INSTRUCTIONS

SPDP Crosslinkers

21650 21651 21857

0279.4

Number	Description
21650	**Sulfo-LC-SPDP**, sulfosuccinimidyl 6-[3´(2-pyridyldithio)-propionamido] hexanoate, 50mg Molecular Weight: 527.57 Spacer Arm Length: 15.6Å
21651	**LC-SPDP**, succinimidyl 6-[3´-(2-pyridyldithio)-propionamido] hexanoate, 50mg Molecular Weight: 425.52 Spacer Arm Length: 15.6Å
21857	**SPDP**, *N*-succinimidyl 3-(2-pyridyldithio) propionate, 50mg Molecular Weight: 312.37 Spacer Arm Length: 6.8Å

Storage: Upon receipt store Product No. 21651 at 4°C protected from moisture. Store Product No. 21650 and 21857 at -20°C protected from moisture. Products are shipped at ambient temperature.

Introduction

The Thermo Scientific SPDP reagents (Figure 1) are a unique group of amine- and sulfhydryl-reactive heterobifunctional crosslinkers. Whether they are used to form amine-to-amine or amine-to-sulfhydryl crosslinks among molecules, the SPDP reagents produce disulfide-containing linkages that can be cleaved later with reducing agents such as dithiothreitol (DTT).

The amine-reactive portion of SPDP reagents is the *N*-hydroxysuccinimide (NHS) ester. Reactions are most commonly performed in phosphate, carbonate/bicarbonate, or borate buffers at pH 7-8. Other buffers can be used provided they do not contain primary amines (or thiols or disulfide reducing reagents – see discussion of the sulfhydryl reactivity). The rate of reaction and degradation by hydrolysis increases with increasing pH; for example, the half-life of the NHS ester is several hours at pH 7 and less than 10 minutes at pH 9. NHS-ester reagents like SPDP and LC-SPDP have limited aqueous solubility and must be dissolved in organic solvent before adding them to a reaction mixture. Sulfo-NHS-ester reagents like Sulfo-LC-SPDP are water-soluble and can be added directly to aqueous reaction mixtures.

The sulfhydryl-reactive portion of SPDP reagents is the 2-pyridyldithio group, which reacts optimally with sulfhydryls between pH 7 and 8.[1] The reaction results in displacement of a pyridine-2-thione group, the concentration of which can be determined by measuring the absorbance at 343nm (see Additional Information section). Reaction buffers must be free of thiols and disulfide reducing agents until quenching or reduction of the 2-pyridyl disulfide is desired.

Two basic strategies can be used to form cleavable crosslinks between proteins with SPDP reagents, depending whether one or neither protein already possesses sulfhydryl groups (-SH) in addition to primary amines (Figure 2). Both conjugation methods result in crosslinks that contain a disulfide bond in the spacer arm, which can be cleaved by reduction with dithiothreitol (DTT) or other reducing agent. In most cases, crosslinks created using SPDP reagents can be cleaved with 25 mM DTT at pH 4.5 without reducing native protein disulfide bonds. However, when preservation of native disulfide bonds is not a concern, cleavage with DTT can be performed most efficiently at pH 7-9.[1]

Crosslinking experiments with SPDP reagents are not limited to those involving proteins. Any of a variety of molecules with primary amines ($-NH_2$) and sulfhydryl groups can be modified or crosslinked using an SPDP reagent (see Additional Information section).

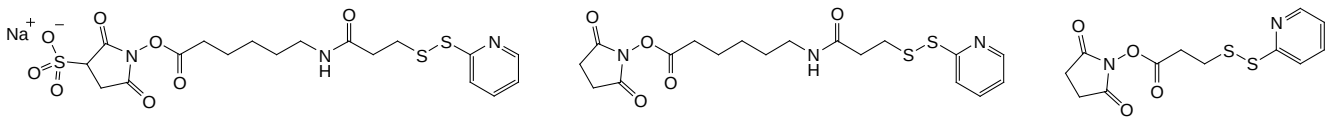

Figure 1. Structures of the SPDP reagents.

