# Unraveling proteoform complexity by native liquid chromatography-mass spectrometry

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# Evaluating the effect of glycation on lipase activity using boronate affinity chromatography and mass spectrometry

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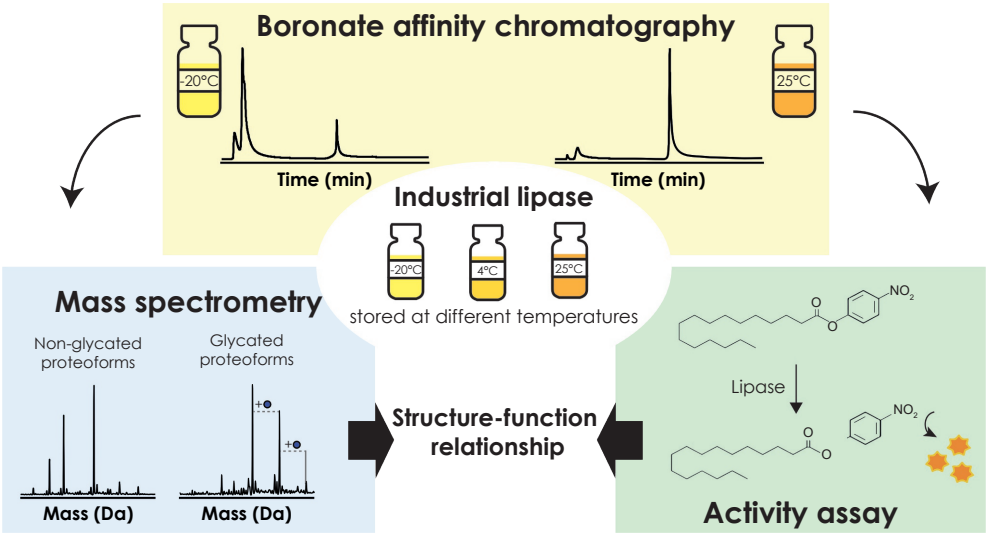
Protein glycation may occur naturally when reducing sugars and proteins coexist, which is often the case for industrial enzymes. This non-enzymatic post-translational modification may result in changed function or stability of the enzyme. To determine the influence of glycation on function of a (temperature-stressed) lipase used for food applications, we developed a method combining boronate affinity chromatography (BAC) to enrich for glycated species followed by mass spectrometry (MS) for structural characterization and functional assessment with activity assays. Using this approach, we demonstrated that storage at -20 °C and 4 °C resulted in minor glycation (below 9% of all assigned proteoforms), whereas storage at 25 °C led to a higher glycation level with up to four sugars per lipase molecule. Remarkably, activity measurements of the BAC fractions revealed that glycation did not reduce lipase activity and stability.



## 8.1 Introduction

Glycation is a post-translational modification (PTM) that has often been observed in dry enzyme products, including an endo-xylanase used for baking [323] and a glucoamylase for glucose syrup production [51, 53]. This PTM occurs via a non-enzymatic reaction between reducing sugars and primary amines of proteins, as originally described by Maillard [45, 324]. While the extent of glycation in these enzyme products is usually low, a range of factors (e.g., availability of reactants, elevated temperatures, basic pH and storage time) can greatly increase the glycation levels [52, 325]. Interestingly, extensive glycation can impact the enzyme functionality, stability and solubility [326]. For instance, in a recent study we demonstrated that glycated proteoforms of an endo-xylanase, induced by an accelerated stability study (storage at 40 °C), showed reduced activity compared to their non-glycated forms [323]. Conversely, glycated glucoamylases, obtained after incubation at 60 °C in the presence of reducing carbohydrates, showed a more efficient conversion of maltose and maltodextrin into glucose [53]. Since glycation can influence the enzyme performance in various ways, it is crucial to develop analytical strategies to monitor potential changes together with their functional impact and thereby, establish structure-function relationships and ensure product quality.

