International Journal of Pharmaceutics 387 (2010) 110–119

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# Recombinant human granulocyte colony stimulating factor pre-screening and screening of stabilizing carbohydrates and polyols

Renata Pavišić[a,*], Karlo Hock[b], Ivana Mijić[b], Anita Horvatić[b], Martina Gecan[a], Mirela Sedić[b], Mirjana Bukvić Krajačić[c], Mario Cindrić[b,**]

[a] Pliva Croatia Ltd, Prilaz baruna Filipovića 29, 10000 Zagreb, Croatia
[b] Institute Ruđer Bošković, Division of Molecular Medicine, Bijenička cesta 54, 10000 Zagreb, Croatia
[c] GlaxoSmithKline Research Centre Zagreb, Prilaz baruna Filipovića 29, 10000 Zagreb, Croatia

ARTICLE INFO

*Article history:*
Received 27 September 2009
Received in revised form 1 December 2009
Accepted 4 December 2009
Available online 18 December 2009

*Keywords:*
r*Hu*G-CSF
Formulation
Stabilizer
Protein and peptide
Screening

ABSTRACT

Protein stabilization by solvent additives is frequently used concept in formulation development, although new technologies implemented over the past decade can improve protein biophysical as well as clinical properties by protein structural design (e.g. PEGylation, acylation, hesylation). The scope of this work was to evaluate the effect of chosen carbohydrate or polyol stabilizer in the formulation; firstly on linear peptide sequences on instable model of r*Hu*G-CSF cleaved macromolecule by novel method named protein and peptide stabilizer pre-screening PPSP (formulated tryptic digest mixture stability evaluation in 54 h) and on overall stability of r*Hu*G-CSF macromolecule by quantifying all relevant degradation parameters. Comprehensive protein stabilizing screening study included conformational analysis of formulated r*Hu*G-CSF protein to obtain information on its secondary structure conformational stability. Protein aggregation induced by modulating conditions in solution (e.g. thermal stress and agitation) was monitored over discrete time periods. Oxidation and deamidation, as well as truncation or hydrolysis were accurately quantified. Together with pre-screening data, obtained by fast and resourceful amino acid sequence degradation analysis by LC–MS, statistical data evaluation of stabilizing contribution of substances selected from group of carbohydrates and polyols was performed. According to the statistical interpretation of obtained results the stabilizers were ranked in the following order: turanose, D-trehalose, lactitol, acetate buffer (non-stabilized sample), xylitol, cellobiitol, sorbitol, D-lyxose, leucrose, sorbitol without polysorbate, cellobiose.



## 1. Introduction

Marginal stability of proteins can be attributed to their molecular complexity and delicate three-dimensional structure. The therapeutic use of proteins without prior stabilization is questionable. It is therefore necessary to develop a final formulation of such properties that will ensure its stability throughout the biopharmaceutical 'life cycle', including manufacturing, shipping, instant administration, or short-/long-term storage prior to administration (Chang and Hershenson, 2002).

A variety of compounds, conditions and microenvironments have been demonstrated to affect the stability of proteins in a solution. Sugars, polyols, and certain amino acids and salts are all known to be protein stabilizers. On the other hand, hydrophobic organic compounds, chaotropic salts such as urea, and guanidine hydrochloride are known to be protein destabilizers. Timasheff and co-workers explain these effects by preferential interaction of proteins with solvent components (Lee and Timasheff, 1981; Gekko and Timasheff, 1981; Arakawa and Timasheff, 1984, 1985), where the protein stabilizers are preferentially excluded from the proteins while destabilizers bind to them. The presence of these co-solutes in protein solution creates thermodynamically unfavourable situation, consequently stabilizing the native protein structure as denaturated would lead to a greater contact surface between protein and the solvent and further increase of the unfavourable effect. Understanding the mechanisms of action of the solvent additives on the protein stability is of the utmost importance while developing a long-term viable formulation (Arakawa et al., 1991, 2001; Chi et al., 2003a).

The scope of this work was to evaluate the effect of chosen formulation stabilizers on r*Hu*G-CSF macromolecule stability. Stabilizers screening, a necessary step in formulation development, was performed by quantifying all relevant degradation parameters. This labour and time consuming screening approach was combined

* Corresponding author. Tel.: +38 513721731; fax: +38 513721256.
** Corresponding author. Tel.: +38 5992183280; fax: +38 514561010.
*E-mail addresses:* renata.pavisic@pliva.com (R. Pavišić), mario.cindric@irb.hr (M. Cindrić).

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doi:10.1016/j.ijpharm.2009.12.015

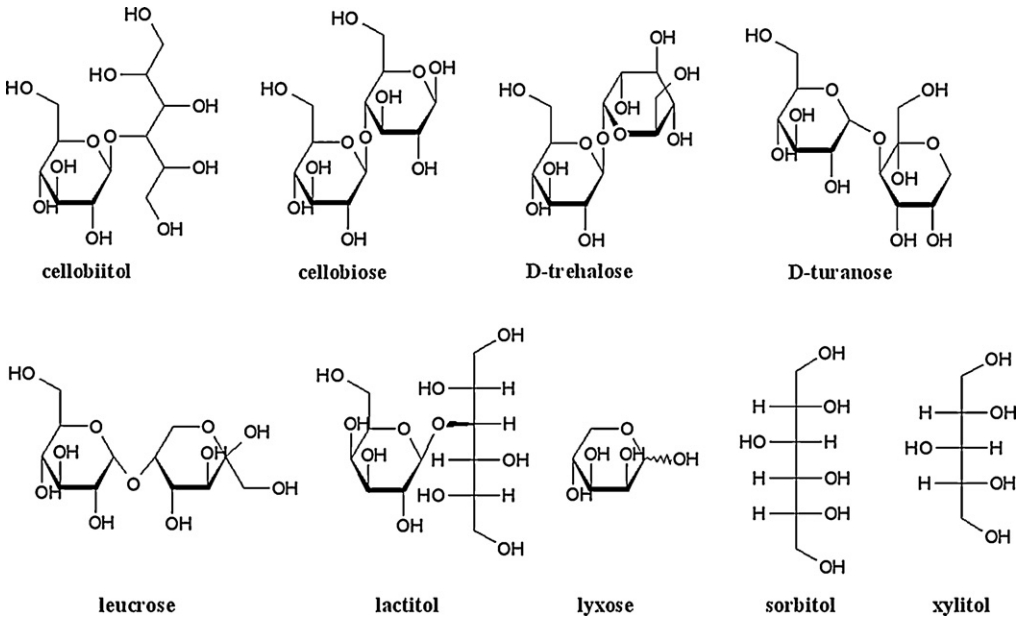


**Fig. 1.** Chemical structures of tested stabilizers.

with rapid pre-screening method linking linear amino acid chain degradation processes and higher order structural stability.

