second time and the peptide further purified by RP-HPLC and recovered as a solid after a third lyophilization.

Although a resin-based method has been reported for the oxidation of peptides [4], we were interested in developing a solution-phase strategy in which reduced peptide eluting directly from the preparative HPLC could be quickly intramolecularly oxidized, thereby significantly limiting the number of manipulations outlined above.

We found that 4,4'-dithiodipyridine (4-PDS) can greatly facilitate the intramolecular oxidation of peptides under the acidic conditions (pH 1.8) typical of mixed solvent systems encountered during RP-HPLC preparative purifications, generally a mixture of standard A (0.1% trifluoroacetic acid in Milli-Q water) and standard B (0.1% trifluoroacetic acid in 9:1 acetonitrile:water). Previous to this report Maruyama et al. [5] reported that 2,2'-dithiodipyridine (2-PDS) could facilitate the formation of intramolecular disulfides at pH 4.0 in a purely aqueous buffer. However, the rate at which nucleophilic thiols add to 2-PDS has been shown to decrease with decreasing solvent dielectric (typical of the mixed solvents used in RP-HPLC), whereas, thiol addition to 4-PDS is little effected [6]. We surmised that 4-PDS added directly to HPLC eluent containing reduced peptide could promote facile intramolecular disulfide bond formation. Peptide I, an arsenic (III) binding helix with a tyrosine residue [7], and peptide II, a redox-sensitive peptide with both tryptophan and methionine residues [8], were used as archetypes for the following protocol.

Peptide I: Ac-YGGKAAACKAACAKAAAA-NH<sub>2</sub>

Peptide II: H<sub>3</sub>N<sup>+</sup>-VTWC $\underline{G}$ H $\underline{C}$ KM-NH<sub>2</sub>

Peptides are cleaved from solid support and side-chain deprotected as described previously [7,8]. Reduced peptides are RP-HPLC purified to homogeneity as seen

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<sup>1</sup> Abbreviations used: RP, reversed-phase; 4-PDS, 4,4'-dithiodipyridine; 2-PDS, 2,2'-dithiodipyridine.

in Fig. 1A (peptide I shown only). Collected fractions from multiple injections are pooled, and the concentration of the reduced peptide is determined using Ellman's reagent [9]. The concentration of pure reduced peptide is typically found to be 10–100  $\mu\text{M}$ , depending on the preparative conditions. A slight excess (1.2 equivalents) of 4-PDS is added from a 10 mM stock in methanol directly to the pooled solution. The reaction progress can be monitored by analytical HPLC, as seen in Fig. 1B, where peptide I was completely oxidized after 2.5h. Peptide II was completely oxidized in under 2min (data not shown). Oxidation rates can be increased by raising the pH or adding additional equivalents of 4-PDS, but the reaction is more prone to mixed disulfide (mono- or disubstituted thiopyridyl peptide) formation and higher-order aggregates under these more aggressive conditions.

Crude oxidation reaction mixtures are concentrated to one tenth their original volume by rotary evaporation, which removes most of the acetonitrile (note: when concentrated by lyophilization, extensive disul-

fide bond shuffling was observed). This concentrated crude (Fig. 1C) is then applied directly onto a C-18 Sep-Pak column, equilibrated with 100% standard A. Elution with 10 column volumes of 100% standard A removes the thiopyridine by-product (Fig. 1D), followed by 10 column volumes of 95% standard A which removes unreacted 4-PDS (Fig. 1E). The oxidized peptide is then eluted with 10 column volumes of the appropriate ratio of standard A and B, in this case of 80% standard A (Fig. 1F). The eluted peptide can then be lyophilized to afford pure oxidized product.

By this method, intramolecular disulfides were formed by the simple addition of 4-PDS to pooled RP-HPLC-purified dithiol model peptides, with little or no side reactions. This procedure eliminates the need for intermediary lyophilization steps common to other oxidation protocols, disfavors dithiol–disulfide exchange reactions due to the very low pH conditions, and saves time and solvent in subsequent purification steps.

