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ABSTRACT

Protein dynamics play a significant role in many aspects of enzyme activity. Monitoring of structural changes and aggregation of biotechnological enzymes under native conditions is important to safeguard their properties and function. In this work, the potential of asymmetrical flow field-flow fractionation (AF4) to study the dynamic association equilibria of the enzyme β-D-galactosidase (β-D-Gal) was evaluated. Three commercial products of β-D-Gal were investigated using carrier liquids containing sodium chloride or ammonium acetate, and the effect of adding magnesium (II) chloride to the carrier liquid was assessed. Preservation of protein structural integrity during AF4 analysis was essential and the influence of several parameters, such as the focusing step (including use of frit-inlet), cross flow, and injected amount, was studied. Size-exclusion chromatography (SEC) and dynamic light scattering (DLS) were used to corroborate the in-solution enzyme oligomerization observed with AF4. In contrast to SEC, AF4 provided sufficiently mild separation conditions to monitor protein conformations without disturbing the dynamic association equilibria. AF4 analysis showed that ammonium acetate concentrations above 40 mM led to further association of the dimers ("tetramerization") of β-D-Gal. Magnesium ions, which are needed to activate β-D-Gal, appeared to induce dimer association, raising justifiable questions about the role of divalent metal ions in protein oligomerization and on whether tetramers or dimers are the most active form of β-D-Gal.

1. Introduction

β-D-Galactosidase (β-D-gal) is a biotechnological enzyme of great interest to the dairy industry. A primary function of this enzyme is catalyzing the hydrolysis of lactose to form glucose and galactose. It is being used for the production of lactose-free dairy products for people suffering from lactose intolerance [1–5]. β-D-Galactosidase can be of animal, plant, or microbial (bacteria, fungi, yeasts) origin. Although bacteria may offer more versatility, yeasts and fungi are preferential sources of β-galactosidase for food biotechnology and pharmaceutical industry [6–8]. Zolnere and Ciprovica [4] summarized and compared the most suitable

commercial β-D-galactosidase enzymes for lactose hydrolysis, emphasizing the variations in optimal conditions for maximal activity. Evidently, enzymes from different microorganisms require different optimal conditions, including pH, temperature, presence of inhibitors or activators, *etc.*, which ultimately govern their final industrial application. For example, β-D-galactosidase from yeast (*Kluyveromyces* species) has proven suitable for the hydrolysis of lactose in milk and sweet whey, whereas the enzyme originating from fungus (*Aspergillus* species) exhibits the highest activity in acid whey [4]. The activity of an enzyme is strongly related to its structure, which can change when the enzyme is exposed to certain conditions. Knowledge on the in-solution native structure, aggregation behavior and chemical composition is essential and requires appropriate analytical techniques.

This study focuses on β-D-galactosidase from *Kluyveromyces* species, has large biotechnology potential [4,9,10]. Extensive re-

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search to determine the optimal conditions for its maximal activity in the hydrolysis reaction has been conducted [3,11–13]. However, its X-ray crystallographic structure was determined only recently [14]. Previously, structural information had been indirectly derived from chromatographic studies, mainly using size-exclusion chromatography (SEC) and native gel electrophoresis (GE) [15]. From the crystallographic data, the enzyme was described to have a tetrameric structure, formed upon association of two dimers ('tetramerization'). The authors predicted a dissociation energy for the tetramer into two dimers of 6 kcal/mol, which was significantly lower than the dissociation energy of the dimers (20 kcal/mol) [14]. Studies performed by ultracentrifugation, chromatographic and electrophoretic techniques reported the dimer being the major component of the enzyme under the examined conditions [14–16]. In all these studies it was hypothesized that the enzyme would be active both in its dimeric and tetrameric forms, with the equilibrium between associated and dissociated dimers depending strongly on the solution conditions [14,15]. More studies are required to elucidate the conditions that govern the association equilibrium and its impact on the activity of the enzyme [14]. The dependence of the higher-order structures of β-D-galactosidase on pH and temperature, and on the presence and concentration of divalent metal ions and various types of salts still need to be assessed.

SEC is one of the most commonly used techniques for size determination and quantitative assessment of the aggregation, including dimers and multimers of proteins [17]. Despite the wide use of SEC, the technique has some well-known limitations [18–21]. The shear forces experienced by the large protein molecules in the narrow channels through the packed bed and interactions with the packing material [22] may affect the aggregation and structure of the enzyme. Additionally, there are restrictions on the buffer types that can be used [20,21]. Moreover, SEC offers limited resolution, especially for very large molecules or molecular aggregates, some of which may be filtered out, either by frits in the system or by the column itself [23]. Given the increasing size and complexity of newly developed biotherapeutic and biotechnological proteins these limitations become significant analytical challenges. A broad set of complementary techniques are required to determine the critical quality attributes of such products.

Asymmetrical flow field-flow fractionation (AF4) is an attractive alternative method [9]. The main advantages of AF4 lie in its versality and ability to resolve higher-order structures. AF4, due to the absence of packing material in the channel, involves very low shear forces and eliminates the risk of filtering effects. In AF4 the analytes are injected in an open ribbon-like channel, and they are separated thanks to a parabolic flow profile based on their diffusion coefficients. The external flow (cross-flow), which is perpendicular to the main parabolic flow, is the main separation force. The cross-flow drives the analytes towards the membrane (accumulation wall) of the channel, resulting in a concentration gradient [24]. Diffusion (or Brownian motion) creates a counteracting motion. Large particles (or molecules) exhibit limited diffusion and they will stay close to the wall, where the lateral flow is slowest. Smaller particles with higher diffusivities will reach equilibrium positions further from the membrane, where the streamlines are faster. As a result, particles are separated according to size, with the largest ones eluting last.

AF4 methods can be optimized by varying a number of parameters, including the cross flow (and its variation in time), the detector flow, injected amount, focusing time, channel thickness, and the composition of the carrier liquid. Both the resolution and the recovery are common goals of this optimization process, but for the characterization of biomacromolecules preservation of the native state (conformation and higher-order structures) is equally important. Many studies have explored the influence of AF4 parameters on protein aggregation. Concerns have been expressed that certain factors, such the focusing step, concentration effect, interactions with the membrane, and sample dilution may affect labile protein aggregates [23–26]. Interactions with the membrane can be avoided by selecting an appropriate ionic strength of the carrier liquid and suitable membrane materials. Frit-inlet injection AF4 (FI-AF4) was introduced to avoid undesirable effects of stopping the flow during the focusing process [27–30]. In FI-AF4 hydrodynamic relaxation may be achieved through a "stop-less" injection. The concept has been applied for the fractionation of lipoprotein particles [31], carbon nanotubes [32], polyion complex self-assemblies [33] and ultra-high-molecular-weight cationic polyacrylamide [34] successfully avoiding adsorption on the membrane and sample self-association.

