Introduction

Biotin-HPDP, or \( N\text{-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide} \), is a unique reagent that offers several key features and advantages for biotinylating proteins. It reacts specifically with thiol groups. The Biotin residue imparted by Biotin-HPDP can be cleaved away from the reacted sulfhydryl to regenerate the protein (or peptide) in its original, unmodified form. Additionally, the reaction of Biotin-HPDP with thiols can be easily followed with spectrophotometry since the reaction's leaving group, Pyridine-2-thione, has a characteristic absorption maximum at 343 nm with an extinction coefficient of 8.08 +/- 0.3 x 10³ M⁻¹ cm⁻¹.¹

When the protein of interest is an antibody, it can be advantageous to biotinylate in a manner that will maintain immunologic activity. Many Biotin derivatives react with primary amine groups and consequently can interfere with antigen binding. Since Biotin-HPDP is reactive towards sulphydryls, this problem can be surmounted. By reducing the immunoglobulin under mild conditions, biotinylation can be isolated to the hinge region. This approach preserves the antigen binding site of the immunoglobulin.

The Biotin binding site of Avidin, reported to be 9 Å below the surface of the avidin molecule, can be sterically hindered when certain amino acids or glycosylated residues are present near the Lysine necessary for Biotin binding.² Optimal antigen binding capabilities can be realized by using a Biotin derivative that has an extended spacer arm, thus reducing this steric hindrance. The spacer arm also improves the complex formation of Biotin with the deep Biotin binding site of Avidin. Biotin-HPDP has such a spacer arm to give a span of 29.2 Å between the reacted sulfhydryl and the attached Biotin group. This affords an increase in sensitivity of detection by Avidin due to the reduction of steric hindrance by macromolecules modified in this manner.

Biotin-HPDP as a cleavable biotinylation reagent has an additional advantage over other cleavable reagents such as NHS-SS-Biotin. After cleaving Biotin away from a protein reacted with NHS-SS-Biotin, each original reacted -NH₂ group will be changed to a -NH₂-(CH₂)₂-SH residue. In summary, the original protein (or peptide) can not be regenerated after cleavage from Biotin. Biotin-HPDP, however, enables the researcher to regenerate the starting protein in its original unmodified form, which is advantageous in many applications.

While the full potential of Biotin-HPDP has not yet been revealed in the literature, Biotin HPDP can be expected to extend the utility of applications developed with NHS-SS-Biotin. These include applications for biotinylating a ligand and using this ligand for receptor purification by pulling the receptor/ligand complex out of a detergent solubilized membrane preparation with immobilized Avidin.³ The fact that Biotin-HPDP is sulphydryl reactive and cleavable opens several possibilities for applications involving reduced IgG or Fab⁻⁻ molecules.

Considerations for the use of Biotin-HPDP

Proteins or peptides to be biotinylated by Biotin-HPDP must have a free sulphydryl group (-SH) available. If this group is not present, it can be introduced into the molecule with Traut's Reagent (Prod. No. 26101). For peptide synthesis, an alternative
is to terminate with a Cysteine residue. Reactions with Biotin-HPDP should be carried out in buffers free of extraneous thiols and consequently excess β-Mercaptoethanol, Mercaptoethylamine, etc., should be removed prior to biotinylation.

Proteins can be biotinylated via sulphydryls on their surfaces. Alternatively, free thiols can be generated from disulfides by incubating with a reducing agent. Mercaptoethylamine (MEA) can be used with IgG or F(ab')2 molecules to cleave the disulfides between the heavy chains while preserving the disulfide linkages between the heavy and light chains. With Mercaptoethylamine cleavage, EDTA is included for its antioxidative effect. Thiol groups in the hinge region of IgG molecules are fairly stable in the presence of EDTA although much less stable in the absence of EDTA than those of Fab'.

The number of thiol groups decreases only slightly in the presence of EDTA. In 0.1 M Sodium Phosphate at pH 6-7, 4°C, the number of free thiols decreased 63-90% and 15-25% in the absence of EDTA and only 7% and 9% in the presence of EDTA with reduced IgG and Fab', respectively, over a 40-hour incubation.