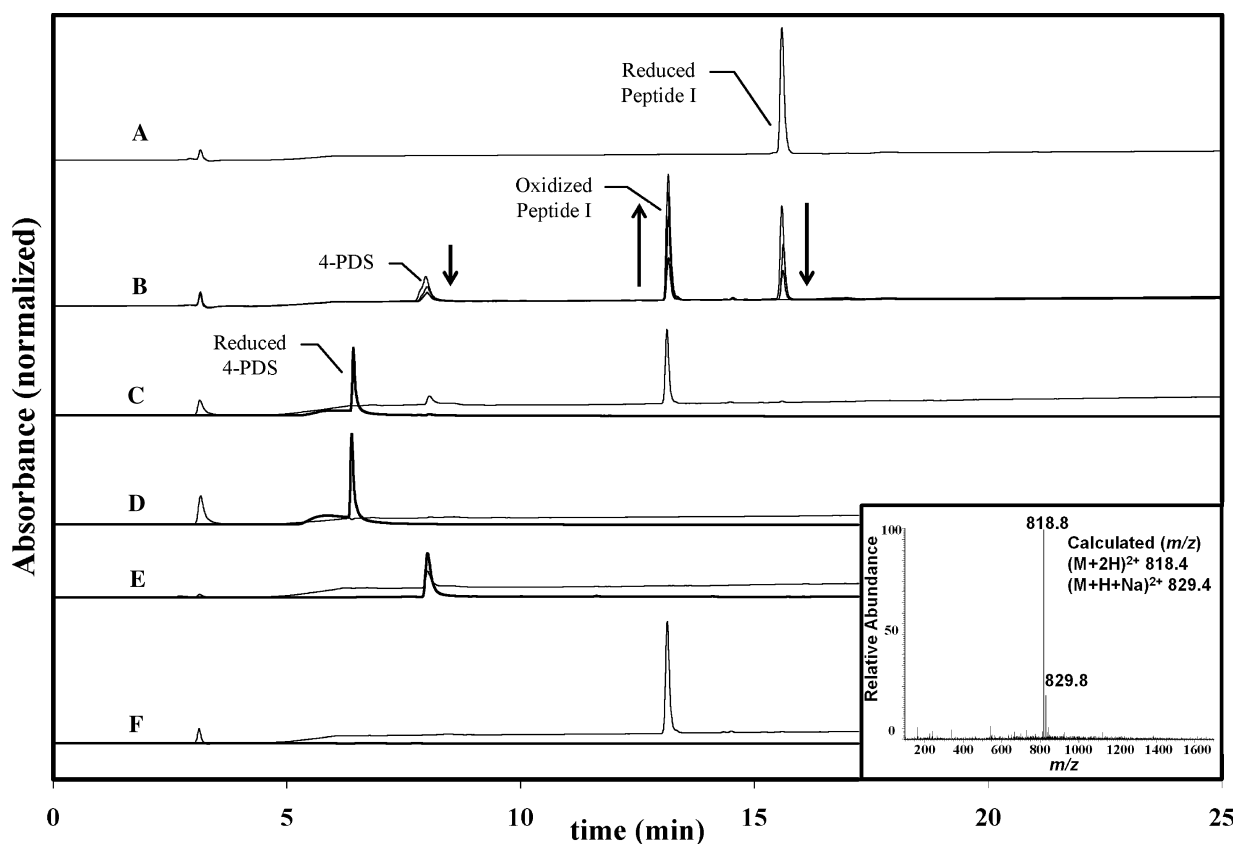


Fig. 1. Assessing reaction progress for the oxidation of peptide I. Linear gradient from 0% standard B to 100% standard B over 50min, monitoring at 220nm (A–F, thin line) and 320nm (C–F, boldfaced line). (A) Pure reduced peptide I. (B) Oxidation monitored over 3h; four time points are shown and arrows indicate observed change in peak intensities with time. (C) Crude oxidized peptide I, after concentration step. (D) Reacted 4-PDS eluted in the first Sep-Pak fraction; 100% standard A, 10 column volumes. (E) Unreacted 4-PDS eluted in the second Sep-Pak fraction; 95% standard A, 10 column volumes. (F) Oxidized peptide eluted in the third Sep-Pak fraction; 80% standard A; 10 column volumes. (Inset) Electrospray ionisation MS of oxidized peptide I is consistent with intramolecular disulfide bond formation.

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