In this study, the dynamic association equilibria between the various species of the enzyme β-D-galactosidase are investigated using AF4 coupled to a triple detection system comprising UV absorbance, differential-refractive-index, and multi-angle light-scattering (UV-MALS-dRI). Three commercially available enzyme products are studied, using different carrier liquid compositions, mainly focusing on type of salt and ionic strength. An important aspect of this work is the evaluation and understanding of possible changes in conformation or association equilibria occurring during the AF4 analysis. The effects of various parameters will be evaluated, including the focusing process, cross-flow rate, and injected amount. To confirm the absence of protein denaturation in AF4, complementary techniques will be used to verify the in-solution state of the protein. FI-AF4 will be used to verify whether the focusing process affected the protein association, while batch-mode dynamic light scattering (DLS) can provide supporting information on the protein oligomerization under the examined conditions. Comparing the potential of AF4 and SEC for studying the dynamic association equilibria may shed light on possible disturbances between the protein species, due to physical stress exerted on the molecules. The overall goal of this study is to evaluate the potential of AF4 to provide structural information on enzymes under conditions resembling those encountered in typical environments.

2. Materials and methods

2.1. Chemicals

Three β-D-Galactosidase samples (β-D-Gal1, β-D-Gal2, β-D-Gal3) from *Kluyveromyces* yeast were used in this study. The samples from external vendors were provided by the DSM Biotechnology Center in formulations containing approximately 50% glycerol. The concentration of the stock solutions was estimated using the Bradford's protein assay [35]. The final concentration of the three samples used for the AF4 and FI-AF4 measurements was approximately 2 mg/mL, unless stated otherwise. Disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, potassium chloride, sodium azide, ammonium acetate and magnesium chloride hexahydrate were all purchased from (Sigma-Aldrich, Schnelldorf, Germany). All carrier-liquid solutions were prepared using ultrapure water (resistivity 18.2 MΩ; Sartorius Arium 611UV; Göttingen, Germany). A phosphate-based eluent (pH 7.0 ± 0.1) containing disodium hydrogen phosphate (6.5 mM), potassium dihydrogen phosphate (3.5 mM), sodium chloride (10, 50, 80, 140, or 200 mM), potassium chloride (2.7 mM) and sodium azide (0.05% by weight) was used. For the investigation of the effect of metal ions, magnesium chloride hexahydrate (2 or 10 mM) was added in a 9 mM phosphate solution containing 25 mM sodium chloride (pH 7.0 ± 0.1). Various concentrations (25, 40, 80, 150 mM) of ammonium acetate (pH 6.9) were also investigated as

carrier-liquid solutions. The final pH was adjusted with ammonium hydroxide (28−30% NH_3 in water).

For comparison purposes, the SEC-UV-MALS-dRI experiments were conducted with comparable mobile-phase compositions as described above for the AF4 experiments. First, experiments were conducted using a phosphate-based mobile phase (100 mM) containing sodium sulphate (100 mM) and sodium azide (0.05% by weight). Additional experiments were performed with phosphate-buffered saline (pH 7.0 ± 0.1) solution, containing sodium chloride (140 mM), potassium chloride (2.7 mM), sodium azide (0.05% by weight) as well as ammonium acetate (100 mM, pH 6.9) as mobile phase.

2.2. Instrumentation

2.2.1. Asymmetrical flow field-flow fractionation (AF4-UV-MALS-dRI)

Experiments were performed using an AF2000 MultiFlow FFF system (Postnova Analytics, Landsberg/Lech, Germany), coupled to an SPD-20A UV/Vis detector operated at 280 nm (PN3212, distributed by Shimadzu Corporation, Kyoto, Japan), a multi-angle light-scattering (MALS) detector (PN3621) and a refractive index detector (PN3150) at a working temperature of 40°C. All components were made available for the project by Postnova. The dimensions of the AF4 channel were 335 mm × 60 mm. The channel had a tip-to-tip length of 275 mm, initial width 20 mm, and final width of 5 mm. Separations were performed in a channel that contained a 350 μm spacer with a maximum width of 20 mm, a minimum width of 5 mm, and a length of 294 mm. A 10-kDa molecular-weight cut-off membrane prepared from regenerated cellulose (Postnova) was used as the accumulation wall. Sample injection was performed at an injection flow (F_{inj}) of 0.20 mL/min for 5 min using a cross-flow rate (F_c) of 3.0 mL/min and subsequent focusing flow rate of 3.30 mL/min. The detector flow rate (F_{out}) was set at 0.50 mL/min. After focusing and during elution F_c was kept constant at 3 mL/min for 25 min, followed by a linear decay over a 5-min period down to F_c = 0.2 mL/min. F_c was then kept constant at 0.2 mL/min for 5 min. Lastly, in a rinsing step, F_c was turned to zero and a laminar flow was maintained through the channel (F_{out} = 0.5 mL/min) during 5 min. The cross-flow rate profile of the AF4 method developed for the separation of the various oligomers of the β-D-Gal products is illustrated in Figure S1.

2.2.2. Frit-Inlet asymmetrical flow-field flow fractionation (FI-AF4-UV-MALS-dRI)

The FI-AF4 experiments were performed using the AF2000 MultiFlow FFF system (Postnova). The channel consisted of the same bottom components as in the standard analytical channel (spacer and ceramic frit). At the top plate a frit of 18.8 mm diameter and with 2 μm pore size is positioned at the tip injection port. A regenerated cellulose membrane of 10 kDa molecular weight cut-off (Postnova) was used.

For the FI-AF4 experiments a 10-uL sample injection was performed at F_{inj} = 0.1 mL/min, a F_c = 3.0 mL/min and a frit-inlet flow (F_{FI}) of 3.2 mL/min. F_{out} was set at 0.30 mL/min. After injection F_c was kept constant at 3 mL/min for 25 min, followed by a linear decay over a 5-min period down to F_c = 0. Lastly, in a rinsing step, F_c was turned to zero and a laminar flow was maintained through the channel (F_{out} = 0.3 mL/min) during 5 min.

