

Innovative Chemicals for Process Intensification in Cell Culture Media

Process guidance for the use of the modified amino acids Phospho-Tyrosine Disodium Salt EMPROVE® EXPERT and Sulfo-Cysteine Sodium Salt Sesquihydrate EMPROVE® EXPERT in fed-batch processes

Introduction

L-Tyrosine is a key amino acid for both cellular metabolism and protein synthesis and its depletion in fed-batch processes has been correlated with a drop in specific productivity¹ and with protein sequence variants.²

This critical amino acid presents an extremely low solubility, especially at neutral pH.^{3, 4} The use of tyrosine di-sodium salt concentrations above 1 g/L in feeds induces precipitation and increases the risk of media instability, mainly through co-precipitation of other amino acids. This may lead to sub-optimal performance due to insufficient supply of nutrients and finally to low performing processes.

L-cysteine is a sulphur-containing amino acid which is oxidized to the dimer L-cystine in the presence of air, oxygen or metal containing catalysts such as copper.⁵

L-cysteine is freely soluble, L-cystine has a reduced solubility in water⁶ and often precipitates in neutral pH feeds.

To overcome these limitations, common fed-batch processes use highly concentrated alkaline feeds which create the need for complex control strategies to minimize pH spikes during feed additions (**Figure 1A**). In order to remove this alkaline feed, tyrosine and cysteine were chemically modified to phospho-L-tyrosine disodium salt⁷ and S-sulfocysteine sodium salt⁸ respectively. The combination of both chemicals allows the integration of both amino acid sources in highly concentrated, neutral pH feeds (**Figure 1B**) and thus provide a unique way to simplify fed-batch processes by enabling the development of stable and neutral pH main feeds.

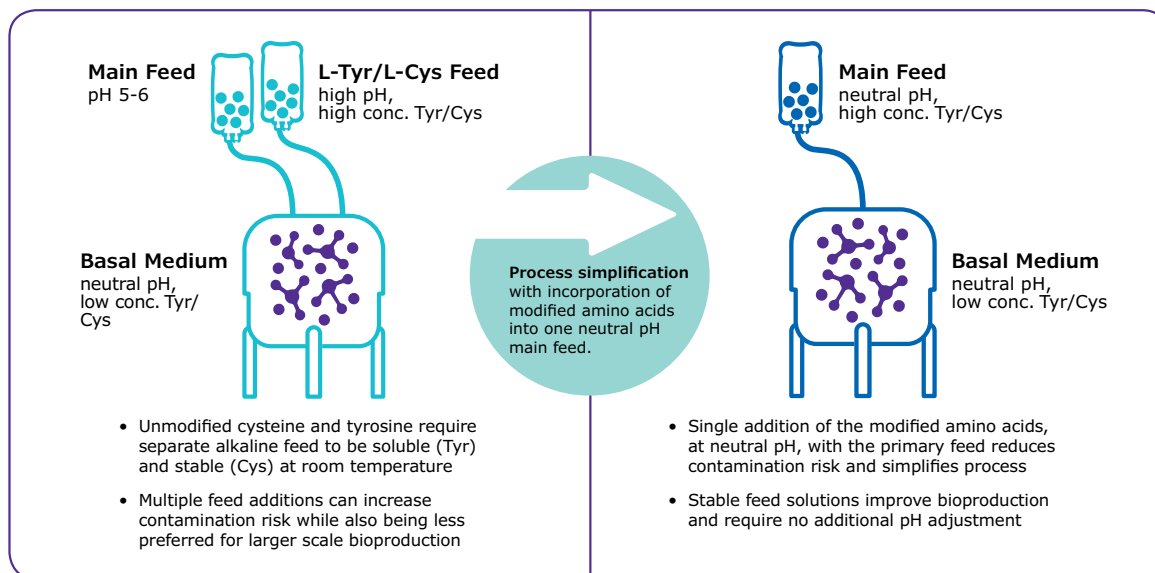


Figure 1. Process simplification with incorporation of modified amino acids into one neutral pH main feed.