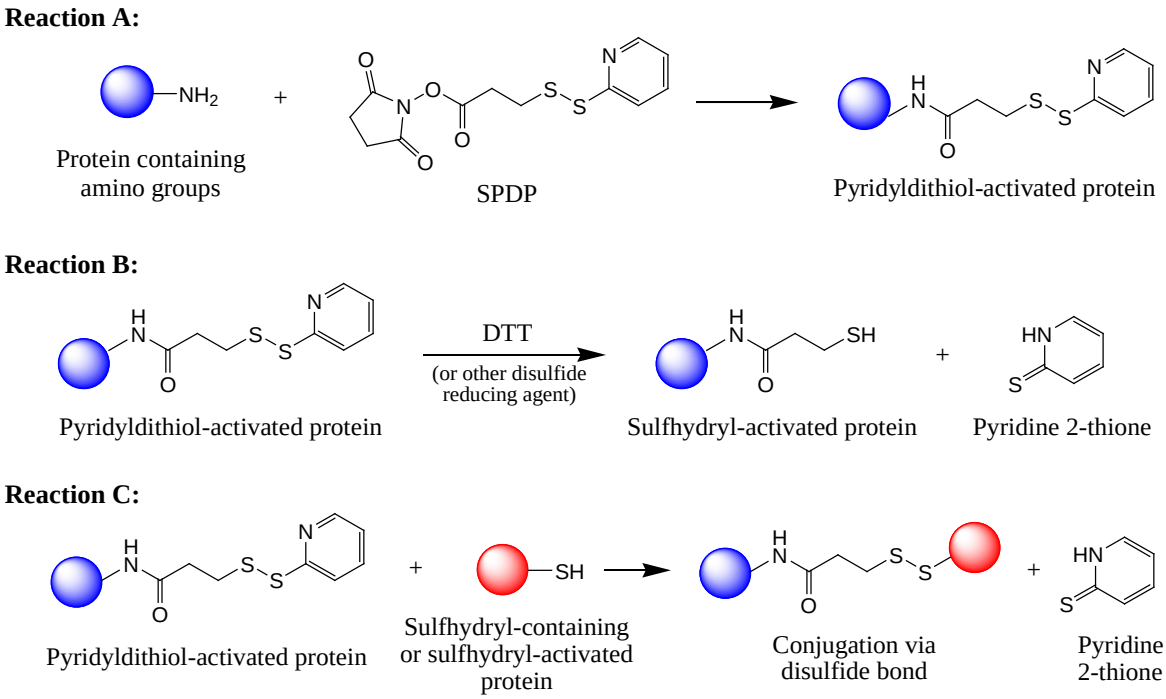

Figure 2. Two protein-to-protein conjugation strategies involving SPDP Reagents. If one protein already contains sulfhydryl groups, then only one protein must be modified by the SPDP reagent and only Reactions A and C are used. If neither protein contains available sulfhydryl groups, then both proteins are modified separately with SPDP reagent (Reaction A), one protein is treated with reducing agent to expose sulfhydryl groups (Reaction B), and finally the two proteins are conjugated (Reaction C).

Procedure for Conjugating Two Proteins, One of Which Contains Sulfhydryls

To conjugate between primary amines on one protein and sulfhydryls (-SH) already present on a second protein, only the first protein must be modified with the SPDP reagent.[2] Then, after desalting the SPDP-modified protein to remove reaction byproducts and excess nonreacted reagent, the sulfhydryl-containing protein is added to make the final conjugate (Figure 2, Reaction 1).

A. Additional Materials Required

- Water-miscible organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF); not necessary for Sulfo-LC-SPDP (Product No. 21650)
- Phosphate buffered saline with EDTA (PBS-EDTA): 100mM sodium phosphate, 150mM NaCl, 1mM EDTA, 0.02% sodium azide, pH 7.5
- Desalting column (e.g., Thermo Scientific Zeba Spin Desalting Columns, Product No. 89891) sufficient to process 1-2mL samples
- Proteins to be conjugated: In the procedure, IgG and β-galactosidase represent the amine-containing and sulfhydryl-containing protein, respectively.

B. Procedure

1. Equilibrate the vial of SPDP Reagent to room temperature before opening.
2. Immediately before use, prepare a 20mM solution of SPDP Reagent:
   - For Sulfo-LC-SPDP (Product No. 21650), dissolve 2mg reagent in 200µL of ultrapure water.
   - For LC-SPDP (Product No. 21651), dissolve 2mg reagent in 235µL of DMSO or DMF.
   - For SPDP (Product No. 21857), dissolve 2mg reagent in 320µL of DMSO or DMF.
3. Add 25µL of the 20mM SPDP solution to 2-5mg IgG dissolved in 1.0mL of PBS-EDTA.
4. Incubate for 30 minutes at room temperature.
5. Equilibrate a desalting column with PBS-EDTA, and buffer exchange the SPDP-modified IgG to remove reaction byproducts and excess nonreacted SPDP reagent.
6. Add 1-3mg of β-galactosidase (~1-3 moles β-gal per mole of IgG) to the IgG solution and incubate the reaction mixture overnight at room temperature.
7. The proteins (IgG and β-galactosidase in this example) are now conjugated.

Procedure for Conjugating Proteins, Neither of Which Contains Sulfhydryls

To conjugate between primary amines of both proteins, each protein must be separately modified with SPDP reagent.[3] The pyridine-2-thione group is then removed from one of the modified proteins using reducing agent. Finally, after removing the reducing agent using a desalting column, the resulting sulfhydryl-modified protein and the SPDP-modified protein are incubated together to make the final conjugate (Figure 2, Reaction 2).