2.2.3. Size-exclusion chromatography (SEC-UV-MALS-dRI)

Size-exclusion chromatography was performed on the same AF2000 MultiFlow FFF system (Postnova) and the using the same detectors as for the AF4 measurements. The TOSOH TSKgel G3000SW$_{XL}$ column (Griesheim, Germany; 300 mm × 7.8 mm i.d., 5-μm particle size, 250-Å pore size) was used in this study. In all

Product	LMw (%)	Dimer (%)	HOS	Recovery (%)
β-Gal1	15	70	10	85
β-Gal2	5	85	2	85
β-Gal3	-	90	5	83

cases an injection volume of 20 μL and an eluent flow rate of 0.5 mL/min were used. Separations were carried out at room temperature.

2.2.4. Dynamic light scattering (DLS)

Measurements were performed at 25°C using plastic disposable UV-cuvette (Brand, Essex, CT) on a Zetasizer Nano-ZS system (Malvern Instruments, Malvern, UK), which detects backscattering at an angle of 173°. β-D-Galactosidase was dissolved in the various salt solutions at a final concentration of 2 mg/mL. DLS values for each sample were averaged over three runs of eleven measurements each. The Z-Average size or Z-Average mean , also known as the cumulants mean or the 'harmonic intensity averaged particle diameter', is considered the primary and most stable parameter obtained from DLS [36].

2.3. Data evaluation

Data acquisition was carried out by AF2000 control software version 2.1.0.1 (Postnova). The molar mass and average-weighted molecular weight (M_w) were calculated using the Zimm model and a refractive index increment (dn/dc) of 0.185. In these calculations, the angles of 7°, 12°, 20° and 158°, 164° were excluded, as their signal-to-noise ratios were too low for accurate measurement.

Recoveries (%) were estimated from the ratios of the peak areas from the UV trace of the separated agglomerated species while applying cross flow, divided by the area obtained when the sample was eluted through the channel at the same outlet flow without cross flow [37]. Only the peaks corresponding to the protein oligomers were integrated. Highly retained sample and higher order structures eluting during the rinsing step (F_c = 0) were not included in the recovery estimation.

3. Results and discussion

3.1. AF4 of β-D-galactosidase under near-native conditions

Initial AF4 experiments were aimed at characterizing three samples of β-D-galactosidase obtained from different commercial sources (β-D-Gal1, β-D-Gal2, β-D-Gal3) to investigate the structural differences. The three samples were analyzed using a constant cross-flow rate (F_c) of 3 mL/min, an outlet flow rate (F_{out}) of 0.5 mL/min, and a saline carrier liquid containing 10 mM phosphate buffer and 50 mM sodium chloride at pH 7.0. Figure 1 shows the UV signals at a wavelength of 280 nm and the MALS signal of the 90° angle for the three analyzed samples. Table 1 summarizes the quantitative information obtained. As can be seen, under the applied conditions a satisfactory sample recovery (*ca.* 80-85%) and sufficient separation between the low-molecular-weight (LMW) species, the dimeric species and the higher-order structures (HOS) were achieved for the three analyzed samples. The main peaks observed for the three samples correspond to the dimer, eluting at approximately 11 min with a molar mass of approximately 2.4×10^5 g/mol (estimated from the combined MALS
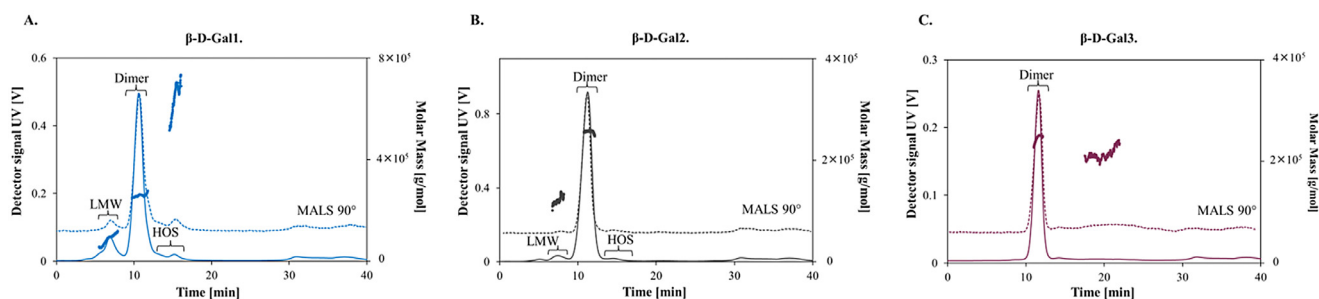
Figure 1. AF4 fractograms of the three examined β-D-Gal products. Carrier liquid: 10 mM phosphate buffer with 50 mM sodium chloride at pH 7.0. Constant F_c of 3 mL/min and F_{out} of 0.5 mL/min were used; Drawn line: UV signals at 280 nm (left-hand axis); Dotted line: MALS signal at 90° angle (arbitrary scale); Data points: estimated molar masses at specific time points (right-hand axis).

and dRI signals). The latter is in line with reported values for the monomer (1.2×10^5 g/mol) [14]. Different amounts of LMW species and of the HOS were observed in the fractograms of the three samples. β-D-Gal1 contained approximately 15% (based on area) of LMW species (ca. 9.7×10^5 g/mol), as well as about 10% of HOS. B-D-Gal2 and β-D-Gal3 were mainly present as the dimer, with less than 10% of LMW species and HOS combined. The MALS trace of β-D-Gal3 exhibited an additional, broad, late-eluting band (16–23 min). This may indicate the presence of larger structures (HOS of increased MW) or unfolded species of higher hydrodynamic radius. The estimated molar mass (data points) in Figure 1c and diffusion coefficients (from AF4 retention times, $D_{dimer}=3.6 \times 10^{-11}$ m^2 s^{-1} ; $D_{unfolded}=1.8 \times 10^{-11}$ m^2 s^{-1}) suggest the presence of unfolded dimeric species in the later eluting peak (16–23 min). Enzyme activity can be significantly influenced by aggregation or oligomerization driven by environmental parameters, such as metal ions, ionic strength, temperature, etc. Therefore, we have studied the effects of: ionic strength, salt type, and presence of metal ions, on the aggregation behavior and oligomerization of these different products.