Mass spectrometry (MS)-based approaches have undoubtedly become key tools for the characterization of PTMs [324]. Unfortunately, the characterization of glycation by MS is often challenging for glycosylated proteins due to the identical mass increment of hexoses added by glycation versus glycosylation, in combination with often low abundance of glycation [47, 308, 318]. Functional assessment of glycation ideally relies on pure glycated species to discern their impact on activity. Therefore, analytical methods should be developed to obtain these pure glycated proteoform fractions without compromising the enzyme functionality. This involves the employment of native separation methods to isolate these species, followed by glycation assessment via MS and activity assays to unravel possible differences in function between glycated and non-glycated proteoforms. For this, charge-based separation techniques, such as ion exchange chromatography and isoelectric focusing [52, 319] are potentially suitable as glycation alters the protein charge profile including the isoelectric point due to the modification of a basic amino acid with a sugar moiety. Unfortunately, these techniques are typically not able to (fully) resolve glycated from non-glycated species [320, 327].

Alternatively, boronate affinity chromatography (BAC) has emerged as a valuable tool for enrichment of glycated proteins under (pseudo-)native separation conditions. BAC separation is based on the formation of a reversible covalent cyclic diester between the *cis*-diol groups of analytes (*e.g.*, glycated proteoforms) and boronic acid ligands of the stationary phase [328]. Currently, BAC is mainly employed to monitor overall glycation levels for (therapeutic) proteins or enrich glycated proteoforms for in-depth structural characterization of these proteins [309, 319]. Moreover, various applications used BAC to analyze endogenous proteins related to diseases, including diabetes [306, 308, 310, 318]. Of note, only few studies used BAC enrichment to investigate the impact of glycation on protein activity. For example, the *in vitro* bioactivity of glycated monoclonal antibodies was investigated by measuring antigen binding affinity of the BAC-separated proteoforms [52, 319]. Moreover, we have recently shown the suitability of BAC in conjunction with MS detection and functional assays for monitoring the influence of glycation on the enzymatic activity of an endo-xylanase [323]. Using this approach, we found reduced activity for glycated proteoforms compared to the non-modified enzyme. The characterized endo-xylanase was a relatively small non-glycosylated protein with low heterogeneity, and application of this approach to larger and more heterogeneous proteins has not yet been demonstrated.

Here, we developed a BAC method to separate non-glycated and glycated proteoforms of a lipase used for food applications. Subsequently, the obtained BAC fractions were structurally and functionally characterized using MS and activity assays revealing the effect of glycation on the enzyme performance. Since reducing sugars may remain in the final product, as a result of the production process, glycation of this lipase can naturally occur during storage. The BAC method was developed using both a low-glycated and heavily-glycated lipase control sample to ensure minimal binding of non-glycated species to the column material, while maximizing the amount of glycated proteoforms retained on the BAC column material. The newly developed BAC method was applied to analyze samples from a stability study, where the enzyme was subjected to different storage temperatures (i.e., -20 °C, 4 °C, and 25 °C) for 1 year. Moreover, we compared the influence of two often applied formulation matrices (wheat or maltodextrin) on the glycation levels after exposure at different storage temperatures. Overall, the use of BAC together with MS detection and activity measurements allowed us to answer questions regarding the relationship between glycation and enzyme function.

## 8.2 Experimental section

### 8.2.1 Materials and samples

Ammonium acetate (≥ 98%), acetic acid (≥ 99%), bovine serum albumin (BSA), D-sorbitol (≥ 99.5%), hydrogen chloride (37%), magnesium chloride (≥ 98%), p-nitrophenyl palmitate (Sigma N2752), L-a-phosphatidylcholine (Sigma 61755), and Tris(hydroxymethyl)aminomethane (≥ 99.8%) were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands). Ammonium bicarbonate (≥ 99.5%) and acetonitrile were purchased from Honeywell Fluka (Steinheim, Germany). 2-propanol and Triton X-10 were supplied by Riedel-De Haen (Seelze, Germany). Sodium hydroxide was obtained from Merck Millipore (Darmstadt, Germany). Digalactosyldiglyceride from oat (DGDG) was prepared by the DSM Biotechnology center (Delft, the Netherlands). NEFA-HR R1 (FUJI FILM Wako Chem 434-91795), NEFA-HR R2 (FUJI FILM Wako Chem 436-91995) and NEFA standard (oleic acid/ Wako NEFA standard). MilliQ (18.2 mΩ) was used from a Purelab ultra (ELGA Labwater, Ede, the Netherlands) or Millipore system (Bedford, MA, USA).