Recombinant methionyl human granulocyte colony stimulating factor (r*Hu*G-CSF) macromolecule is extremely sensitive to agitation, pH, ionic strength, temperature, light, freezing, etc. (Herman et al., 1996), thus making the protein macromolecule almost ideal for the stabilizer screening study. Studied model protein r*Hu*G-CSF, is a non-glycosylated, 175 amino acid, 18.8 kDa protein produced in *Escherichia coli*. Cytokine macromolecule r*Hu*G-CSF belongs to a distinct structural class of growth factors and folds into a four-helical bundle with a left handed twist and overall dimensions of 45 Å × 26 Å × 26 Å (Hill et al., 1993). The structure of r*Hu*G-CSF is stabilized by two disulfide bonds, both of which are required for activity. The molecule also contains one free cysteine residue (Ricci et al., 2003). When dissolved only in water, r*Hu*G-CSF produced both in mammalian cell (one *O*-linked glycan on $Thr_{133}$ glycosylated, known as Lenogastrim) and *E. Coli* (non-glycosylated, known as Filgrastim) share the same extreme instability relative to formulated pharmaceutical product. Production of PEGylated form could potentially overcome this problem, resulting in a hybrid polymer–biopolymer macromolecule with clinical and biophysical properties superior to the non-stabilized r*Hu*G-CSF molecule (Piedmonte and Treuheit, 2008).

The formulation matrix of tested prototype r*Hu*G-CSF formulations contained single, tested non-specific stabilizer selected from the group of sugars and polyhydric alcohols: trehalose, xylitol, cellobiitol, turanose, cellobiose, leucrose, lactitol, lyxose or sorbitol (Fig. 1). The non-ionic surfactant polysorbate 80 and acetate buffer pH 4.0 were present in tested formulations as in currently marketed r*Hu*G-CSF produced in *E. Coli* (Neupogen by Amgen®) which assures long-term stability of product stored at 2–8 °C (Herman et al., 1996).

Optimal conditions in the tested formulations were set to avoid the environment known to destabilize r*Hu*G-CSF, as the goal of the study was to isolate and monitor only the extent of stabilization induced by single tested non-specific stabilizer. Since pH of the solution is an important factor that can influence on conformational stability of protein/peptide, the pH of the tested formulations was kept at 4.0 (Ricci et al., 2003; Narhi et al., 1991), as the secondary structure of protein, namely alpha helices integrity is preserved at low pH where the free cysteine is protonated (Arakawa et al., 1993). Furthermore, a low salt environment (meaning no addition of agents that would increase ionic strength), was used in test formulations to minimize aggregation, as the literature data for r*Hu*G-CSF indicate that increasing of the ionic strength by the addition of 150 mM NaCl induced aggregation at pH of 3.5 (Chi et al., 2003b).

Overall, eight formulations besides marketed one were evaluated with two control samples used to facilitate interpretation of results: non-formulated sample composed solely of protein in acetate buffer pH 4.0 and sample composed of protein in acetate buffer pH 4.0 stabilized by sorbitol only; both prepared without addition of polysorbate.

In order to get complete overview of protein behaviour in prepared r*Hu*G-CSF samples, the stabilizer screening study was performed by means of monitoring protein conformation by circular dichroism (CD) spectroscopy, protein aggregation by size exclusion chromatography (SEC HPLC) and field flow fractionation (FFF), oxidation and deamidation by reverse phase chromatography (RP HPLC) following exposure to agitation, elevated temperature, forced oxidation and freeze/thaw cycling simulating stresses: all of which are conditions a product may encounter during its lifecycle.

The pre-screening experiment was conducted to evaluate connectivity between linear amino acid chain degradation processes and higher order structural stability (e.g. aggregation and secondary structure) in different formulations. Protein fragments stability was monitored by peptide mapping liquid chromatography/mass spectrometry (LC/MS) analysis, method not yet used for this purpose and its results were compared with other screening methods.

Based on the results of statistical analysis of collected data the overall stabilizing effect of the selected substances, was estimated with the aim to both determine the optimal stabilizer, and to compare the response pattern of different stabilizers across a variety of parameters essential for the product stability.

## 2. Materials and methods

### 2.1. Materials

Recombinant methionyl human granulocyte colony stimulating factor (r*Hu*G-CSF), expressed in *E. Coli*, was produced by

PLIVA (Zagreb, Croatia). Stabilizers D(+)-trehalose dihydrate, xylitol, minimum 99% and cellobiitol, minimum 98% were purchased from Sigma Aldrich (St. Louis, USA). D(+)-Turanose, ~99%, D(+)-cellobiose, ≥99.0%, D-leucrose, ≥98.0%, D-lactitol monohydrate, purisis, ≥99.0% were purchased from Fluka (Buchs, Switzerland) and D-lyxose, 99% were purchased from Fluka (Milwaukee, Switzerland). Sorbitol, Ph. Eur. quality, was purchased from Roquette (Lestrem, France). Polysorbate 80, Ph. Eur. quality, was purchased from J.T. Baker (Phillipsburg, USA). Acetic acid glacial, Ph. Eur. quality, and sodium hydroxide, Ph. Eur. quality, were purchased from Merck (Darmstadt, Germany). Water for injections, produced by PLIVA (Zagreb, Croatia), was used for preparation of formulations.

### 2.2. Preparation of protein formulations

r*Hu*G-CSF formulations were prepared by ultrafiltration of concentrated r*Hu*G-CSF solution (protein concentration 2.9 mg/ml in NaCl solution) into formulation placebo. The ultrafiltration step was repeated three times at 5000 g, using Millipore Centrifugal Filter Device Centricon YM-10, 10,000 MW cut-off. Each formulation placebo contained single stabilizer; D(+)-trehalose dihydrate, xylitol, cellobiitol, D(+)-turanose, D(+)-cellobiose, D-leucrose, D-lactitol monohydrate, D-lyxose, or sorbitol in a concentration of 50 mg/ml and 10 mM sodium acetate buffer used to adjust the pH to 4.0 (the buffer was prepared by titration of acetic acid with sodium hydroxide solution). Following ultrafiltration step, polysorbate 80 was added to each of thus prepared nine samples to achieve final concentration of 0.04 mg/ml. The samples were made to starting volume using formulation placebo making protein concentration 2.9 mg/ml in the tested samples.

Two control samples without the addition of polysorbate 80 were prepared. The non-stabilized sample was prepared by ultrafiltration of concentrated r*Hu*G-CSF solution into 10 mM acetate buffer pH 4.0 (serving as formulation placebo for this sample) which was further used for dilution to starting volume resulting in protein concentration of 2.9 mg/ml in the sample. The sample stabilized by sorbitol only was prepared in the same way as the non-stabilized sample using sorbitol formulation placebo for ultrafiltration and final dilution steps.