3.2. Effect of sodium chloride concentration on β-D-galactosidase

A known limitation of AF4 is overloading. This can be influenced by the carrier-liquid composition. Therefore, it is important to investigate the overloading phenomena and its possible effect on the denaturation. In his critical overview, Wahlund [38] specifically emphasized the importance of examining possible overloading as part of AF4 method development, by varying the injected mass by a factor of 5 to 10. Overloading leads to distorted peaks and shifting of the peak maximum. When such phenomena are observed, the sample load should be decreased until the retention time and the peak symmetry remain constant. Overloading and aggregation phenomena were investigated at 10 and 140 mM sodium chloride in a phosphate-based (PBS) eluent, with sample injected amounts varying from 20 to 200 μg. The resulting elution profiles are shown in Supplementary Material (Figure S2 A, B). No shift in the retention time, nor distortion of the peak of the dimeric species were observed when varying the injected amount at 10 mM sodium chloride (10 mM) in PBS eluent (Figure S 2A). In contrast, at 140 mM sodium chloride in PBS eluent and above 40 μg amount injected, the retention time of the dimeric species shifted to higher retention time and the peak shape notably altered, indicating possible overloading. Subsequent experiments for studying the effects of increasing sodium chloride concentration were conducted by injecting 40 μg of enzyme.

The optimal ionic strength for characterization studies by AF4 varies strongly with the proteins to be analyzed. Literature suggests that neutral pH and an ionic strength values of 50 to 100 mM may be a good starting point [37,39,40]. In this work, the effects of

ionic strength of the carrier liquid on the retention and stability of β-D-galactosidase were investigated by varying the concentration of NaCl (10, 50, 80, 140, 200 mM) in a phosphate-based buffer (10 mM) of near-physiological pH. β-D-Gal1 sample was used as a test sample, as it showed various oligomers in comparison to the other samples (Figure 1), allowing information to be obtained on both stability and separation. An increase in ionic strength of the carrier liquid led to a shift towards higher retention times of the peak corresponding to the dimeric species, while the peaks corresponding to the LMW species were not significantly affected (Figure 2). An increase of the peak width of the dimeric species was also observed, especially at higher salt concentrations (140 and 200 mM) and the separation between the dimer and the HOS was hampered. Recoveries were 80-85% regardless of the ionic strength of the carrier liquid. This suggests that the peak broadening is not caused by protein-membrane interactions. The shift of the dimer peak towards higher retention times suggests a change in the diffusion coefficient, which may indicate conformational changes of the dimeric species or aggregation at high ionic strength.

However, when comparing the molar mass provided by the MALS detector for this sample at both low and high concentration values of sodium chloride tested, protein aggregation appears evident (Figure 2B). At 10 mM sodium chloride, the dimeric species were predominantly present, whereas at 200 mM sodium chloride the peak is almost bimodal, indicating the presence of more than one specie. The estimated molar mass at the beginning of the peak (11–14 min) suggests the presence of dimeric species, followed by a steep increase for later-eluting species (15–18 min). The increased aggregation or association of proteins at higher ionic strength conditions may be due to "salting-out" effects, and can significantly impact the enzyme activity [12,41].

3.3. Effects of type and concentration of salts in the carrier liquid on the association equilibria

3.3.1. Ammonium acetate

A significant aspect of this work was to study the effects of different carrier liquids on the equilibrium between the associated and dissociated dimers of β-D-galactosidase. The AF4 method using a phosphate-based eluent containing sodium chloride provided a good separation between the various species of the investigated β-D-Gal products but showed signs of protein aggregation at high salt concentrations. Therefore, the possibility of using an ammonium acetate carrier liquid was studied. Figure 3 depicts the AF4 elution behavior of β-D-Gal1 using the sodium chloride/phosphate-based eluent in comparison to an ammonium-acetate carrier liquid at identical ionic strength (80 mM) and pH (6.9±0.1). Clearly, when using ammonium acetate, the separation between the dimer peak and the HOS is lost, leading to a broad bimodal peak. Moreover, the estimated molar masses at each time
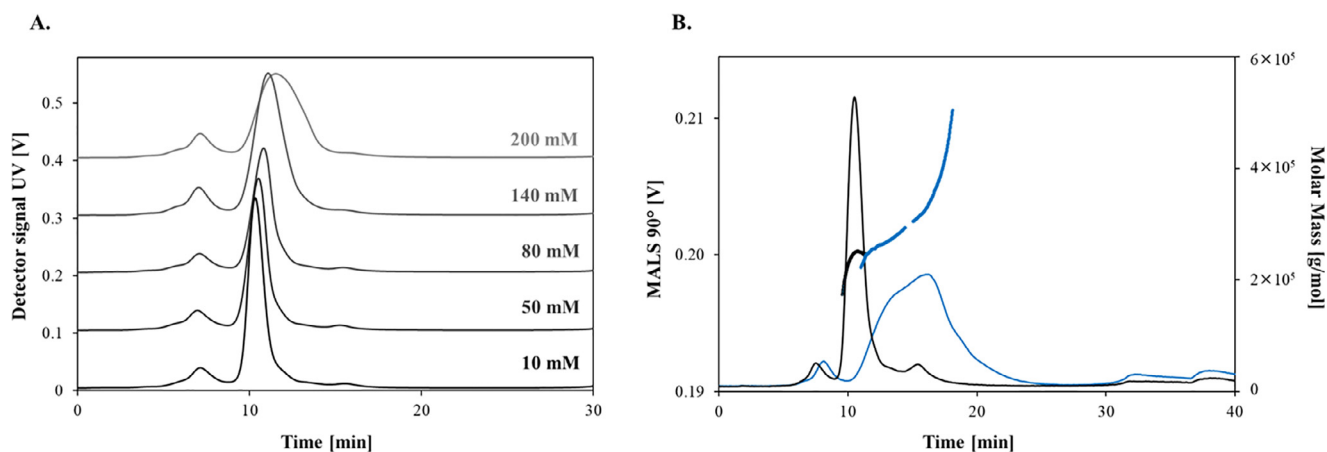
A.

B.

Figure 2. A) AF4-UV fractograms showing the effect of increasing concentrations of sodium chloride (10, 50, 80, 140, 200 mM) in a phosphate-based carrier liquid on the elution profile of the β-D-Gal1 product (40 μg amount injected). B) AF4-MALS traces and molar-mass estimates obtained at low (10 mM; black trace) and high (140 mM; blue trace) sodium chloride concentrations.
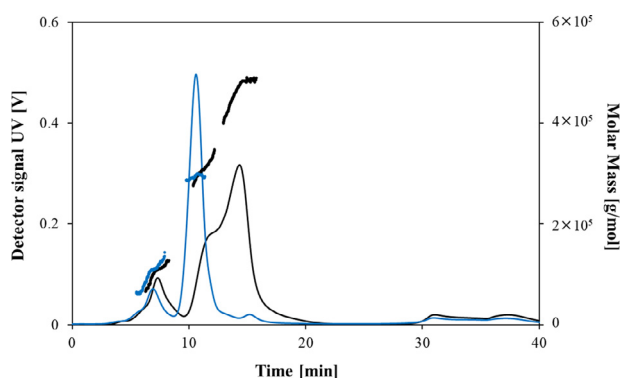

Figure 3. β-D-Gal1 AF4 fractograms obtained when analyzed using a sodium chloride/phosphate-based eluent (blue) and an ammonium acetate eluent (black) at comparable ionic strength (80 mM) and pH (6.9$\pm$0.1). Injected amount 40 μg. Constant F_c of 3 mL/min and F_{out} of 0.5 mL/min were used.

point suggested a major shift of the equilibrium from dimers to HOS in the ammonium-acetate eluent. The molar mass of the main peak eluting around 14 min is very close to the molar mass of the tetrameric species (4.8 $\times$ 10^5 g/mol).