Benefits

Modified amino acids allow development of neutral pH, highly concentrated and stable cell culture media formulations

- Easily soluble tyrosine derivative Phospho-Tyrosine
- Stable cysteine derivative Sulfo-Cysteine enabling
 - Reduction of recombinant protein fragmentation
 - Decrease in trisulfide bond content

Simplified process

- Fewer feed stream preparations
- Easier process configuration
- Less contamination risks
- Prevention of local caustic shocks due to high pH

Performance

1. Solubility and stability

In water, the solubility of phospho-L-tyrosine disodium salt (PTyr) was evaluated at 53 g/L, more than 100-fold higher than the solubility of L-tyrosine (0.38 g/L). In concentrated feeds, PTyr₂Na⁺ exhibited a solubility of 70 g/L in contrast to L-tyrosine, which was not soluble and to L-tyrosine disodium salt for which the maximum solubility was <1 g/L.

No degradation or precipitation of phospho-tyrosine was observed during the first six months in liquid feed, and no free tyrosine generated from potential oxidation reactions was detected.

S-Sulfocysteine sodium salt (SSC) was soluble up to 1.3 M in water at room temperature and 50 mM SSC were soluble in the pH 7.0 feed. In water, SSC was stable at acidic and neutral pH, whereas a spontaneous dissociation leading to cysteine release and cystine formation was observed at alkaline pH.

There was no visible precipitation or change in color during three months storage in neutral pH feed at 15 mM SSC. There was no significant decrease of the SSC concentration in the feed stored at 4 °C or room temperature protected from light.

SSC is known to interact with other thiol-containing molecules like cysteine, glutathione or monothioglycerol. To ensure SSC stability, we recommend removing any thiol containing components from target feed formulations.

Reduce costs

- Save preparation time and efforts for various feed containers

Efficient process

- Reduced volume to be processed in upstream and downstream

Reliable supply

- Large manufacturing capacities ensure reliable supply for our customers

Full transparency

- We provide comprehensive documentation with our Improve[®] dossier to support your GMP processes.

2. Performance in Fed-batch processes

The performance of both modified amino acids was tested in fed-batch using several CHO cell clones producing different IgG1s.^{8,9} In all processes tested so far, equimolar concentrations of both cysteine and tyrosine proved to be the best concentrations for both PTyr and SSC in the feed. However, specific cell line requirements may require a titration experiment for both amino acids independently.

In addition, our studies highlighted the essential role of the tyrosine concentration in the medium used in the fed-batch process.⁷ Since PTyr is cleaved in the cell culture media by cellular phosphatases, the kinetic of Tyr release may vary between cell lines. Therefore, we recommend monitoring the tyrosine content in the spent medium during the entire fed-batch. Since a minimum of 1 mM tyrosine seems to be required to ensure efficient transport of the amino acid into cells,² we recommend to adjust the tyrosine concentration in the medium in order to exceed that concentration at any time during the process. In the process used in our studies, this resulted in an adjustment of the tyrosine concentration in the medium from 1 mM to 2.5 mM.

Finally, in our hands, phospho-tyrosine cannot be used as replacement of tyrosine in the medium. For sulfo-cysteine, the replacement of cysteine in the medium lead to different results depending on the clone. Our recommendation is to avoid modifying the cysteine concentration in the medium.

Results obtained in a standard Fed-batch process using a CHO cell line producing an IgG1 are presented in **Figure 2**.