A. Additional Materials Required

- Water-miscible organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF); not necessary for Sulfo-LC-SPDP (Product No. 21650)
- Phosphate buffered saline with EDTA (PBS-EDTA): 20mM sodium phosphate, 150mM NaCl, 1mM EDTA, 0.02% sodium azide, pH 7.5. **Note:** If acetate buffer will be used in section C, do not exceed 20mM sodium phosphate in this buffer. Otherwise 20-100mM sodium phosphate can be used.
- Acetate buffer: 100mM sodium acetate buffer, 100mM NaCl, pH 4.5
- Desalting columns (e.g., Zeba™ Spin Desalting Columns, Product No. 89891) sufficient to process 1-2mL samples
- Dithiothreitol (DTT), Product No. 20290
- Two proteins to be conjugated

B. SPDP-Modify Both Proteins in Separate Reactions

Note: Perform this section of the procedure separately for both proteins.

1. Equilibrate the vial of SPDP Reagent to room temperature before opening.
2. Immediately before use, prepare a 20mM solution of SPDP Reagent:
   - For Sulfo-LC-SPDP (Product No. 21650), dissolve 2mg reagent in 200µL of ultrapure water.
   - For LC-SPDP (Product No. 21651), dissolve 2mg reagent in 235µL of DMSO or DMF.
   - For SPDP (Product No. 21857), dissolve 2mg reagent in 320µL of DMSO or DMF.
3. Add 25µL of the 20mM SPDP solution to 2-5mg protein dissolved in 1.0mL of PBS-EDTA.
4. Incubate for 30-60 minutes at room temperature.
5. Equilibrate two desalting columns with PBS-EDTA, and buffer exchange the SPDP-modified proteins to remove reaction byproducts and excess nonreacted SPDP reagent. Alternatively, the samples can be dialyzed using a Thermo Scientific Slide-A-Lyzer Dialysis Cassette and concentrated using Slide-A-Lyzer® Concentrating Solution.
6. At this point, both proteins are SPDP-modified and one of them must be reduced (section C) before conjugation.

C. Reduce SPDP Disulfides in One Protein

1. Choose only one of the two SPDP-modified proteins for this section of the procedure. Use the protein whose function or activity is least dependent on native disulfide bonds.
2. Dissolve 23mg DTT in 1mL acetate buffer or PBS-EDTA (makes 150mM solution). Use acetate buffer to avoid reducing native disulfide bonds in the protein.
3. Add 0.5mL DTT solution per 1mL of SPDP-modified protein (results in 50mM DTT).
4. Incubate for 30 minutes.
5. Equilibrate a desalting column with PBS-EDTA, and desalt the protein (now sulfhydryl-modified) to remove the DTT.

D. Conjugate the Activated Proteins

1. Mix the SPDP-modified and sulfhydryl-modified proteins and incubate for 18 hours at room temperature or 4°C.
2. To isolate conjugate from the two unconjugated proteins, use an appropriate size exclusion chromatography method.

Additional Information

A. Please visit the web site for additional information on this product including the following items:

- Tech Tip #1: Attach a protein onto glass, silica or quartz surface using a cleavable crosslinker
- Tech Tip #26: Prepare a reversible and measureable sulfhydryl-reactive affinity column

B. Protocol For Pyridine-2-Thione Assay to Determine Level of SPDP-modification

1. Dilute 100µL of SPDP-modified and desalted protein to 1mL with PBS.
2. Measure and record the absorbance at 343nm of the protein sample compared to PBS-EDTA blank (test in triplicate).
3. Add 10µL of 15mg/mL DTT to the 1mL SPDP-modified protein sample, mix.
4. After exactly 15 minutes, measure and record the absorbance at 343nm of the reduced sample.
5. Calculate the change in absorbance: $\Delta A343$ = (Ave. A343 after DTT) – (Ave. A343 before DTT)
6. Calculate molar ratio of SPDP to Protein using the following equation:

$$\frac{\Delta A}{8080} \times \frac{\text{MW of Protein}}{\text{mg/mL of Protein}} = \text{moles of SPDP per mole of Protein}$$

Where the value 8080 reflects the extinction coefficient for pyridine-2-thione at 343 nm: 8.08 x 10^3 $M^{-1}cm^{-1}$.

Cited References

1. Carlsson, J., et al., (1978). Protein thiolation and reversible protein-protein conjugation. *Biochem J* **173:** 723-7.
2. Cumber, A. J., et al., (1985). Preparation of antibody-toxin conjugates. *Meth Enzymol* **112:** 207-25.
3. Neurath, A. R. and Strick, N. (1981). Enzyme-linked fluorescence immunoassays using β-galactosidase and antibodies covalently bound to polystyrene plates. *J Virol Meth* **3:** 155-65.