The lipase samples were provided by DSM (Delft, the Netherlands). All samples contained between 15-25% enzyme. The formulation matrix was either wheat flour or maltodextrin. For the BAC method development, two lipase control samples with varying degree of glycation were used, *i.e.,* one stored at -20 °C (low level of glycation) and one stored for years at room temperature (high level of glycated proteoforms), both formulated in wheat flour. To monitor the effect of glycation on function, samples from a stability study were used, where the lipase was stored at different temperatures (*i.e.,* -20 °C, 4 °C or 25 °C) for 1 year. Prior to BAC, the samples were dissolved in milliQ water (300 mg/mL), followed by centrifugation at 14140 rcf for 15 min. The supernatant was collected and directly injected on the BAC system without further pretreatment. For the maltodextrin-formulated samples, the fluorescence of these samples was measured both at excitation 295 nm/emission 350 nm and excitation 365 nm/emission 440 nm.

### 8.2.2 Boronate affinity chromatography

The BAC method development was performed on an Acquity UPLC H-class system from Waters (Milford, MA, USA) equipped with a pump, degasser, column oven, autosampler and a photodiode array detector. The fraction collection of the samples was done using an Agilent HPLC 1100 system (Waldbronn, Germany) with degasser, autosampler, quaternary pump, column thermostat and diode array detector. For the BAC separation, a TSKgel Boronate-5PW column (7.5 x 75 mm, 10µm) from Tosoh Bioscience

(Montgomeryville, PA, USA) was used. The optimized mobile phases consisted of 250 mM ammonium acetate and 120 mM Tris at pH 8.6 (A) and 250 mM ammonium acetate and 400 mM sorbitol at pH 8.6 (B). The pH of the mobile phases was adjusted with concentrated sodium hydroxide or hydrochloric acid. A step-gradient was performed with first 15 min 100% A followed by flushing the column with 100% B for 20 min. Thereafter, the column was cleaned and equilibrated with 100 mM acetic acid (for 10 min) and mobile phase A (for 10 min), respectively. The column temperature was set to 20 °C, the flow rate to 0.7 ml/min and the UV wavelengths to 260 and 280 nm. For MS characterization and activity measurements, 100 µl sample was injected and fractions of the peaks were collected. Prior to MS analysis, the samples were deglycosylated by incubation with 10 µl PNGase F (Roche Diagnostics, Mannheim, Germany) overnight at 37 °C. In parallel to MS detection, the fluorescence signal of the maltodextrin-formulated fractions was measured at excitation 295 nm/emission 350 nm and excitation 365 nm/emission 440 nm.

### 8.2.3 Liquid chromatography coupled to mass spectrometry

The deglycosylated lipase control samples (non-separated samples as well as BAC-separated peaks) were characterized using reversed phase liquid chromatography (RPLC) coupled to MS using an Acquity I-class instrument (Waters), composed of a binary pump, autosampler, and column thermostat. An Acquity UPLC Protein BEH300 C4 column (2.1 x 50 mm, 1.7 µm) from Waters was employed. The mobile phases were 0.1% formic acid in milliQ (A) and 90% acetonitrile with 0.1% formic acid (B). The gradient started at 3% B for 2 min followed by a linear increase from 17% B to 44% B in 8 min. Thereafter, the column was cleaned by decreasing the %B from 90% to 10% in 1 min, which was repeated three times. Finally, the column was re-equilibrated at 3% B for 2.8 min. The separation was performed at 75 °C with a flow rate of 0.4 ml/min and an injection volume of 10 µl. The RP separation was coupled to the Synapt G2-S (Waters, Wilmslow, UK) operated in positive-ion mode. In the ESI source, the voltage was set to 1 kV, the nebulizing gas pressure to 6 bar, the dry gas to 900 l/h and source temperature to 120 °C. The scan time was 1 s and *m/z* range was from 500 to 3500. Deconvolution of mass spectra was done in MassLynx (Waters).