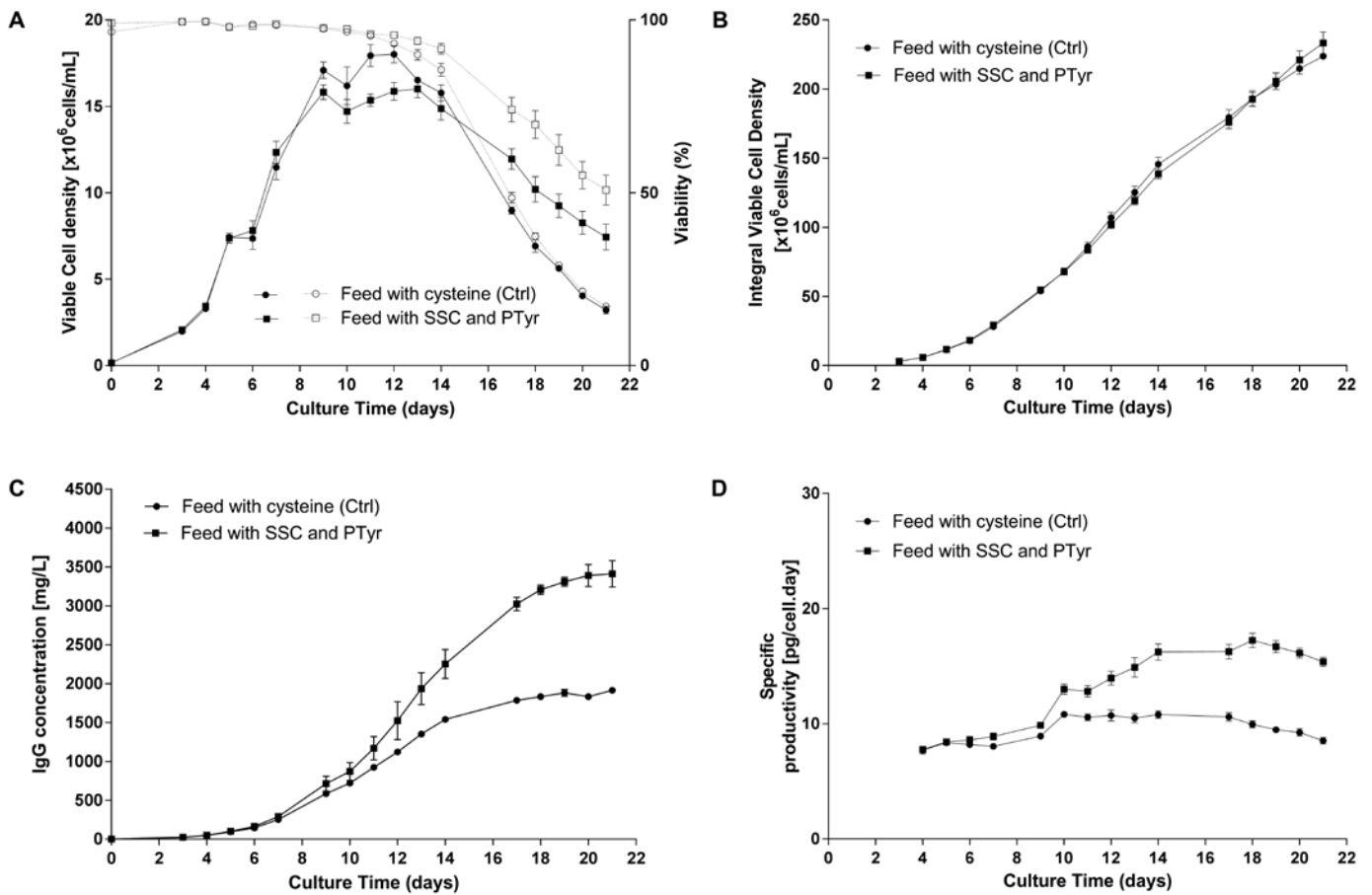


Figure 2. 1.2 L bioreactor fed-batch experiment using a feed containing either cysteine and tyrosine (alkaline pH) or SSC and PTyr (neutral pH). Feed was added at 3% (v/v) at day 3 and 6% (v/v) at days 5, 7, 9 and 14. (A) Viable cell density (plain lines) and viability (dotted lines), (B) Integral viable cell density, (C) IgG concentration in the supernatant measured by a turbidometric method, (D) Specific productivity.