Different concentrations of ammonium acetate (25, 40, 80, 140 mM) were tested to study the effects on the equilibrium between the β-D-galactosidase species in detail (Figure 4). A change in the percentages based on the UV peak area (at 280 nm) of the LMW, dimeric and tetrameric species for β-D-Gal1 with increasing ammonium-acetate concentration was observed (Figure 4 B). As seen in Figures 4A and 4B, at low concentrations of ammonium acetate (25 – 40 mM), the equilibrium is tilted towards the dimeric species. At concentrations above 40 mM association of the dimers is induced, and the presence of tetrameric species is evident. The overlaid fractograms obtained by using 40 mM and 140 mM ammonium acetate and the estimated molar-mass values obtained are shown in Supplementary Material, Figure S3. This is in contrast to the results obtained with the sodium chloride/phosphate-based eluent, in which a similar effect was only observed at higher concentrations of sodium chloride (above 140 mM).

The focusing step in AF4 may induce protein-protein or self-interaction, as well as interactions of the analyte with the membrane [24]. To eliminate the possibility of experimental conditions contributing to the association of the dimers, the effects of focusing time, cross flow, focus flow, and sample concentration were studied. Varying the focusing time from 2 to 10 min while using

140 mM ammonium acetate as carrier liquid, did not reveal a significant effect on the equilibrium between dimeric and tetrameric species (Supplementary Material, Figure S4). To investigate the influence of the injected amount (20, 40, 100, 200 μg), the injection volume was kept constant while varying the sample concentration at 40 and 140 mM ammonium acetate (Supplementary Material, Figure S2 C,D). Overloading phenomena started to occur when the injected amount exceeded 100 μg, both at low and at high ionic strength. However, the association at 140 mM ammonium acetate did not appear to be influenced by the injected amount.

The effect of the cross-flow rate (1.5, 2, 3, 3.5 mL/min) was also investigated at low and at high ionic strength. It is known that lowering the cross-flow rate leads to lower resolution [37]. On the other hand, higher cross-flow rates may affect the recovery, as the analytes are forced closer to the membrane during the entire analysis, which may lead to protein-membrane interactions [37]. The fractograms shown in Figure S5 (supplementary material) provided no indication of any loss in recovery or shift in equilibrium between the dimeric and tetrameric species upon increasing the cross-flow rate, neither at low nor at high ionic strength.

To verify that the stop-flow and focusing process do not induce unwanted structural changes or shift the dynamic equilibrium, the tetramer formation was also investigated using frit-inlet AF4 (FI-AF4). In this case, hydrodynamic relaxation is achieved during injection without stopping the flow to the detector. Thus, the procedure of sample injection and hydrodynamic relaxation do not involve halting the sample elution [30]. Various flow conditions were investigated with FI-AF4-UV-MALS-dRI (results not shown) using carrier liquid conditions at which tetramerization occurs (140 mM an ammonium acetate). Although the resolution between the different species was lower than that achieved with analytical AF4, the presence of the tetrameric species was verified by MALS (Figure 5). This confirms that their occurrence observed in the fractograms obtained by conventional AF4 (Figure 3) is not caused by the stop-flow relaxation process. The fractograms obtained at two exemplary cross flow rate conditions (2, 3 mL/min) suggest a slight increase in resolution with increasing cross flow. The estimated molar masses increase during the ascending slope of the later eluting peak, indicates coelution of dimeric and tetrameric species.

Lastly, all three β-D-Gal samples were analyzed by AF4 and FI-AF4 using 140 mM ammonium acetate as carrier liquid (Figure 6). Comparison of these fractograms with those obtained using the sodium chloride/phosphate-based carrier liquid (Figure 1) reveals a shift in the aggregation equilibria from dimers to tetramers for β-D-Gal1 and β-D-Gal3. In contrast, the equilibrium does not
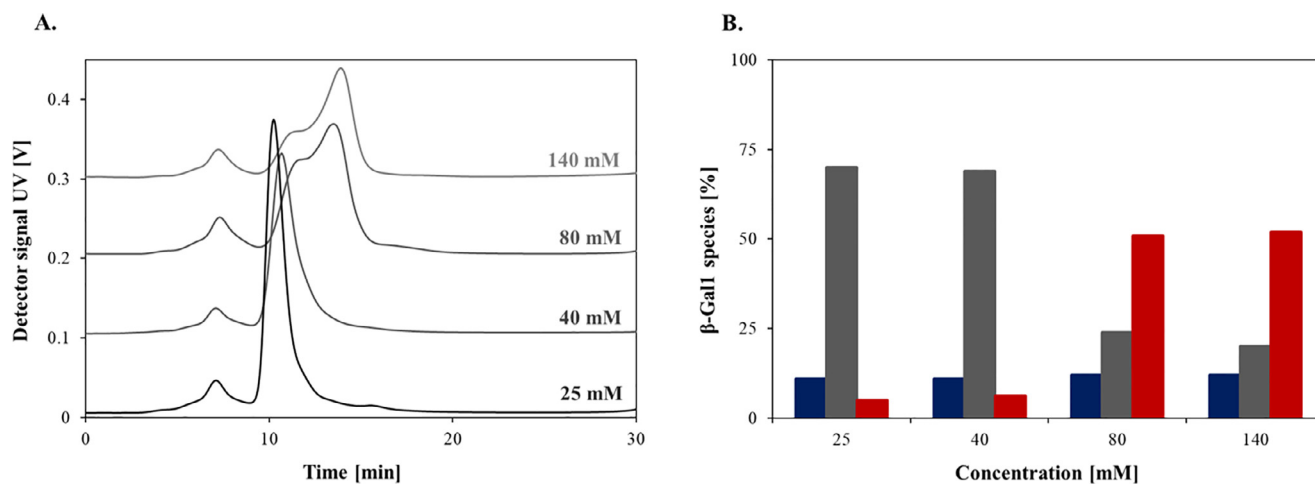
Figure 4. A) Monitoring the shift of the equilibrium between the β-D-Gal1 species at various concentrations of ammonium acetate (25, 40, 80, 140 mM). B) Relative contents based on the area of the LMW (blue), dimer (grey) and tetramer (red) signals at the different concentrations of ammonium acetate.
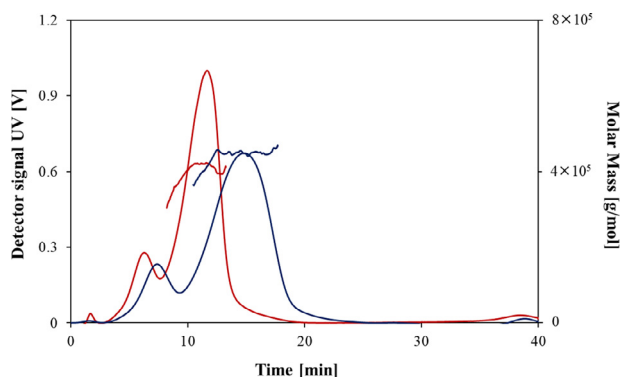