The BAC peaks of the temperature-stressed materials were measured with SEC-UV-MS. These measurements were performed with a biocompatible Ultimate 3000 instrument (Thermo Fisher Scientific) equipped with a quaternary pump, autosampler, column thermostat, and variable wavelength detector. A TSKgel UP-SW3000 (4.6 x 150 mm, 2 µm) was obtained from TOSOH

(Griesheim, Germany). As mobile phase, 100 mM ammonium acetate at pH 6.8 was used. The flow rate was 0.25 ml/min and the UV wavelengths were 260 and 280 nm. Prior coupling to Impact qTOF-MS (Bruker, Daltonics, Bremen Germany), the flow rate was reduced with a factor 100 via a post-separation flow splitter. The Captive Spray source was operated in positive mode with a capillary voltage of 750 V. The nebulizer gas pressure, dry gas flow rate and dry gas temperature were 0.4 bar, 3.1 l/min, and 220 °C, respectively. The insource collision energy to 150 V. The voltage in the collision cell was set to 20 V and the collision cell RF was 2100 Vpp. The *m*/z-range was 1000-6000. For the deconvolution of the mass spectra the maximum entropy algorithm was used in the DataAnalysis software (v5) from Bruker Daltonics.

### 8.2.4 Activity assays

For the functional characterization, the activity of different samples was determined before and after BAC separation in triplicate. The different assays were performed using a Konelab Arena 30 (ThermoFisher, Manchester, UK) equipped with a sample disk, reagent disk, cuvette loader, incubator and photometer. The general lipase activity was measured using the chromogenic substrate p-nitrophenyl palmitate (3 mg/ml in 2-propanol). Under vigorously stirring, 3.5 ml of substrate solution was dropwise added to 46.5 ml 100 mM Tris buffer with 1% Triton X-100 (pH 8.5). In the Konelab analyzer, 5 µl sample (activity between 0.04 - 0.3 units/ml) was mixed with 120 µl substrate solution followed by incubation at 37 °C for 5 min. During this incubation time, the change in absorption was measured at 405 nm and the slope of the linear part of the obtained curve was used as measure for the activity. Calibration was performed using a lipase standard. For this assay, one unit was defined as the amount of enzyme that liberates 1 µmol p-nitrophenol per min under the assay conditions.

Phospholipase and galactolipase activity of the start material and collected BAC fractions were also measured. First substrate solutions (0.7% L-a-Phosphatidylcholine- and 0.7% digalactosyldiglyceride solutions) were prepared in 60 mM acetate buffer containing 1.4% Triton X-100 (pH 5.5). In the Konelab analyzer, 40 µl of these substrate solutions was pre-incubated at 37°C for 2 min and subsequently mixed with 10 µl sample (diluted in 60 mM acetate buffer pH 5.5 to an activity between 0.015-0.075 U/ml). After 20 min incubation, 130 µl NEFA-R1 was added and incubated for 2 min followed by addition of 65 lL NEFA-R2. After a subsequent incubation time of 3 min, the absorption was measured at 540 nm. Sample blanks were measured by reversing the addition of sample and reagents: 130 µl NEFA-R1 at 0 min, 10 µl sample at 1.1 min, 40 µl substrate at 2.2 min, 65 µl NEFA-R2 at 4.2 min and

final absorption measurement at 7.2 min. A calibration line was prepared by measuring oleic acid solutions of 0.2, 0.4, 0.6, 0.8 and 1.0 mM prepared from the NEFA oleic acid standard. By plotting absorbance (sample - sample blank) versus the known oleic acid concentrations of the standard solutions, the slope was calculated via linear regression. The amount of activity in the unknown samples was calculated as follows: Activity in U/ml = (Abs sample - Abs blank)/(a x t) x df, where a is the slope of the calibration line, t is the incubation time (20 min) and df is the dilution factor. For these measurements, one unit is defined as the amount of enzyme that liberates one µmol of free fatty acid per min under the defined assay conditions.