The example protocols listed below can be adapted as necessary for specific applications. Biotin-HPDP can be dissolved in a variety of dipolar aprotic solvents, with Dimethylsulfoxide (DMSO) being a typical choice. Biotin-HPDP would be slightly more soluble in Dimethylformamide (DMF). The biotinylation reaction can be carried out in a variety of buffers and at a pH range of 6-9. In addition to using Dithiothreitol, Biotin can also be cleaved away from the biotinylated protein by using 100 mM β-Mercaptoethanol or 1% Sodium Borohydride. Note, however, that these two reducing agents may reduce the disulfide bonds between the heavy and light chains of IgG and Fab'. Additionally, Sodium Borohydride, being a very strong reducing agent, may destroy functional groups on proteins or peptides.

**Representative Protocols**

**Materials and Stock Solutions**

4 mM Biotin-HPDP stock solution. Prepare by adding 2.2 mg Biotin-HPDP to 1.0 ml of dry Dimethylformamide (DMF).

Note: Gentle warming of the Biotin-HPDP/DMF mixture to 37°C and vortexing or sonication will assure complete dissolution. This stock can be aliquoted and stored frozen.

Phosphate-Buffered Saline/EDTA Buffer, PBS-EDTA. The composition of this buffer is 20 mM Sodium Phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.4.

Desalting Column. Such as Pierce Prod. No. 43243, with a 6,000 MW cut-off or equivalent.

Mercaptoethylamine•HCl. Pierce Prod. No. 20408. Used to reduce IgG.

Dithiothreitol. Pierce Prod. No. 20290. Used to cleave Biotin from the reacted protein.

Immobilized Avidin Column. Pierce Prod. No. 20362. Used to bind the biotinylated proteins and to sequester the Biotin after cleavage from the proteins.

**Example Protocols**

I. **Biotinylation of β-D-galactosidase**

1. Dissolve 4 mg of β-D-galactosidase in 2.0 ml PBS-EDTA.
2. Add 0.1 ml of the Biotin-HPDP/DMF solution to 2.0 ml of the β-D-galactosidase solution.
3. Vortex well and incubate for 90 minutes at room temperature.

   **Note:** the progress of the reaction can be monitored by following the change in absorbance at 343 nm due to the release of Pyridine-2-thione.

4. Purify the Biotin-S-S-galactosidase by applying the mixture to a 15 ml desalting column (15 cm x 1.5 cm, 6,000 MW cut-off) that has been pre-equilibrated with PBS-EDTA. Discard the column effluent. Add 2 ml of PBS-EDTA and again discard the column effluent. Add 5 ml of PBS-EDTA and collect the effluent as a single fraction. This fraction will contain Biotin-S-S-galactosidase.

II. **Biotinylation of IgG with Biotin-HPDP Preparation of reduced IgG**

1. Dissolve 40 mg of IgG in 2.0 ml of 0.1 M Sodium Phosphate buffer, 5 mM EDTA, pH 6.0.
2. Add 14.0 mg of 2-Mercaptoethylamine•HCl, mix and incubate at 37°C for 90 minutes.

3. Cool the solution to room temperature and perform a buffer exchange to remove excess Mercaptoethylamine. Apply the mixture to a 15-ml desalting column (15 cm x 1.5 cm, 6,000 MW cut-off) that has been pre-equilibrated with PBS-EDTA buffer. Discard the column effluent. Add 2 ml of PBS-EDTA and again discard the column effluent. Add 5 ml of PBS-EDTA.

4. Collect effluent as a single fraction. This fraction will contain reduced IgG.

**Reaction of Biotin-HPDP with reduced IgG**

1. Add 0.1 ml of the Biotin-HPDP stock solution to 2.0 ml of the reduced IgG solution from Step 4 above.

2. Vortex well and incubate for 90 minutes at room temperature. 

   **Note:** the progress of the reaction can be monitored by following the change in absorbance at 343 nm due to the release of Pyridine-2-thione.

3. Purify the Biotin-S-S-IgG by applying the mixture to a 15-ml desalting column (15 cm X 1.5 cm, 6000 MW cut-off) that has been pre-equilibrated with PBS-EDTA. Discard the column effluent. Add 2 ml of PBS-EDTA and again discard the column effluent. Add 5 ml of PBS-EDTA and collect effluent as a single fraction. This fraction will contain Biotin-S-S-IgG.

**References**