### 2.3. Protein stabilizer pre-screening by peptide mapping LC/MS

Samples were diluted to protein concentration of 1.5 mg/ml using the corresponding formulation placebos (see Section 2.2), desalted through Microcon YM-10 tube and re-diluted with appropriate volume of digestion solution to starting volume. Digestion solution was a mixture of Tris–acetic buffer (pH 8.5, 0.1 M)/formulation placebo (2:1, v/v). After ultrafiltration and r*Hu*G-CSF digestion solution preparation the final solution was digested with trypsin in a mixture containing 250 μl of protein (1.5 mg/ml) and 5 μl of trypsin (1 mg/ml). The digestion mixtures were incubated for 18 h at 37 °C. After digestion reduction of disulfide bridges was performed by adding 50 μl of DTT (0.01 M) into the digestion mixture. Obtained samples were placed into boiling water for a minute and left to stand at 25 °C for 1620, 2441 and 3253 min prior to analysis. Quantitative analysis of peptides produced by trypsin cleavage was performed on Q-TRAP mass spectrometer (Applied Biosystems) connected to an Agilent 1100 Series LC System (Wilmington). A gradient HPLC method was employed to separate trypsin fragments. Mobile phase A consisted of 0.1% TFA (v/v) in water and mobile phase B consisted of a mixture acetonitrile/water (90:10, v/v) with addition of 0.1% TFA (v/v). Waters Symmetry 300 C18 column (4.6 mm × 150 mm, pore size 30 nm, particle size 5 μm) maintained at 30 °C was used for the analysis. The column was equilibrated for 5 min with channel A at flow rate of 0.75 ml/min. Peptides were eluted over the next 85 min with gradient raising linearly from 0% to 40% B at flow rate of 0.75 ml/min and then linearly to 90% B and gradient flow rate raising linearly to 1.25 ml/min in 30 min. Column was rinsed for 5 min with linear gradient raising from 90% to 100% B at constant flow rate of 1.25 ml/min. After that, elution conditions were returned to 0% B and flow rate was decreased to 0.75 ml/min (initial conditions) during 5 min. Initial conditions were kept for the last 5 min equilibrating the column for the following injection. Autosampler temperature was set to 25 °C. Injection volume was 50 μl. Flow splitter for the MS analysis (LC Packings, Dionex, CA, USA) with split ratio of 1:4 was employed. ESI source of the mass spectrometer was operated in positive ion mode under following conditions: source temperature 450 °C, scan rate 1000 amu/s, LIT fill time 40 ms, declustering potential 30 V, entrance potential 10 V, collision energy 10 eV.

### 2.4. Protein conformation monitoring by circular dichroism (CD) spectroscopy

Conformational analysis of protein was carried out by CD measurements. CD spectra were recorded on a Jasco J-810 spectropolarimeter (Tokyo, Japan) equipped with a Peltier temperature controller Jasco PFD 425S. Far-UV spectra were collected using a 1-cm path length quartz cuvette. Thermal transitions were examined by monitoring of ellipticity at 222 ± 2 nm ($\Theta_{222}$), at a rate of 1 °C/min between 50 and 90 °C. Sensitivity setup was standard (100 mdeg), data pitch at 0.1 °C, response 1 s. Samples were first diluted to 50 μg/ml in formulation placebo and further diluted to 20 μg/ml in water (Milly Q) prior to measurement. Noise reduction and data analysis were performed using Spectra Analysis Program. Degree of protein denaturation (*Tm* determination) was assessed using Spectra Analysis Program – Denatured Protein algorithm.

### 2.5. Protein aggregation monitoring by size exclusion chromatography (SEC HPLC)

Dimer, oligomers and aggregate amounts were determined by size exclusion chromatography on an Agilent 1100 HPLC system (Agilent Technologies) using TSKgelG3000SWXL 7.8 mm × 3000 mm and 5 μm particle size column (Tosoh Bioscience, Stuttgart, Germany) with diode array detection at 215 nm (Bw 4 nm) with reference wavelength at 450 nm (Bw 80 nm). The column temperature was maintained at 30 °C. Mobile phase used was 50 mM ammonium hydrogen carbonate (pH 7.0) at flow rate 0.5 ml/min. Samples were diluted to about 0.4 mg/ml using formulation placebo solutions and 20 μl was injected for analysis. The total time for chromatographic elution was 30 min. Samples, while in the autosampler tray, were maintained at 7 °C.

### 2.6. Protein oxidation, deamidation and truncation monitoring by reverse phase chromatography (RP HPLC)

The level of oxidized, deamidated and truncated forms was determined by RP HPLC. Analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies) using Phenomenex Jupiter $C_4$ column (4.6 mm × 250 mm), 5 μm particle size, and 300 Å pore size. Detection was performed with diode array detection at 215 nm. The column temperature was maintained at 60 °C. Mobile phase A: 0.1% TFA/10% MeCN (v/v) and mobile phase B: 0.1% TFA/80% MeCN (v/v) were used at the flow rate 0.6 ml/min. Separation was achieved using linear gradient from 66% to 73% mobile phase B in 35 min followed by 15 min linear gradient from 73% to 90% mobile phase B after which returns to 66% mobile phase B. Samples were diluted to concentration of about 0.2 mg/ml using formulation placebo. Forced oxidation study was performed by adding solution of hydrogen peroxide to final concentration of about 0.02%, mixed

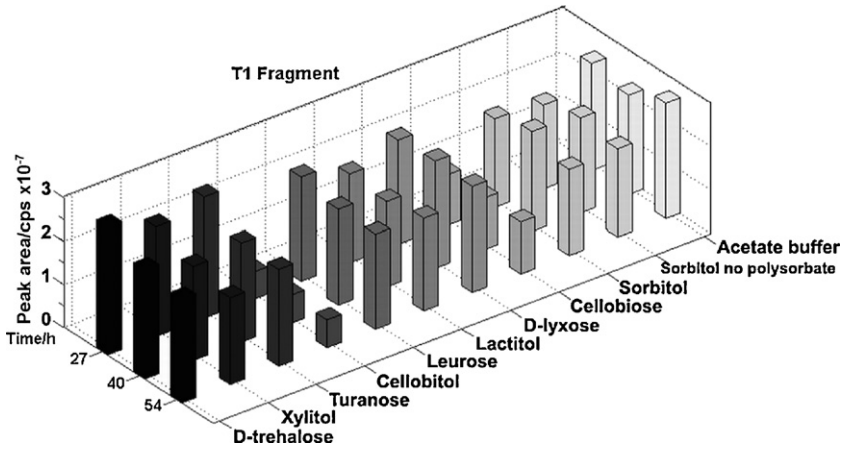


**Fig. 2.** Stability of r*Hu*G-CSF T1 fragment in tested samples at time points 27, 40 and 54 h determined by mass spectrometry.

and incubated at 25 °C for 30 min. The reaction was then quenched with about 1.5 mg of L-methionine. The injection volume was 50 μl. The total time for the method was 60 min. Samples, while in the autosampler tray, were maintained at 10 °C.

### 2.7. Statistical data evaluation

The performance of different stabilizers under each of the tested conditions was ranked within a respective condition so as to make the results for all tests cross-comparable. The overall results were weighted so as to reflect the fact that the LC/MS measurements on the same fragment were taken at three different time points. The performance rankings of different stabilizers were then compared using Kruskal–Wallis ANOVA. Post hoc multiple comparisons were used to determine individual differences in performance among respective stabilizers, and Kendall coefficient of concordance was used to determine the degree of agreement among the performed tests in ranking of the stabilizers (Siegel and Castellan, 1988).

## 3. Results and discussion

### 3.1. Protein/peptide stabilizers pre-screening (PPSP) by peptide mapping LC/MS

Protein/peptide stabilizers pre-screening (PPSP) relies on the idea of rapid detection of degradation sites of a fragmented protein. Modern analytical techniques for protein analysis are often not able to accurately pinpoint the most critical degradation site in protein without fragmenting the protein in smaller pieces. Smaller protein fragments are more amenable to analysis than large protein macromolecules which generally give limited amount of information after intact molecule analysis. Depending on macromolecule size and the number of protein sites susceptible for degradation, early beginning of degradation process might remain undetected.

In this method the protein was firstly fragmented to peptides by enzymatic treatment and quantified in time after 27, 40, and 54 h. Quantification was performed accurately by triple quadrupole mass spectrometer in opposite to UV/VIS absorbance by selecting mass window of 0.5 Da. For example deamidation of asparagine and glutamine residues with mass shift of 1 Da for each amino acid clearly detectable by mass spectrometer would stay undetected by UV/VIS, refractive index, or fluorescence detection.