The protein concentration in the samples was determined using a bicinchoninic acid (BCA) assay (Sigma). A calibration line was prepared using different bovine serum albumin solutions with concentrations of 0, 0.01, 0.02, 0.04, 0.08, 0.1, 0.25, 0.4, 0.6, 0.7, 0.85, and 1.0 mg/ml. The working reagent was prepared by mixing 50 parts reagent A (BCA solution, Sigma B-9643) with one part reagent B (4% Copper (II) sulfate.5 $H_2O$ (w/v) solution, Sigma C-2284) (v/v). Using the Konelab analyzer, 15 µl sample was added to 180 µl working reagent. Thereafter, the samples were incubated at 37 °C for 30 min followed by measurement of the absorbance at 540 nm.

For all activity assays, the specific activity (U/mg protein) was calculated by dividing lipase activity by the protein concentration. The average specific activity of the non-separated lipase samples and the BAC fractions were calculated from the three independent measurements. Thereafter, a t-test test for a significant difference between two independent samples was performed.

## 8.3 Results and discussion

In this study, we investigated the effect of glycation on the functionality of a commercially available lipase. This lipase has a backbone mass of 28 kDa with four possible N-glycosylation sites (**Fig. S1**) from which up to three sites are actually occupied with high mannose type N-glycans [229]. Besides extensive glycosylation, proteoforms with different C-terminal processing were described for this enzyme. Specifically, the most abundant variants were the mature form (M) (i.e., complete sequence without truncation), the mature form lacking tryptophan (M-W) and the mature form lacking tryptophan and serine (M-SW). The combination of the glycosylation site occupancy, number of mannoses per glycan and C-terminal processing leads to a heterogeneous pool of (glyco)proteoforms (**Fig. S2**).

### 8.3.1 BAC method optimization

For the optimization of the BAC method, we used two lipase control samples with different levels of glycation, namely low- and high-glycated control samples. The low-glycated control sample was stored at -20 °C and therefore, no to minor glycation was expected. The high-glycated control sample consisted of lipase standard stored at room temperature for approximately 2 years which induced glycation. The glycation levels in both samples were assessed using MS. Since differences in size of high mannose N-glycans result in the same mass differences as glycation, the samples were deglycosylated prior to MS analysis. In the low-glycated control sample, no species with a mass increment of 162 Da were detected indicating the absence or very low abundance of glycated species (**Fig. 1a**; **Table S1**).

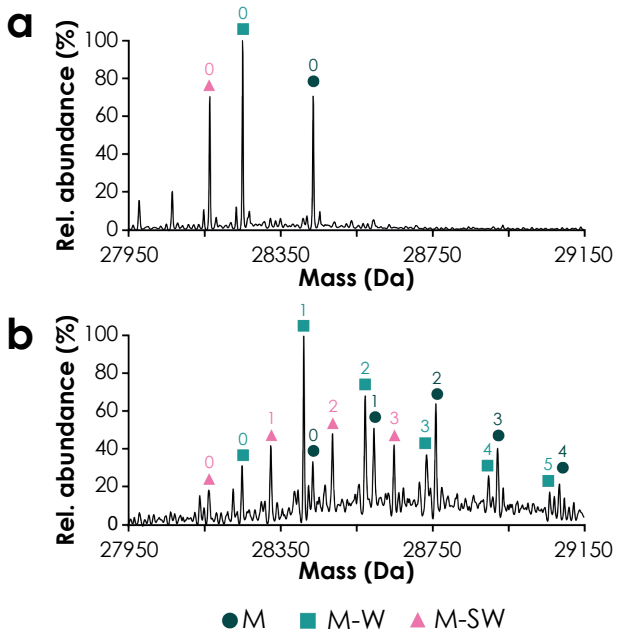



**Figure 1** | Zero charge deconvoluted mass spectra of the two lipase standards measured with RPLC-MS, including low-glycated control sample (**a**) and high-glycated control sample (**b**). The symbols indicate different sequence variants (*i.e.,* C-terminal or N-terminal truncation). The number of hexoses is indicated above each annotated peak. Mass spectra of both samples without removal of the glycosylation can be found in **Figure S2**. All masses and assignments can be found in **Table S1**.