Figure 5. Fl-AF4-UV-MALS analysis of β-D-Gal1 using 140 mM ammonium acetate as carrier solution and utilizing F_c at 2 mL/min (red) or 3 mL/min (blue) with F_{out} 0.3 mL/min.

3.3.2. Magnesium (II) chloride

The importance of certain divalent metal cations, such as Mg^{2+} and Mn^{2+}, on the stability and activity of β-D-galactosidase have been extensively discussed [1,13,42]. Divalent metal ions have proven to be important for achieving maximal catalytic efficiency of the enzyme [13,43,44]. Although the importance of Mg^{2+} and Mn^{2+} for optimal activity is well documented, little is known about the influence of these ions on the structure of the enzyme.

The versatility of AF4 with respect to mobile-phase composition allows investigation of a great diversity of conditions that are not compatible with other techniques. To investigate the effect of the Mg^{2+} divalent ions on the structure of β-D-galactosidase, magnesium chloride was added to the carrier liquid in concentrations of 2 and 10 mM. Results showed that a higher concentration of magnesium (II) chloride induces the association of the dimers (Figure 7A). Both the later elution and the estimated molar mass (approximately 4.8×10^5 g/mol) confirmed the presence of tetrameric species. The previously proposed effect of divalent ions on the formation of the tetramer is thereby confirmed by AF4-MALS.

To determine whether the tetrameric structure is stable in the absence of Mg^{2+} ions, β-D-Gal1 was incubated in 10 mM magnesium chloride and then analyzed with an AF4 carrier liquid containing no or 10 mM magnesium chloride in the PBS. As depicted in Figure 7B, when analyzing the incubated β-D-Gal1 in the absence of Mg^{2+}, the equilibrium shifts back towards the dissociated
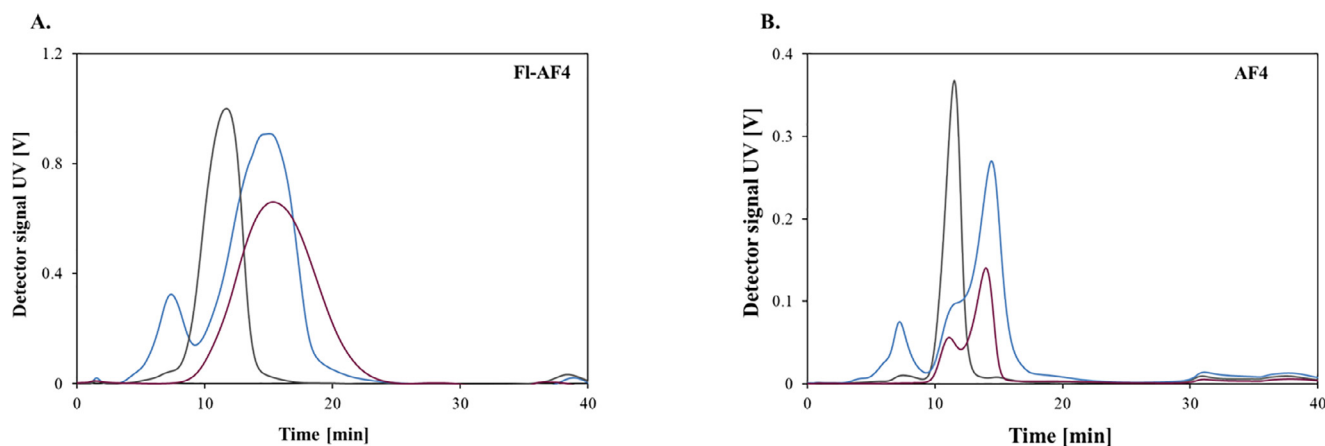
seem to be affected for β-D-Gal2. Batch-mode DLS experiments confirmed increasing in-solution aggregation for β-D-Gal1 and β-D-Gal3 with increasing ammonium acetate concentration. However, the z-average diameter of β-D-Gal2 remained unchanged over the examined concentration range (Supplementary Material, Figure S6).

Figure 6. Comparison between Fl-AF4 (A) and AF4 (B) fractograms of the three products at 140 mM ammonium acetate: β-D-Gal1 (blue β-D-Gal2 (grey), β-D-Gal3 (purple).
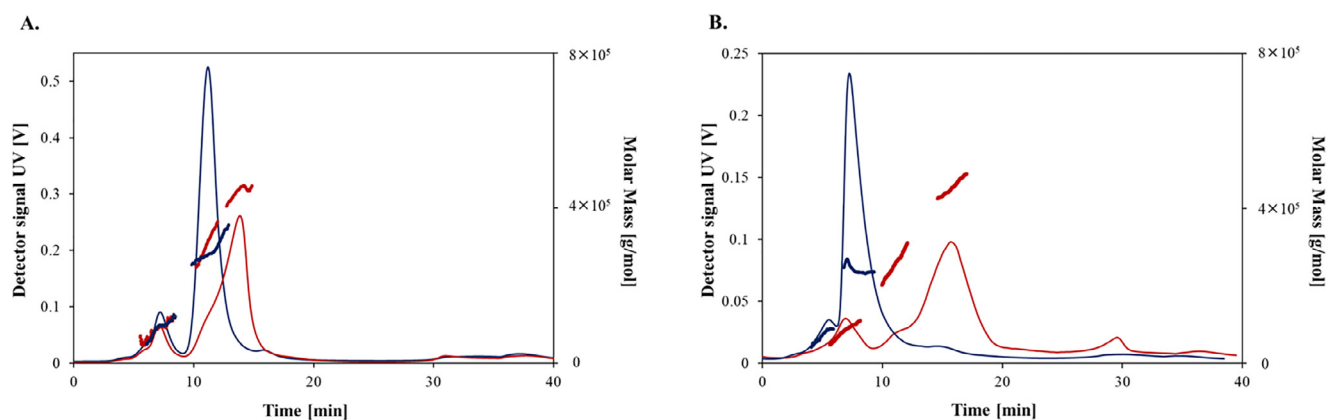
A.