Protein was stabilized by formulation placebo at the very beginning of digestion process since digestion solution was a mixture of Tris–acetic buffer (pH 8.5, 0.1 M) and formulation placebo (2:1, v/v). Fragment degradation obtained in different formulations revealed degradation progress (Figs. 2–9) for each formulation vs. T1–T10 fragments evanescing in time. Linear sequence of amino acids and the location of disulfide (–S–S–) bridges in protein are presented in Table 1 (disulfide bridges were in –SH reduced form after the addition of DTT to peptide mixture).

T1, T4, T9 and T10 r*Hu*G-CSF fragments demonstrated highest stability under the study conditions, somewhat reduced stability was noted for T8 fragment, while stability of T2, T5 and T6 fragments was generally poor. Low stability of fragments T2, T5, T6

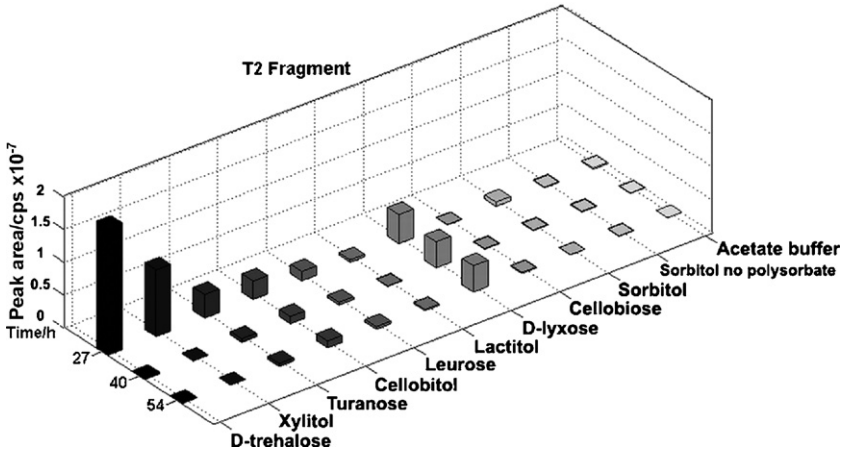


**Fig. 3.** Stability of r*Hu*G-CSF T2 fragment in tested samples at time points 27, 40 and 54 h determined by mass spectrometry.

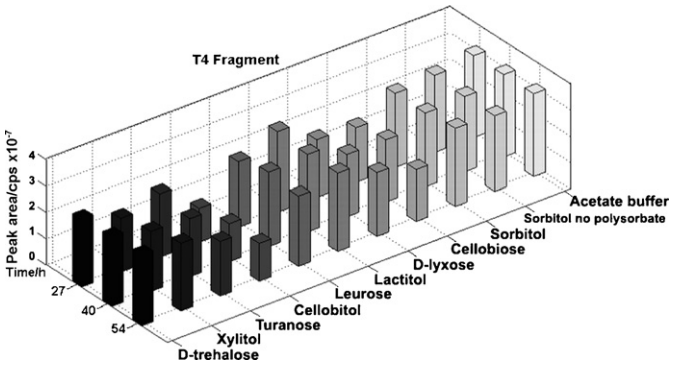


**Fig. 4.** Stability of r*Hu*G-CSF T4 fragment in tested samples at time points 27, 40 and 54 h determined by mass spectrometry.

could potentially be due to presence of cysteine in peptide backbone. Kinetic analysis performed for named cysteine-containing fragments up to 27 h (data not shown) exhibited first-order kinetic curve expected for in solution peptide hydrolysis. Curve steepness calculated in 27 h time period on eight time points (linear portion of the curve) could predict further degradation and stabilizer efficiency in slowdown of cystein-containing peptide hydrolysis. Cellobiitol and cellobiose were consistently the least effective in stabilizing r*Hu*G-CSF fragments T1, T4 and T8–T10. Interestingly, some stabilizers (primarily trehalose, but also xylitol, turanose and lyxose) were found to have stabilizing effect on these peptides implying that they stabilize the oxidized cysteines. T3 and T7 fragments were not analysed since they consist of single amino acid.

### 3.2. Protein conformation monitoring by CD

Thermally induced unfolding experiments were carried out to assess thermal stability of r*Hu*G-CSF in the tested formulations. Conformational changes were monitored by circular dichroism spectroscopy. The ellipticity at 222 nm ($\Theta_{222}$), a standard measure of helical content of a protein, was used to estimate the secondary structural change of protein. By determining denaturation temperature (*Tm*) values, the stabilizing effect of tested formulations, namely the effect of added stabilizers, was determined. Although r*Hu*G-CSF remains native and compact in some aspects of structural integrity in formulated solutions ranging from pH 2 to 7 (Kovlenbach et al., 1997), its conformational stability is pH-dependent (Narhi et al., 1991). The pH value of the tested formulations was set at pH 4.0, where conformational stability of r*Hu*G-CSF is optimal (Krishnan et al., 2002; Narhi et al., 1991). In this manner, it was possible to evaluate the stabilization effect of a single tested stabilizer.

Temperature dependency of ellipticity at 222 nm ($\Theta_{222}$) was recorded for each formulation tested. The denaturation temperature (*Tm*) of r*Hu*G-CSF formulations, determined as the point of inflection of thus obtained curve (Fig. 10), was within the narrow temperature range starting from 74.5 to 78.3 °C (Fig. 11).

The *Tm* value for non-stabilized r*Hu*G-CSF (acetate buffer pH 4.0) was 74.5 °C, whereas the addition of stabilizers in the formu-

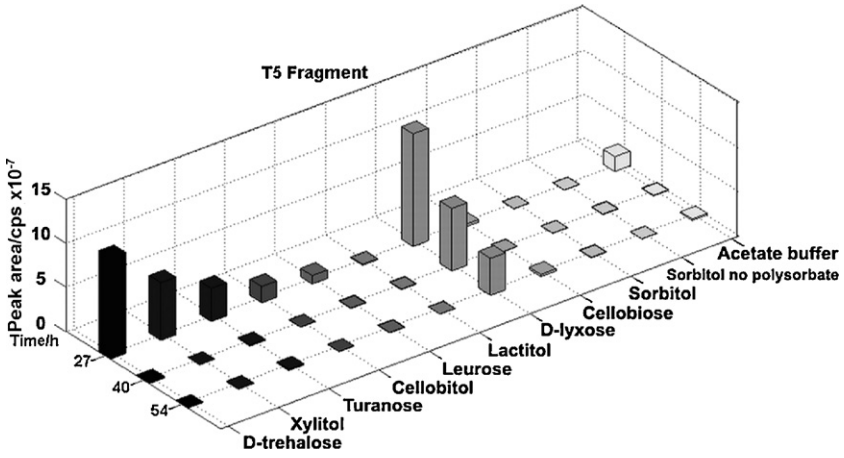


**Fig. 5.** Stability of r*Hu*G-CSF T5 fragment in tested samples at time points 27, 40 and 54 h determined by mass spectrometry.