3. Impact on the monoclonal antibody

The use of PTyr and SSC in concentrated feeds did not result in a detectable modification in the amino acid sequence^{7, 8} or the glycosylation or charge variant profile of a model IgG1 (**Figure 3 A and B**).

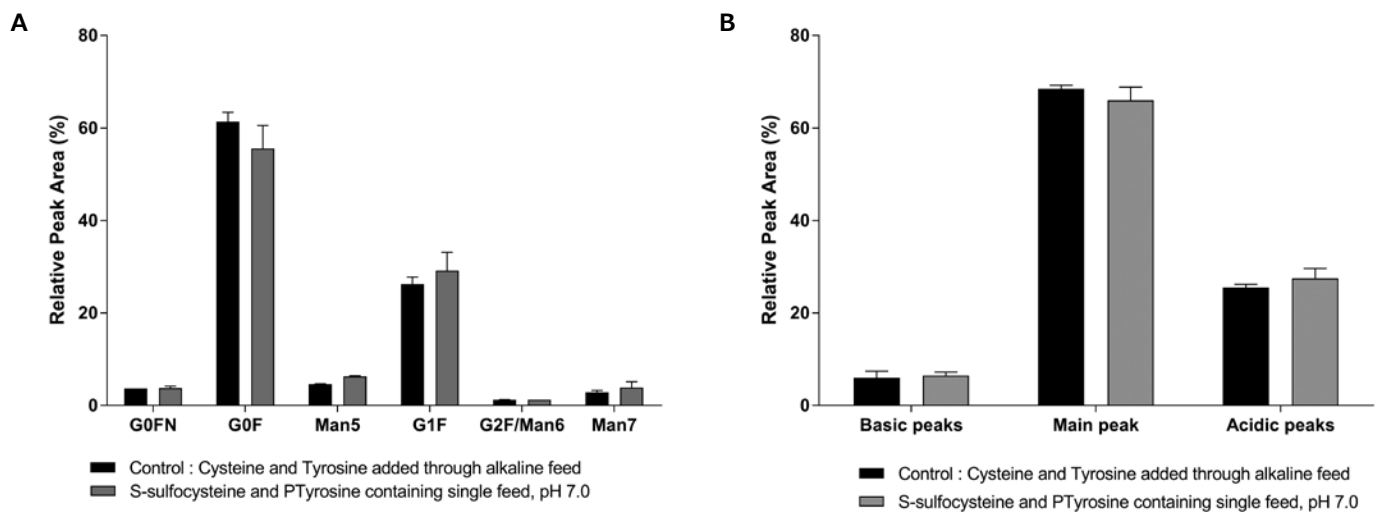


Figure 3. Impact of SSC-containing feed on the quality attributes and sequence of the monoclonal antibody. (A) Glycosylation pattern determined using the 2-AB labeling method. (B) Charge variant distribution obtained using IEF.

In depth analysis of the antibody allowed us to demonstrate a reduction in the IgG heterogeneity when SSC was used in the process.⁹ Indeed, the use of this modified amino acid resulted in a reduced fragmentation of several IgG1s as well as in a reduced level of trisulfide bond linkages between the heavy chain and the light chain of the mAb (Figure 4).