B.

Figure 7. A) Comparison of β-D-Gal1 fractograms obtained with 2 mM (blue) and 10 mM (red) magnesium chloride present in a 10 mM PBS carrier liquid. B) fractograms of a β-D-Gal1 sample incubated in 10 mM magnesium chloride and analyzed in the presence (red; 10 mM) and absence (blue) of Mg^{2+} ions in the carrier liquid. Constant F_c of 3 mL/min and F_{out} of 0.5 mL/min were used.

dimeric species, suggesting that an excess of Mg^{2+} is necessary for the tetramer to be stable.

In light of the above results, the tetramerization after incubation with magnesium chloride of the three different products, was studied and the results were compared. Association of the dimers was apparent for products β-D-Gal1 and β-D-Gal3, but not for β-D-Gal2, which is in accordance with the behavior observed with increased concentration of ammonium acetate (Supplementary Material, Figure S7). The tetramerization of lactase in the presence of magnesium chloride was also evaluated with the Fl-AF4-UV- -MALS-dRI system and with batch-mode DLS (Supplementary Material, Figure S6 and Figure S7). The hydrodynamic z-average diameters of the three products were estimated in the presence of 10 mM magnesium chloride (Figure S6) and compared with the values obtained at an elevated ammonium acetate concentration (up to 200 mM). Because the type of salt and the ionic strength may affect the apparent size, BSA was used as a control to evaluate the influence of these two factors. For BSA, no significant influence of the salt type and ionic strength was observed. The z-average diameters of products β-D-Gal1 (approximately 14.5 nm) and β-D-Gal3 (approximately 16.6 nm) in a solution containing 10 mM magnesium chloride and in a solution containing 200 mM ammonium acetate were quite similar, with in-solution aggregation or oligomerization indicated under these conditions. In contrast, and as expected from the AF4 results where aggregation was not observed, the z-average diameter of β-D-Gal2 remained smaller (approximately 12.8 nm) and appeared unaffected by the presence of Mg^{2+} ions. The DLS results confirmed that AF4 and Fl-AF4 were providing "soft" separation conditions, preserving the labile associated dimeric species (tetramers) if they are present in a solution. This underlines the potential of AF4 and Fl-AF4 to provide detailed structural insights in the actual active conformation of enzymes at conditions resembling their natural environments.

3.4. AF4 vs. SEC to study association equilibria

SEC is the reference size-based separation technique to monitor purity levels and quantify aggregation in the quality-control (QC) process of β-D-galactosidase. SEC experiments were performed at conditions comparable to those used for AF4, to investigate whether it is feasible to study the association equilibria in the presence of a stationary phase material. Initial SEC-UV-MALS-dRI experiments were conducted using a phosphate-based mobile

phase containing 100 mM sodium sulphate, and 0.05% w/w sodium azide (ionic strength 600 mM, pH 6.8). A comparison between the various species observed by AF4 and SEC is presented in Figure 8. Two essential conclusions can be drawn from this figure. HOS cannot be clearly resolved under the applied SEC conditions, as the resolution decreases when the elution times approach the exclusion limit of the column used (TSKgel G3000SW$_{XL}$). Another important observation is that a fraction of the monomer is eluting just after the dimer for all the investigated products as revealed by MALS. This is in sharp contrast with the AF4 results, which revealed the dominance presence of dimeric species in all cases, with no monomeric structures being detected.

Although dissociation during SEC analysis may be questioned for β-D-Gal1, this is not the case for the other two products. For β-D-Gal2 and, especially, β-D-Gal3 the elution times and the molar-mass estimated from MALS suggested a notable increase in the amounts of monomeric species observed (Figure 8). The exerted physical stress on the protein structures while passing through the narrow channels of the stationary phase and the potential occurrence of unwanted interactions between the protein and the stationary phase material may cause changes in the protein conformation [45]. β-D-Galactosidase has a pI of approximately 5.9. At pH 6.8 the protein is negatively charged, while some of the silanol groups of the stationary phase may still be deprotonated and negatively charged. The ionic strength (600 mM) of the examined buffer may not suffice to prevent electrostatic interactions to the extent that disruption of the protein structure can be avoided. However, dissociation of the dimer may be primarily caused by the shear forces imposed on the labile protein structures.