Besides, we could confirm the presence of the previously reported variants with different C-termini (i.e., M, M-W and M-SW) and also detected N-terminal truncated proteoforms (**Table S1**). In the high-glycated control sample, several glycated proteoforms were detected with up to five hexoses attached to the protein (**Fig. 1b**; **Table S1**). The glycation level was estimated by comparing the intensity of the glycated proteoforms to the total intensity of all assigned

proteoforms showing that around 87% of the proteoforms were glycated. For this sample, the M, M-W, and M-SW variants exhibited a similar ratio as for the non-glycated sample.

To obtain BAC separation of glycated proteoforms from non-glycated variants, several method parameters were optimized that were previously shown to be important, including mobile phase pH, salt concentration and temperature [309, 319, 329]. The most stable interaction between the glycated proteins and the BAC column material is formed at a pH above the $pK_a$ value of the boronate ligands (pH > 8.2) [328]. Additionally, the pH of the mobile phases should be within the range where the enzyme is stable to permit further functional characterization which for this lipase is between pH 5 and 9 leading to the choice of 250 mM ammonium acetate at pH 8.6 for the separation. However, using only this buffer almost all proteoforms (93%) of the low-glycated control sample were retained on the BAC column (**Fig. 2a**) indicating that secondary interactions contributed greatly to the retention. To reduce the influence of these interactions, we added different concentrations of Tris (0-200 mM) to the mobile phase, where 200 mM Tris resulted in retention of only 3% of the proteoforms for the low-glycated control sample and 67% for the high-glycated control sample (based on the UV response recorded at 280 nm). Since the high-glycated control sample is expected to contain around 87% glycated proteoforms, Tris concentrations of 150 mM and higher probably already suppress the specific cis-diol interaction. Therefore, we selected 120 mM Tris for the BAC mobile phase, where the relative binding peak areas were 13% for the low-glycated control sample and 79% for the high-glycated control sample.

To determine the composition of the proteoforms present in the non-retained (or non-binding) peak and retained (or binding) peak, we collected fractions and analyzed them with MS (**Fig. 3**; **Table S2**). Overall, the majority of the proteoforms in non-binding peaks were non-glycated and proteoforms in the binding peaks were mainly glycated. While with direct intact MS analysis (**Fig. 1b**), no glycation was detected for the low-glycated control sample, using BAC enrichment we were able to detect glycated proteoforms with one or two hexose residues attached to the protein comprising ~6% of all assigned proteoforms (**Fig. 3a**). For the high-glycated control sample, species with two glycations were most dominant, but also proteoforms with none, one, three or four glycations were detected in the binding peak (**Fig. 3b**). Besides these glycated forms, minor amounts of non-glycated species eluted in the binding peaks (i.e., 7% for the low-glycated control sample and 8% for the high-glycated control sample). Interestingly, from these non-glycated

proteoforms mainly the mature enzyme was detected indicating that the C-terminal amino acid might have an influence on BAC retention. The higher hydrophobicity of tryptophan on the C-terminus (M form) compared to a serine (M-W) or phenylalanine (M-SW) could result in increased hydrophobic interactions with the column material. Hydrophobic interactions in BAC may be suppressed by addition of organic solvents to the mobile phase [330]. Unfortunately, these solvents can affect the nativity of protein hampering further functional characterization.