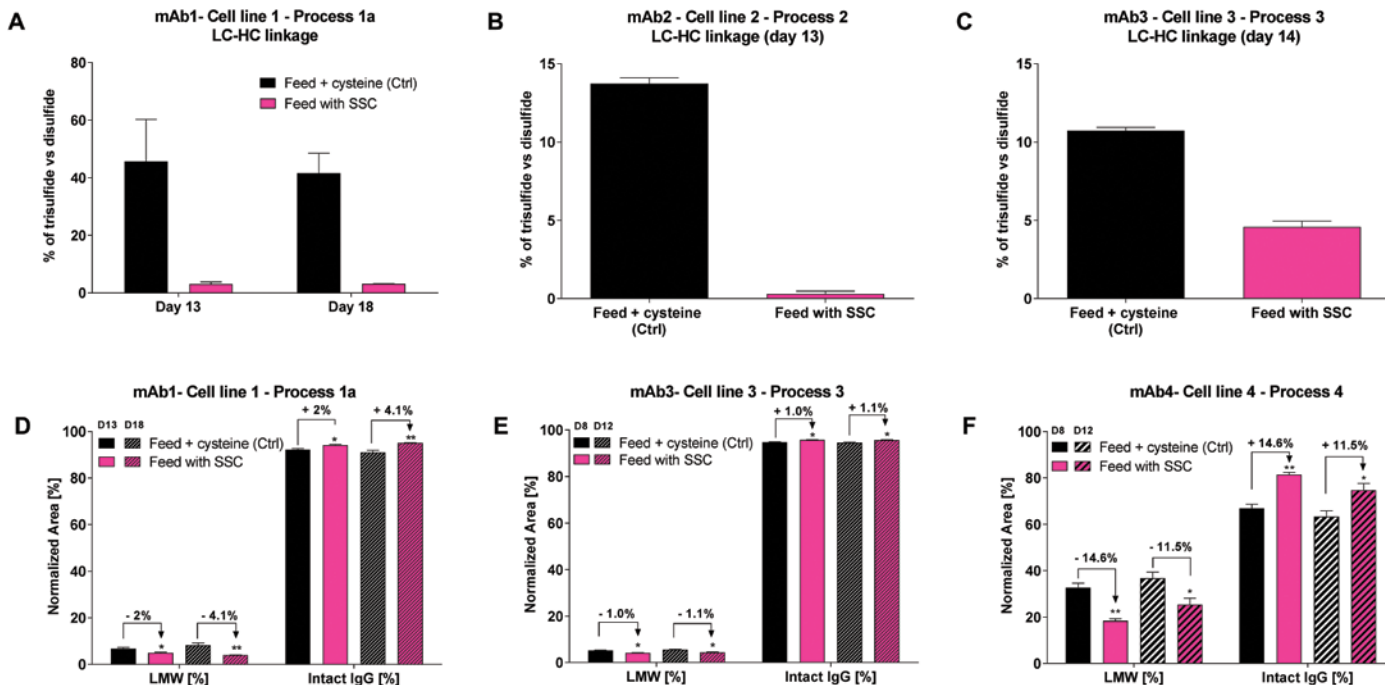


Figure 4. Relative quantification of the amount of trisulfide linkages as well as low molecular weight forms for three different mAbs.

A to C: Trisulfide bonds between light chain and heavy chain of four IgGs were compared with the disulfide linkages and quantified using LC-MS. D to F: Fragments were separated according to their size under non-reduced conditions using CE-SDS. Statistical differences were assessed by Kruskal-Wallis and Dunn's multiple comparison tests. p-values of less than 0.05 (*) and 0.01 (**) were considered significant.

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Specifications

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Safety Data Sheet

The current safety data sheets can be retrieved from the website: **SigmaAldrich.com**

Packaging, Storage and Ordering Information

L-Cysteine-S-sulfate Sodium Salt Sesquihydrate EMPROVE® EXPERT

Cat. No.	Packaging Unit	Packaging Material
1.37116.0100	100 g	0.25 L PE wide mouth bottle
1.37116.1000	1 kg	1.8 L PE wide mouth bottle
1.37116.5000	5 kg	8 L PE wide mouth bottle

Product should be stored between +15 °C and +25 °C. Minimum 24 months shelf life when stored in unopened original packs in this way.

Phospho-L-Tyrosine Disodium Salt EMPROVE® EXPERT

Cat. No.	Packaging Unit	Packaging Material
1.37119.0100	100 g	Outer HDPE tub, inner glass bottle
1.37119.1000	1 kg	Outer HDPE drum, two inner LDPE liners
1.37119.9010	10 kg	Outer HDPE drum, two inner LDPE liners

Product should be stored between +2 °C and +8 °C. Minimum 12 months shelf life when stored in unopened original packs in this way.

References

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