The disclosed methods, in contrast, can be performed at high concentrations on a small or large (*e.g*, industrial) scale to provide properly refolded complex proteins. The ability to refold proteins at high concentrations and at large scales can translate into not only enhanced efficiency of the refold operation itself, but also represents time and cost savings by eliminating the need for additional equipment and personnel. Accordingly, a method of refolding proteins present in high concentrations could translate into higher efficiencies and cost savings to a protein production process.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a series of plots depicting depicting the effect of thiol-pair ratio and redox buffer strength on product-species distribution; Figure 1a depicts the effect of a 5 mM buffer strength; Figure 1b depicts the effect of a 7.5 mM buffer strength; Figure 1c depicts the effect of a 10 mM buffer strength; Figure 1d depicts the effect of a 12.5 mM buffer strength; Figure 1e depicts the effect of a 15 mM buffer strength and Figure 1f depicts the effect of a 20 mM buffer strength.

Figure 2 is a series of plots depicting the effect of the degree of aeration on the species distribution under fixed thiol-pair ratio and thiol-pair buffer strength.

Figure 3 is an analytical overlay of a chemically controlled, non-aerobic refold performed at 6 g/L and optimized using an embodiment of the described method performed at 1L and 2000L.

SUMMARY OF THE INVENTION

In a first aspect, the present invention refers to a method of refolding a complex multimeric protein expressed in a non-mammalian expression system and present in a volume at a concentration of 2.0 g/L or greater comprising:

(a) contacting the protein with a refold buffer comprising a redox component comprising a final thiol-pair ratio having a range of 0.001 to 100 and a thiol-pair buffer strength of 2 mM or greater and one or more of:

(i) a denaturant;

(ii) an aggregation suppressor; and

(iii) a protein stabilizer;

to form a refold mixture; wherein the thiol-pair ratio is calculated according to Equation 1:

$$\frac{[\text{reductant}]^2}{[\text{oxidant}]}$$

wherein the thiol-pair buffer strength is calculated according to Equation 2:

$$2[\text{oxidant}]+[\text{reductant}]$$

wherein said thiol-pair ratio and said thiol-pair buffer strength are varied in a full or partial factorial matrix; and

wherein the thiol-pair ratio value and thiol-pair buffer strength value used in said refold buffer are those respective values resulting in a higher yield of the desired form of said complex multimeric protein than is obtained prior to said varying of said thiol-pair ratio and said thiol-pair buffer strength;

(b) incubating the refold mixture; and

(c) isolating the protein from the refold mixture.

Also described is a~~A~~ method of refolding a protein expressed in a non-mammalian expression system and present in a volume at a concentration of 2.0 g/L or greater comprising: (a) contacting the protein with a refold buffer comprising a redox component comprising a final thiol-pair ratio having a range of 0.001 to 100 and a redox buffer strength of 2 mM or greater and one or more of: (i) a denaturant; (ii) an aggregation suppressor; and (iii) a protein stabilizer; to form a refold mixture; (b) incubating the refold mixture; and (c) isolating the protein from the refold mixture.

In various ~~embodiments~~examples the redox component has a final thiol-pair ratio greater than or equal to 0.001 but less than or equal to 100, for example within a range of 0.05 to

50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50 and a Thiol-pair buffer strength equal to or greater than 2 mM, for example greater than or equal to 2.25 mM, 2.5 mM, 2.75 mM, 3 mM, 5 mM, 7.5 mM, 10 mM, or 15 mM, wherein the thiol-pair buffer strength is effectively bounded at a maximum of 100 mM. Restated, in terms of ranges, the thiol buffer strength can be between 2 and 20 mM, for example between 2.25 mM and 20 mM, 2.5mM and 20 mM, 2.75 mM and 20 mM, 3 mM and 20 mM, 5 mM and 20 mM, 7.5 mM and 20 mM, 10 mM and 20 mM, or 15 mM and 20 mM, to form a mixture.

In one ~~embodiment~~example of a refold buffer, the refold buffer comprises urea, arginine-HCl, cysteine and cystamine in Tris buffer. In a further ~~embodiment~~example the components are present in the refold buffer in proportions described in Example 3.

In another ~~embodiment~~example of a refold buffer, the refold buffer comprises urea, arginine HCl, glycerol, cysteine, and cystamine in Tris buffer. In a further ~~embodiment~~example the components are present in the refold buffer in proportions described in Example 4.

In some ~~embodiments~~examples, the protein is initially present in a volume in a non-native limited solubility form, such as an inclusion body. Alternatively, the protein is present in the volume in a soluble form. The protein can be a recombinant protein or it can be an endogenous protein. The protein can be a complex protein such as an antibody or a multimeric protein. In another ~~embodiment~~example, the protein is an Fc-protein conjugate, such as a protein fused or linked to a Fc domain.

The non-mammalian expression system can be a bacterial expression system or a yeast expression system.

The denaturant in the refold buffer can be selected from the group consisting of urea, guanidinium salts, dimethyl urea, methylurea and ethylurea. The protein stabilizer in the refold buffer can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes. The aggregation suppressor can be selected from the group consisting of arginine, proline, polyethylene glycols, non-ionic surfactants, ionic surfactants, polyhydric alcohols, glycerol, sucrose, sorbitol, glucose, Tris, sodium sulfate, potassium sulfate and osmolytes. The thiol-pairs can comprise at least one component selected from

the group consisting of glutathione-reduced, glutathione-oxidized, cysteine, cystine, cysteamine, cystamine and beta-mercaptoethanol.

In various ~~embodiments~~examples, the purification can comprise contacting the mixture with an affinity separation matrix, such as a Protein A or Protein G resin. Alternatively, the affinity resin can be a mixed mode separation matrix or an ion exchange separation matrix. In various aspects, the incubation can be performed under aerobic conditions or under non-aerobic conditions.

DETAILED DESCRIPTION OF THE INVENTION

The relevant literature suggests that when optimizing various protein refolding operations, the refold buffer thiol-pair ratio has been purposefully varied and as a result the thiol buffer strength was unknowingly varied across a wide range of strengths (see, *e.g.*, Lilie, Schwarz & Rudolph, (1998) *Current Opinion in Biotechnology* 9(5):497-501, and Tran-Moseman, Schauer & Clark (1999) *Protein Expression & Purification* 16(1):181-189). In one study, a relationship between the thiol pair ratio and the buffer strength was investigated for lysozyme, a simple, single-chain protein that forms a molten globule. (De Bernardez *et al.*, (1998) *Biotechnol. Prog.* 14:47-54). The De Bernardez work described thiol concentration in terms of a model that considered only the kinetics of a one-way reaction model. However, most complex proteins are governed by reversible thermodynamic equilibria that are not as easily described (see, *e.g.*, Darby *et al.*, (1995) *J. Mol. Biol.* 249:463-477). More complex behavior is expected in the case of large multi-chain proteins containing many disulfide bonds, such as antibodies, peptibodies and other Fc fusion proteins. Until the present disclosure, specific relationships had not been provided for thiol buffer strength, thiol-pair ratio chemistry, and protein concentration with respect to complex proteins that related to the efficiency of protein production. Consequently, the ability to refold proteins in a highly concentrated volume has largely been an inefficient or unachievable goal, leading to bottlenecks in protein production, particularly on the industrial scale.

Prior to the present disclosure a specific controlled investigation of the independent effects of thiol-pair ratio and thiol-pair buffer strength had not been disclosed for complex proteins. As described herein, by controlling the thiol-pair buffer