In an attempt to use SEC to study the equilibrium between the dimeric and tetrameric species as was observed with AF4 when using ammonium acetate at concentrations exceeding 40 mM, comparable conditions (100 and 200 mM) were used in SEC experiments. The SEC-UV-MALS-dRI results confirmed the dominant presence of dimeric species for the three investigated products at 100 mM ammonium acetate (Supplementary Material, Figure S8). This contrasts with the AF4 results, according to which the tetrameric species were dominant in β-D-Gal1 and β-D-Gal3 at concentrations exceeding 40 mM ammonium acetate (Figure 4). Increasing the concentration of ammonium acetate to 200 mM, led to a shift of the observed equilibrium towards the tetramer, as shown in Figure 9. The results underline the advantages of AF4 over SEC for analysis of labile protein structures.
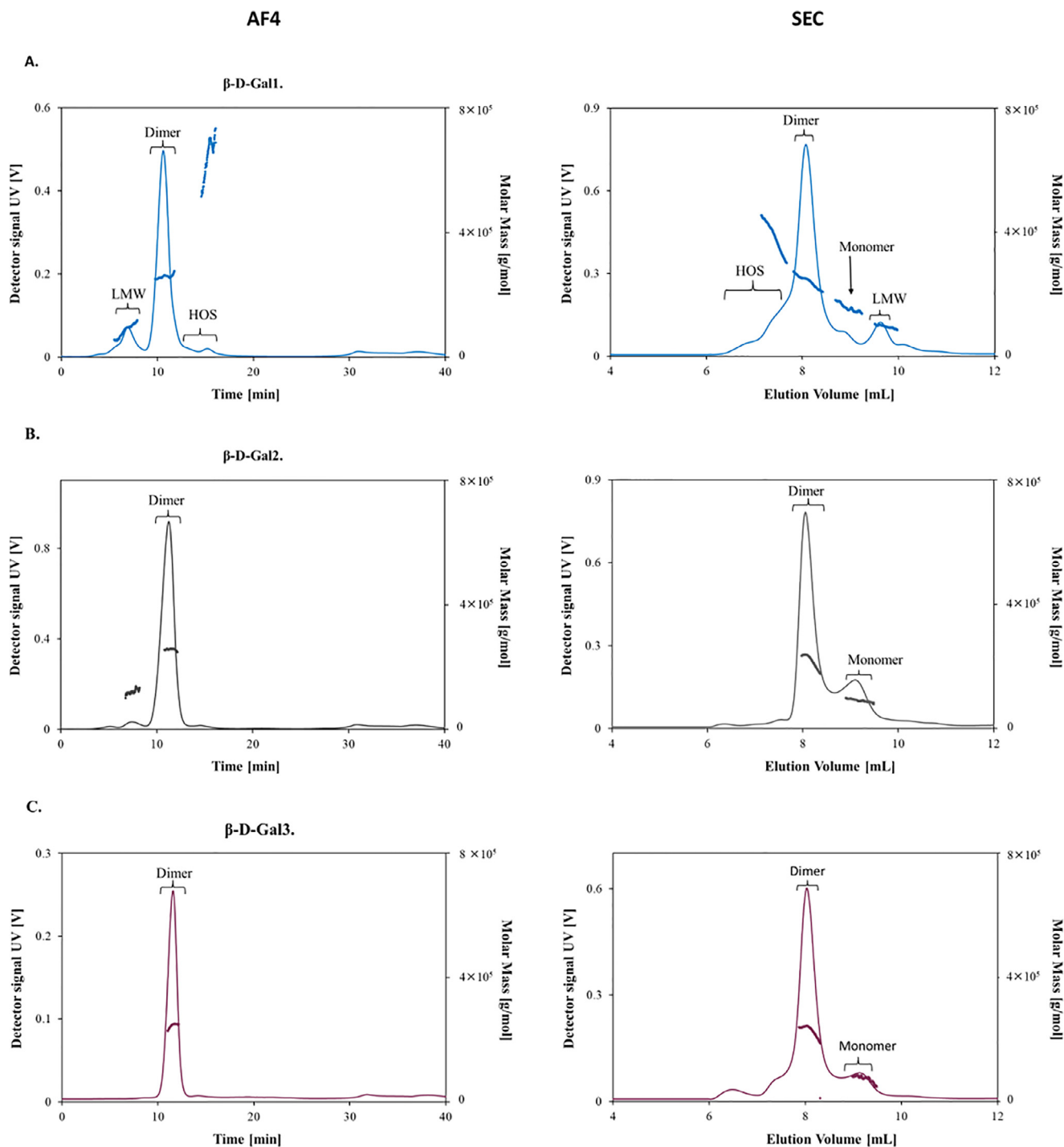
AF4

SEC

A.

β-D-Gal1.

B.

β-D-Gal2.

C.

β-D-Gal3.

Figure 8. AF4 fractograms (left) are SEC chromatograms (right) of the three β-D-galactosidase products. SEC column: TSKgel G3000SWXL (300 mm × 7.8 mm i.d., 5-μm particle size, 250-Å pore size)

A.

β-D-Gal1.
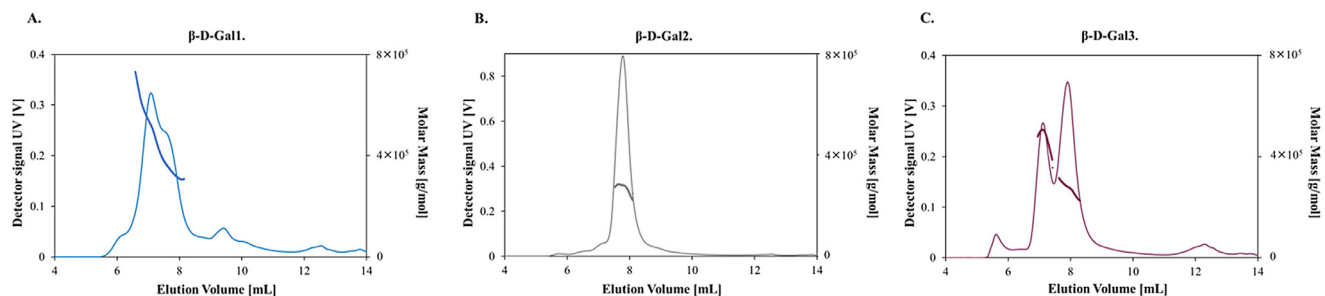
B.

β-D-Gal2.

C.

β-D-Gal3.

Figure 9. SEC-UV chromatograms and the respective molar-mass estimates for the three β-D-Gal products. Mobile phase: 200 mM ammonium acetate; Column: TSKgel G3000SWXL (300 mm × 7.8 mm i.d., 5-μm particle size, 250-Å pore size).

4. Conclusions

AF4 was used to study the effects of a number of carrier liquid conditions (type of salt, ionic strength) on the dynamic association equilibria of the biotechnological enzyme β-D-galactosidase. Three commercial products of this enzyme were investigated. The effect of three different salts (sodium chloride, ammonium acetate, magnesium (II) chloride) at various concentrations were investigated. Elevated concentrations of ammonium acetate (above 40 mM) and magnesium chloride (above 10 mM) were found to shift the equilibrium from dimeric to associated dimeric species (tetramer, approximately 4.8×10^5 g/mol) for two of the examined products, as revealed by multi-angle light scattering. It was verified that the tetramer formation in the presence of ammonium acetate or magnesium chloride was not induced by key parameters of the AF4 separation (focusing process, cross flow rate, injected amount). Frit-inlet (FI) AF4, which employs hydrodynamic relaxation without stopping the flow, supported this conclusion. Congruous structural information was obtained by AF4, FI-AF4 and DLS, confirming that the analytical techniques provided "soft" separation conditions, preserving the labile protein-association equilibria. In contrast, SEC required higher ionic-strength conditions to avoid unwanted interactions between the stationary phase and the analytes, while the flow through narrow channels exerted physical stress on the protein structures. As a result, supramolecular protein structures were found not to be preserved during SEC analysis. A next challenge is to investigate the coupling of AF4 with high-resolution MS (ICP-MS, ESI-MS) to obtain simultaneous structural and compositional information.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461719.

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