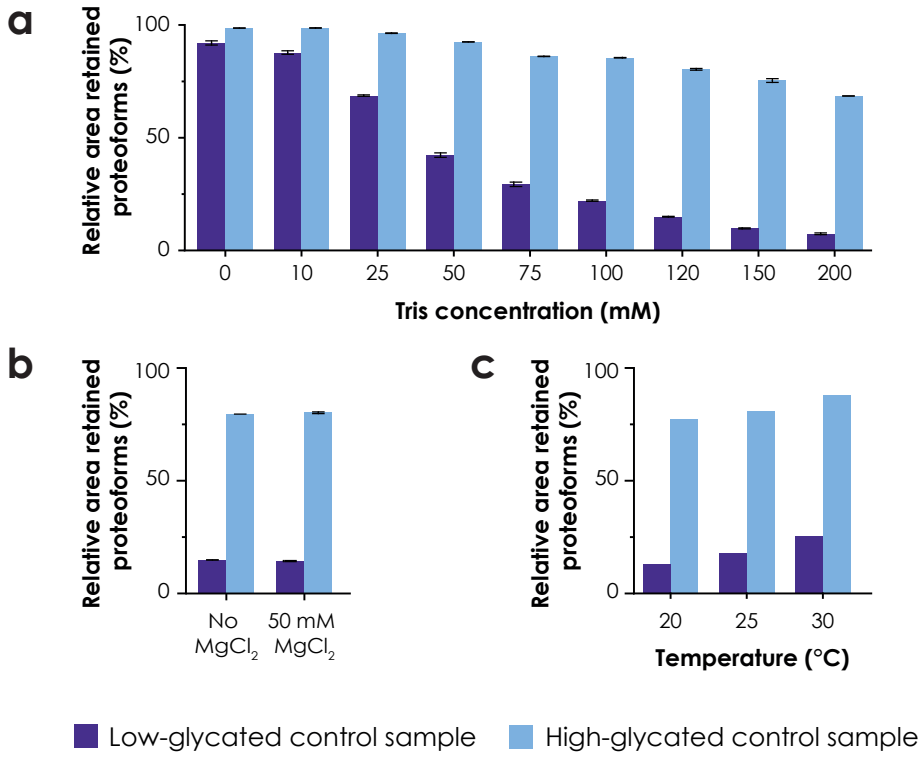



**Figure 2** | Optimization of the BAC mobile phase composition and separation temperature. The effect of different parameters on the UV peak area (recorded at 280 nm) of the binding peak was assessed for the low-glycated control sample (dark blue) and high-glycated control sample (light blue). In particular, the effect of shielding agent Tris (**a**), presence of $MgCl_2$ (**b**), and column temperature (**c**). The peak areas of the measurements evaluating the Tris and $MgCl_2$ concentration were measured in duplicate (error bars represent standard deviation) while evaluation of temperature was performed by single measurements.

To further stabilize the *cis*-diol interaction as well as to limit possible electrostatic interactions, the addition of $Mg^{2+}$ ions to the mobile phase has been reported [330]. However, we observed similar elution profiles for mobile phases with and without $MgCl_2$ (**Fig. 2b**) indicating that either electrostatic interactions do not play a significant role or that the mobile phase buffer (250 mM ammonium acetate) is already sufficient to suppress electrostatic

interaction. The elution of glycated species was achieved by the addition of 400 mM D-sorbitol the 250 mM ammonium acetate buffer (pH 8.6). The reduction of sorbitol concentration from 400 to 200 mM still allowed elution of glycated species but resulted in peak broadening, which was undesirable for fraction collection (**Fig. S3**).

Besides, the separation temperature may have a profound effect on the BAC separation. We compared the relative area of the binding peaks of both samples measured at 20, 25 and 30 °C (**Fig. 2c**) revealing that higher column temperatures led to increased binding peaks. Specifically, we observed an increase from 13% to 25% for the low-glycated control sample and from 78% to 88% for the high-glycated control samples when changing the separation temperature from 20 to 30 °C. An explanation could be that higher temperatures alter protein conformation, such as partly unfolding, resulting in additional nonspecific retention. Altogether, the optimized separation was performed at 20 °C and by using mobile phases composed of 250 mM ammonium acetate with 120 mM Tris at pH 8.6 (binding buffer) and 250 mM ammonium acetate and 400 mM sorbitol at pH 8.6 (elution buffer).