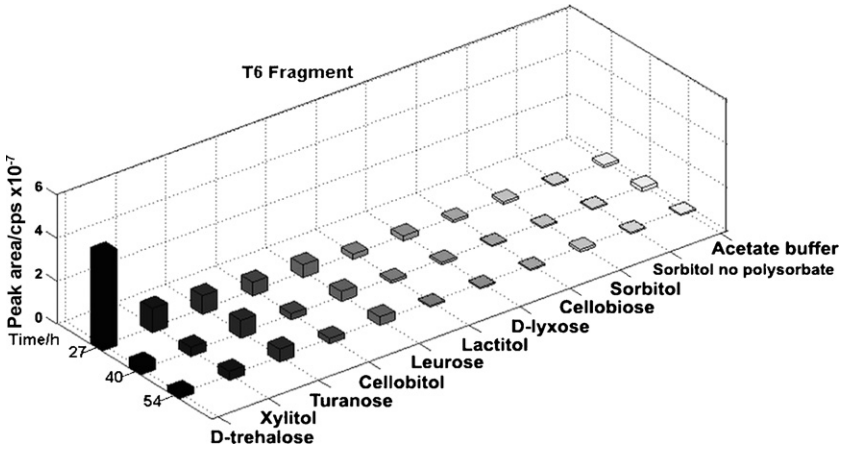


**Fig. 6.** Stability of r*Hu*G-CSF T6 fragment in tested samples at time points 27, 40 and 54 h determined by mass spectrometry.

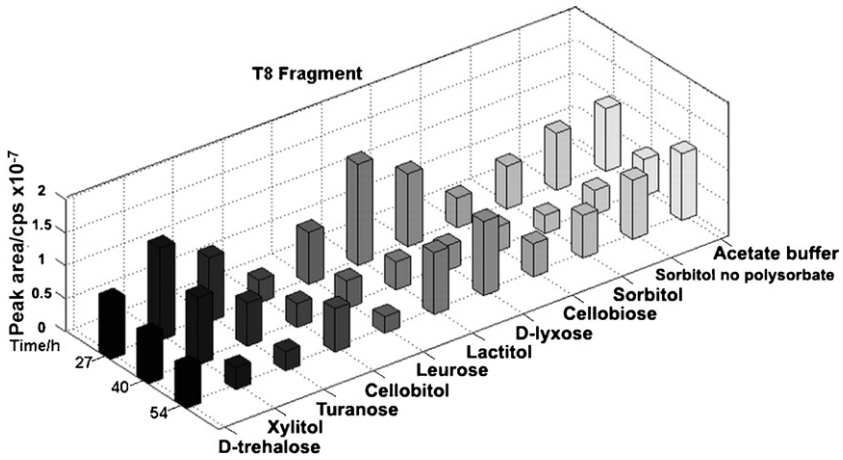


**Fig. 7.** Stability of r*Hu*G-CSF T8 fragment in tested samples at time points 27, 40 and 54 h determined by mass spectrometry.

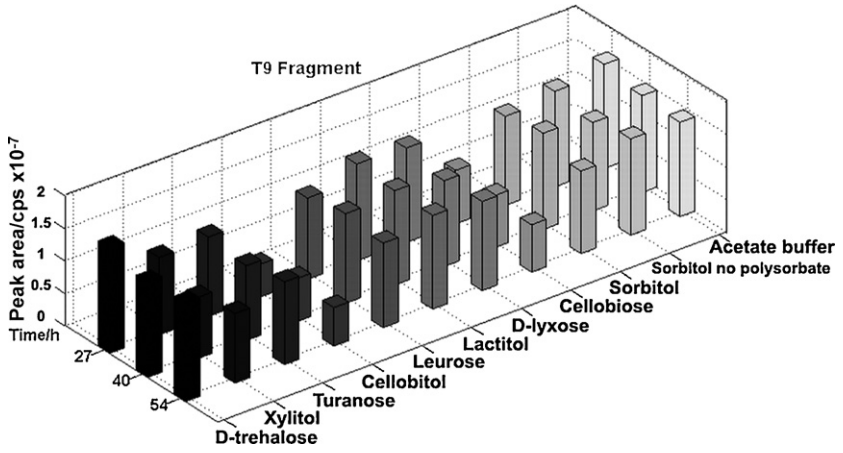


**Fig. 8.** Stability of r*Hu*G-CSF T9 fragment in tested samples at time points 27, 40 and 54 h determined by mass spectrometry.

lation resulted in the increase of observed *Tm* values indicating apparent improvement in thermal stability of protein. When ranking the added substances according to their stabilizing effect, the fact that non-stabilized r*Hu*G-CSF sample contains acetate buffer alone while stabilized formulations in addition to acetate buffer and tested stabilizer contain 0.004% polysorbate 80 must be considered, knowing that presence of small quantities of polysorbate in the formulation can increase thermal degradation (Wang, 1999; Treuheit et al., 2002). Thermal stability of native protein was therefore altered in two ways, by addition of polyols and carbohydrates in high concentration (stabilizing effect, stabilizer depended) and by addition of polysorbate 80 (generally destabilizing effect). The sample stabilized with sorbitol, with and without presence of polysorbate, illustrates this well with the difference of *Tm* of approximately 3.6 °C. Finally, although the error associated with far-UV CD measurement is typically 5–10% indicating that mea-

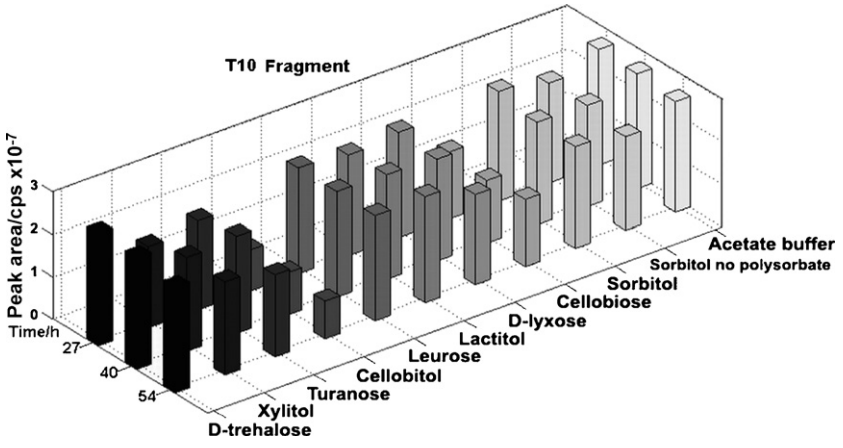


**Fig. 9.** Stability of r*Hu*G-CSF T10 fragment in tested samples at time points 27, 40 and 54 h determined by mass spectrometry.

**Table 1**
*In silico*, theoretically calculated and observed monoisothopic masses of r*Hu*G-CSF tryptic fragments T1–T10 (reduced disulfide bridges) used for stability estimation.

| Fragment | Amino acid sequence[a] | m/z (theoretical monoisotopic) | Charge | m/z (observed) |
|---|---|---|---|---|
| T3 | K | 147.1 | 1 | N/A |
| T7 | R | 175.1 | 1 | N/A |
| T9 | VLR | 387.3 | 1 | 387.3 |
| T10 | HLAQP | 565.3 | 1 | 565.3 |
| T5 | LC*ATYK | 698.4 | 1 | 698.3 |
| T1 | MTPLGPASSLPQSFLLK | 894.0 | 2 | 893.9 |
| T8 | AGGVLVASHLQSFLEVSYR | 1017.0 | 2 | 1016.9 |
| T4 | IQGDGAALQEK | 565.3 | 2 | 565.3 |
| T2 | CLEQVR | 747.4 | 1 | 747.4 |
| T6 | LC*HPEELVLLGHSLGIPW APLSSC*PSQALQLAGC*LS QLSHGLFLYQGLLQALEG ISPELGPTLDTLQLDVADF ATTIWQQMEELGMAPAL QPTQGAMPAFASAFQR | 1615.2 | 7 | 1615.3 |

[a] An asterix symbol (*) refers to free cystein residues after disulfide bridge reduction.

