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Protocol for Reduction of MSSM

A 1 mg sample of MSSM contains 5 mg total solid of which 4 mg is NH_4^+ TFA^- salt from HPLC purification.

Our studies indicate that this salt improves the stability of the product (particularly for shipping) and hence it was not removed.

The following four-step procedure can be used to provide pure MSH from MSSM:

Step 1) Dissolve MSSM: To reduce a 1 mg MSSM sample dissolve the white solid in 300 µl of 25 mM $NH_4^+CO_3^-$ pH 7.8 or 25 mM HEPES pH 8.0 or 25 mM Tris•HCl pH 8.0.

Step 2) Reduce with DTT: Add dithiothreitol (DTT) to a concentration of 20 mM from a freshly prepared 1M stock solution. Allow to reduce at room temperature for 20 min. Acidify the solution with 10% TFA in H_2O or 10% HCl in H_2O to pH 3-5 using pH strip as an indicator. The mildly acidic pH will minimize the oxidation of the thiol during extraction of the DTT.

Step 3) Extract DTT with ethyl acetate (EtOAc): Mycothiol is very hydrophilic and is not soluble in EtOAc, DTT on the other hand will partition between both layers. Extract the reduced MSH in a 1.5 ml polypropylene microcentrifuge tube (ideally using a tube with a screw cap) at least 5 times with an equal volume EtOAc. For each extraction, centrifuge the sample for 1 min at 5000×g to separate the phases. Remove the EtOAc phase using a pipettor (aspirator not recommended) and monitor the DTT content of the EtOAc phase using the DTNB assay by shaking 5 µl of EtOAc solution with 100 µl of 0.2 mM DTNB working stock.[1] Determine the desired final DTT content of the MSH stock and assume it will be the same as the EtOAc phase DTT content (the partition coefficient of DTT is 1). We recommend <0.1 mM residual DTT in a 10 mM MSH stock (typical after 5 extractions). Note that each DTT generates 2 equivalents of TNB anion with an extinction coefficient 14.3 mM^{-1} at 412 nm.

Step 4) Remove the EtOAc: Next, remove final traces of EtOAc using a speedvac and simultaneously decrease the sample volume to 0.2 mL. The theoretical final MSH concentration is 10 mM at 0.2 mL. The final MSH solution concentration can be measured using the DTNB assay.

Step 5) Storage: Small aliquots of reduced MSH should be frozen at -80°C in vials charged with an argon atmosphere for later use.

[1]DTNB assay: working stock of DTNB is a freshly made 0.2 mM DTNB (from a 10 mM stock in 100 mM Tris•HCl pH 8.0 stored at 4°C) in 100 mM Tris•HCl pH 8.0. Dilute the thiol-containing sample to 0.1 mM RSH into the DTNB working stock according to the method of Ellman. Read the solution in a spectrophotometer at 412 nm and assume an extinction coefficient of 14.3 mM^{-1}. GL Ellman, *Arch. Biochem. Biophys*. **82**, 70 (1959).