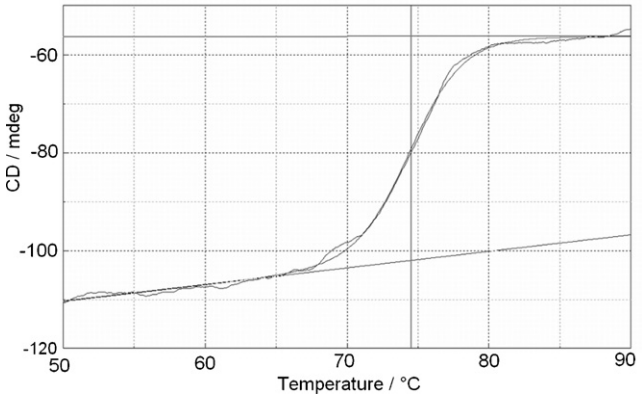


**Fig. 10.** Temperature dependency of ellipticity at 222 nm ($\Theta_{222}$) of r*Hu*G-CSF in non-stabilized sample (acetate buffer pH 4.0). The *Tm* value is determined as the point of inflection of this curve.

sured difference between samples does not appear to be significant, highest increase in thermal stability for r*Hu*G-CSF can be noted when using cellobiose, lactitol, lyxose, xylitol and cellobiitol in the formulation.

### 3.3. Protein aggregation monitoring by SEC HPLC

Prevention of protein aggregation is one of the major concerns in modern protein formulation having in mind that oligomers, higher oligomers and aggregates are known for their immunogenic potential (Hermeling et al., 2004; Rosenberg, 2006; Li et al., 1995; Lèon et al., 1998). Such physical degradation induced by agitation or thermal stress, which can occur during routine manufacture (passage through filters and pumps, filling on high speed filling machines, etc.), shipping and storage (accidental freezing or exposure to elevated temperature) must be stopped at the beginning of the process or in the worst case only minimized.

r*Hu*G-CSF aggregates are formed as a result of protein–protein interaction caused by short-term hydrophobic auto-association (approximate size 200 kDa) or disulfide bonds interactions (1000–4000 kDa). These data were obtained by orthogonal analytical method (field-flow fractionation, data not shown).

It is well known that protein aggregation is highly dependent, among other factors, on the presence of co-solutes in the protein environment (Chi et al., 2003b), i.e. addition of disaccharide sucrose to r*Hu*G-CSF has decreased its aggregation rate (Krishnan et al., 2002) as opposite to non-formulated r*Hu*G-CSF that would produce large amount of aggregates after freeze–thaw cycling at −20 °C or during long-term storage. The efficiency of selected stabilizers in preventing protein aggregation was investigated by measuring the amount of dimer and higher molecular weight impurities in tested formulations upon preparation and after subjecting them to stress conditions known to promote protein degradation; incubation at elevated temperature (40 °C) and physical agitation (vortex mixing).

The level of impurities in tested samples at starting time point prior to exposure to stress conditions was uniform and below significant values (<1%) (Fig. 12).

#### 3.3.1. Thermal stress

Lyxose and leucrose exhibited least stabilizing effect against thermally induced aggregation with 17.9% and 15.8% of total impurities measured after one month of incubation at 40 °C. Cellobiose and turanose samples with approximately 11% of impurities followed by sorbitol and trehalose with approximately 8%, lactitol and xylitol with approximately 7%, down to cellobiitol with approximately 5% of total impurities demonstrated superior stabilizing

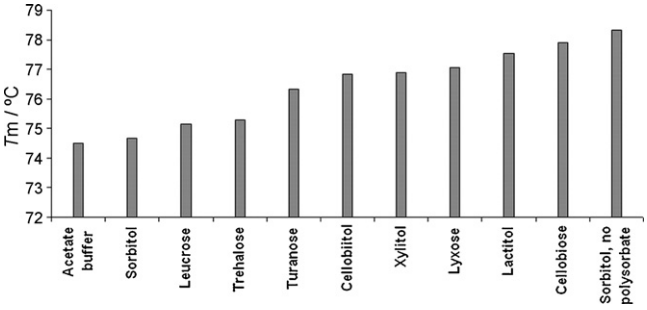


**Fig. 11.** The *Tm* temperature determined by thermal denaturation studies for tested r*Hu*G-CSF formulations.

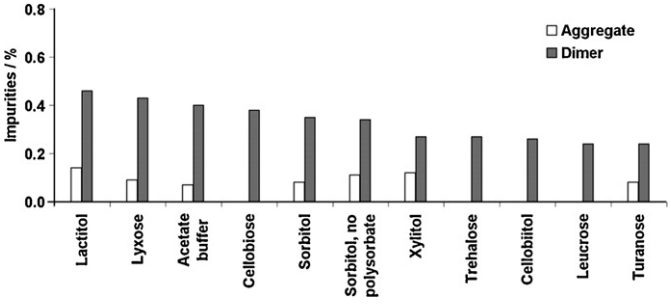


**Fig. 12.** The amount of dimers and aggregates in tested r*Hu*G-CSF samples prior to exposure to stress conditions (start analysis) determined by SEC HPLC.

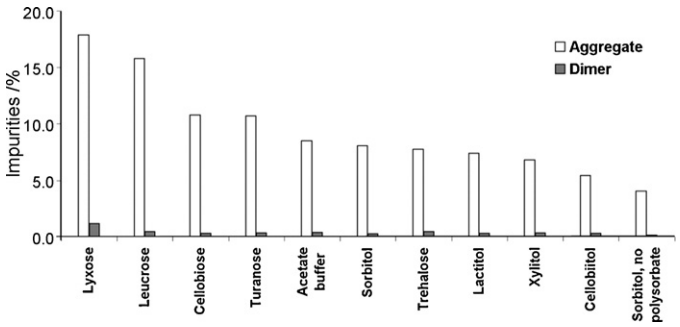


**Fig. 13.** The amount of dimers and aggregates in tested r*Hu*G-CSF samples incubated at 40 °C for one month determined by SEC HPLC.

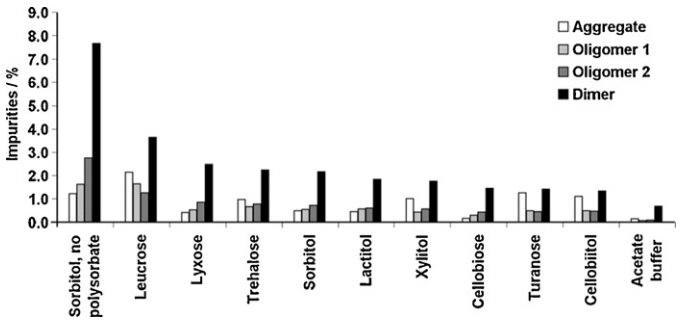


**Fig. 14.** The amount of dimers, aggregates, oligomers in tested r*Hu*G-CSF samples subjected to agitation determined by SEC HPLC.

effect (Fig. 13). Presence of stabilizer sorbitol and deficiency of polysorbate 80 in the r*Hu*G-CSF vicinity demonstrated to be highly efficient in inhibiting aggregation (4.0% of total impurities) owing this to already discussed effect of polysorbate 80 on the thermal stability of protein. From structural point of view, stabilizers that demonstrated highest stabilizing effect sorbitol, lactitol, xylitol and cellobiitol, with exception of trehalose, were from the group of polyols, characterized with linear part of molecule (Fig. 1) which is possibly responsible for more successful creation of microenvironment in protein degradation-sensitive regions preferring its native rather than unfolded state.

Freeze–thaw stress performed by freezing at temperature −20 °C and thawing at 2–8 °C, one cycle, indicated that all formulations had comparable cryoprotective effect on r*Hu*G-CSF protein as the amount of total impurities measured by SEC HPLC did not exceed 0.2% for any of the formulation tested. The results of performed freeze–thaw experiment demonstrate that all formulations had comparable cryoprotective effect on rHuG-CSF protein as the amount of total impurities measured by SEC HPLC did not exceed 0.2% for any of the formulation tested. While preferential exclusion mechanism for the stabilization of proteins in aqueous systems applies equally well to protein cryopreservation, these thermodynamic arguments for protein stabilization are no longer applicable whenever water is eliminated from the system as would be the case during lyophilization process. During drying, the major stress that must be overcome is the removal of the protein's hydration shell. By looking at structure of the stabilizes tested (Fig. 1) we can hypothesize that each one has the potential to satisfy the hydrogen-bonding requirements of the polar groups on dried proteins, and thus serve as water substitutes for dried proteins. Potential stability issue in the lyophilized form would be the absence of polysorbate 80 in two of the formulations tested, as its presence in the formulation can prevent protein denaturation during lyophilization, presumably by competing with proteins for the ice–water interface.

### 3.3.2. Agitation and aggregation

Agitation induced protein aggregation is presumably the consequence of protein interactions with surfaces such as air–water or vial–water interfaces (Charman et al., 1993). Agitation, one of the most common physical stresses that can cause protein aggregation was used by applying vortex mixing of samples immediately prior to analysis to assess protective effect of tested formulations on r*Hu*G-CSF agitation induced aggregation (Fig. 14). Formulations stabilized by stabilizers leucrose, lyxose, trehalose and sorbitol had more than 2% of dimers, while those containing lactitol, xylitol, cellobiose, turanose, and cellobiitol had between 1.3–1.8% of dimers. Absence of polysorbate 80 in the formulation stabilized by sorbitol resulted in highest measured amount of dimer (7.7%). Interestingly, all of the tested samples have higher impurity levels than sample containing acetate buffer alone. It could be expected knowing that formation of oligomers and aggregates by agitation is in the most of the cases reversible process. Density of formulated solutions in contrast to acetate buffer solution is much higher (50 mg of stabilizers/ml) and number of molecular collisions produced by agitation is more probable in dense solution. However, differences noticed among differently formulated solutions can also reveal susceptibility of each formulation to inhibit or provoke unwanted collisions.

It is worthwhile to notice that despite different driving forces of r*Hu*G-CSF agitation and thermally induced stress the position of highest ranking stabilizers (cellobiitol) and lowest ranking stabilizers (lyxose and leucrose) were in both experiments the same.

### 3.4. Protein oxidation, deamidation and truncation monitoring by RP HPLC

Methionine oxidation has been recognized as an important cause of inactivation of therapeutic proteins and peptides during bioprocessing and storage (Wang, 1999). Methionine oxidation can typically be divided in two categories site specific and non-site specific (Li et al., 1995). Site specific oxidation is induced by transition metal ions (e.g. residual amounts of tungsten originating from syringe manufacturing process, (Rosenberg, 2006)) and therefore can be inhibited by adding chelating agents such as EDTA, citric acid, etc. in different recombinant protein production phases. Non-site specific oxidation is induced by light or by contaminant oxidants like hydrogen peroxide. Any harmless and non-toxic excipient that can limit and reduce the exposure of methionine residues to potential oxidants may be used to protect against non-site specific oxidation.

Forced oxidation study of r*Hu*G-CSF was performed by spiking the tested formulations with high concentration of hydrogen peroxide, incubating the sample at 25 °C and finally quenching the reaction by addition of L-methionine. The amount of oxidized r*Hu*G-

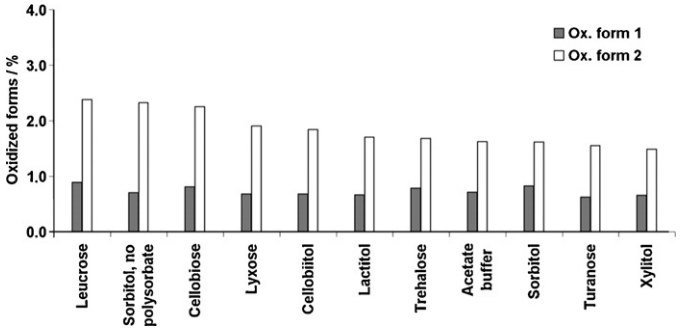


**Fig. 15.** The amount of oxidized form 1 and oxidized form 2, in tested r*Hu*G-CSF samples subjected to forced oxidation conditions determined by RP HPLC.

CSF formed was measured by RP HPLC (Fig. 15). Presence of two oxidized forms (oxidized form 1 and oxidized form 2) was observed and they both consist of monooxidized Met127 or Met138. Any of four methionines present in r*Hu*G-CSF potentially might be oxidized (Lèon et al., 1998; Yin et al., 2004); however, probability for methionine oxidation is not equal for all four methionines, as it depends on methionine microenvironment, i.e. first adjacent amino acid in chain or closeness of potential oxidizers in three-dimensional protein conformation (Lu et al., 1999).

No apparent difference between the tested formulations was noted in the amount of r*Hu*G-CSF oxidized form 1, whereas the amount of oxidized form 2 differed between samples stabilized by different substances. Literature data (Lu et al., 1999) suggest that Met138 is solvent-accessible and its surrounding microenvironment may be critical for G-CSF function. Consequently, the amount of oxidized form 2, ranging from 1.5% for sample stabilized by xylitol and turanose to 2.4% for sample stabilized by leucrose, could be indication of Met138 oxidation. Similarly, the presence of constant levels of oxidized form 1 in tested formulation can probably be attributed to oxidation of Met127 which is less accessible to the solvent. Although the focus of the experiment was on oxidation, different r*Hu*G-CSF modifications as i.e. deamidation, truncation and structurally rearranged forms were observed and quantified.

In this case no connection between structural class of stabilizer (polyols and sugars) could be noted regarding their effectiveness in preventing oxidation.

### 3.5. Statistical data evaluation

Pre-screening data obtained by LC/MS peptide mapping experiment divided stabilizers in three groups: turanose, trehalose, lactitol, lyxose, leucrose, and acetate buffer as top stabilizing candidates, xylitol, sorbitol and sorbitol without polysorbate as border cases and cellobiose and cellobiitol as outliers (Fig. 16A). Furthermore, statistical evaluation of data acquired by comprehensive and time consuming screening study that included quantitative analysis of dimers, oligomers, aggregates and oxidized, deamidated, truncated and altered r*Hu*G-CSF variants is presented on Fig. 16B. Turanose, trehalose and lactitol remained in the group of top stabilizer candidates. Cellobiitol was added to that group in opposite to pre-screening data analysis results and xylitol changed the position from border to top. Sorbitol, cellobiose and acetate buffer defined their position as border cases and sorbitol without polysorbate, lyxose and leucrose showed little stabilizing effect.

Overall stabilizing effect of selected substances was estimated with the aim of determining both the optimal stabilizer, and to compare the response pattern of different stabilizers across a variety of parameters essential for the protein stability. According to the overall performance, i.e. the lowest cumulative rank, the stabilizers were ranked in the following order (from best to worst): turanose, trehalose, lactitol, acetate buffer (non-stabilized sample), xylitol, cellobiitol, sorbitol, lyxose, leucrose, sorbitol without polysorbate, cellobiose (Fig. 16C). The performance of the tested stabilizers was significantly different for the tested conditions (Kruskal–Wallis $H(10, 561) = 65.94$, $p < 0.001$). However, top-scoring candidates turanose and trehalose were only significantly superior to leucrose, sorbitol without polysorbate, and cellobiose. Lactitol and acetate buffer were also significantly better than sorbitol without polysorbate and cellobiose but not leucrose, and xylitol was only better than cellobiose ($p < 0.05$ for the listed comparisons). There was a significant degree of agreement among the performed tests in how the stabilizers performed overall with Kendall $W = 0.118$, $\chi^2$ (10, $N = 51$) = 60.05, and $p < 0.001$ (Siegel and Castellan, 1988), suggesting consistency in ranking of the stabilizers. In other words, the ordering of the stabilizers' performance was correlated among the tested conditions, with the top-ranking stabilizers achieving consistently better scoring.

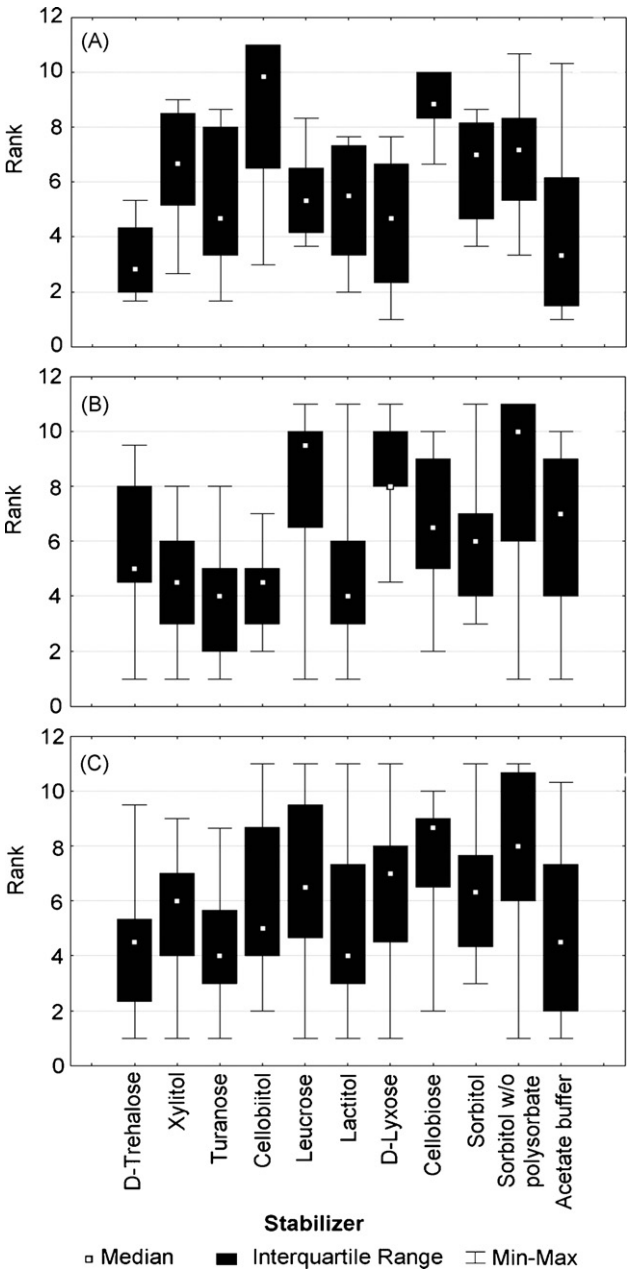


**Fig. 16.** Performance of various stabilizers under tested conditions. Median rank and interquartile range reflect the cumulative performance of each stabilizer. (A) Stabilizer pre-screening ranking, (B) stabilizer ranking without pre-screening data evaluation and (C) cumulative ranking.

## 4. Summary and conclusions

The effect of selected carbohydrate and polyols stabilizers on r*Hu*G-CSF molecule was evaluated firstly employing pre-screening method based on peptide mapping LC/MS analysis and complemented by comprehensive screening study of protein stability under degrading conditions using a battery of analytical test methods. Based on pre-screening data turanose, trehalose, lactitol, lyxose, leucrose, and acetate buffer are top formulation candidates, xylitol, sorbitol and sorbitol without polysorbate are border cases and cellobiose and cellobiitol are outliers (Fig. 16A). Comprehen-

sive screening results confirmed ranking of turanose, trehalose and lactitol as top stabilizers and indicated two more: cellobiitol and xylitol. Sorbitol, cellobiose and acetate buffer remained border cases and sorbitol without polysorbate, lyxose and leucrose showed little stabilizing effect (Fig. 16B).

Comparison of intact protein stability and the protein excised pieces cannot be directly compared but PPSP can be statistically incorporated in overall data evaluation as any other stability indicative method (Fig. 16C). It is expected knowing that such sensitive method as LC/MS is capable of rapid detection of even the smallest chemical changes, a process that may not even be relevant in intact protein where its native conformation protects certain parts of protein backbone as they are buried deeply inside the protein's three-dimensional structure. However, named method employed for the first time for stabilizer evaluation showed valuable contribution to overall protein stability assessment. The method can pinpoint the weakest parts of the macromolecule and help us answering why some degradation process is more preferred than the other in chosen formulation (e.g. the least stabile fragments T2, T5 and T6 contain cysteines in which oxidation state can be directly correlated to stabilizer selection under certain pH conditions).

The results of the current comparisons thus suggest that the stabilizers should be ranked in the following order: turanose, D-trehalose, lactitol, acetate buffer (non-stabilized sample), xylitol, cellobiitol, sorbitol, D-Lyxose, leucrose, sorbitol without polysorbate, cellobiose when looking at the overall performance. However, since individual measures of protein stability are rarely equally important for the final utility of the product, one should consider performance under individual tests as well as the overall score so as to best meet the specific needs for a given product.

Finally, there was no solid statistical connection found between stabilizers within the same structural group (cellobiitol and lactitol are disaccharide polyols, sorbitol and xylitol are monosaccharide polyols, lyxose is a monosaccharide sugar and cellobiose, trehalose, turanose and leucrose are disaccharide sugars). Interestingly, looking at the top three stabilizers turanose, trehalose and lactitol it can be noted that the first two are disaccharide sugars while lactitol belongs to group of disaccharide polyols. Overall, tiny stabilizer structural differences might cause huge changes in protein stability as can be seen from the experimental study results. As structurally different stabilizers yielded similar effects we hypothesize that they act by being preferentially excluded from the surface of the protein, thus creating such thermodynamic conditions that favour protein native state.

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