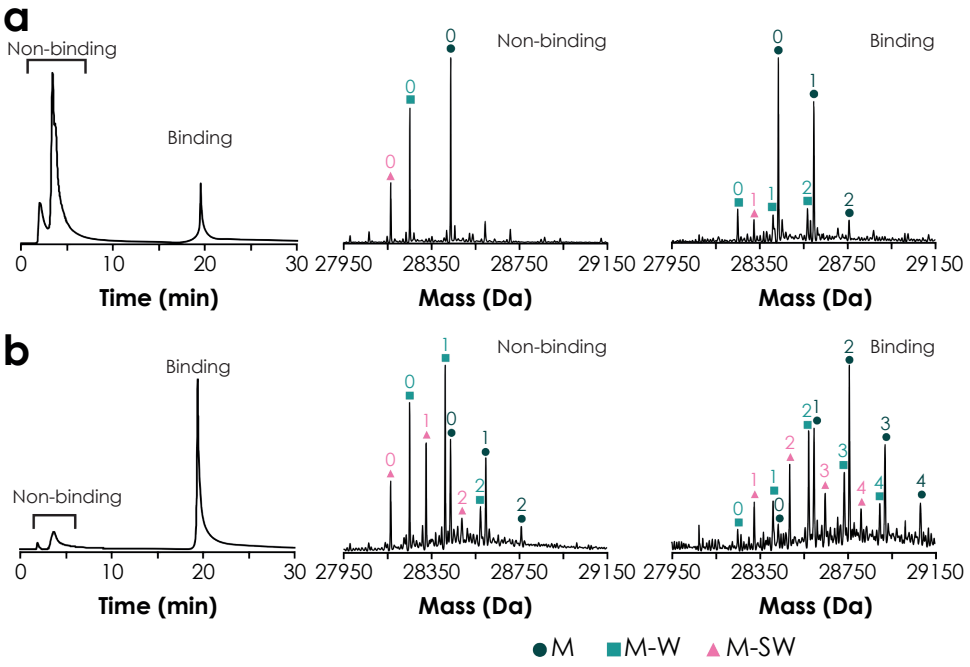



**Figure 3** | BAC separation of the low-glycated control sample (**a**) and high-glycated control sample (**b**). For both samples, the UV chromatogram recorded at 280 nm and obtained mass spectra of the non-binding (upper spectrum) and binding peak (lower spectrum) are displayed. In the MS data, the three most abundant C-terminal truncated variants (*i.e.*, M, M-W, and M-SW) are assigned. The number above the assigned masses represent the number of hexoses per protein. Details on the assigned proteoforms can be found in **Table S2**.

### 8.3.2 Effect of different storage temperatures on lipase proteoforms activity

Since BAC is a native separation technique, activity of resolved proteoforms, in this case glycated species, could be assessed. For this purpose, we induced glycation by subjecting lipase samples (formulated with wheat flour or maltodextrin) to a storage temperature of 25 °C for 1 year. Additionally, the same lipase samples were stored at -20 °C and 4 °C, where the latter was chosen as it is the recommended storage temperature of the lipase product. These three samples were first separated with BAC followed by SEC-UV-MS of the collected peaks for structural characterization. In parallel, lipase activity of the BAC fractions on three substrates, p-nitrophenyl palmitate, phospholipid (PC), and galactolipid (DGDG), was determined. Where the p-nitrophenyl palmitate provides information on the general lipase activity, the other two natural substrates are more specifically reflecting food application performance. To allow comparison of proteoform function, we first determined whether the experimental BAC conditions maintained lipase activity. We observed activity of the starting material dissolved in the BAC mobile phase for all substrates (**Fig. S4**). To minimize interfering matrix compounds (e.g., salts, sugars, small peptides or even insoluble particles and aggregates) during the separation, we performed centrifugation to remove large particles followed by filtration using spin filters with 10 kDa cut-off to reduce interferences of small molecules (salts or sugars) or peptides. However, during this final step a substantial loss of activity and protein amount was encountered (**Fig. S4**). Therefore, the lipase samples separated by BAC were measured without additional sample filtration.

All lipase samples showed similar separation profiles compared to the control samples with a non-binding peak at 4.2 min (peak 1) and a binding peak at 20.8 min (peak 3) (**Fig. 4a**; **Table S3**). The majority of the proteoforms of samples stored at -20 °C and 4 °C eluted in peak 1 (up to 70% of the assigned proteoforms). Peak quantification was based on protein concentrations determined in the BCA assay rather than on UV response at 280 nm, since the latter was influenced by the C-terminal truncated variants (M-W and M-SW lack one tryptophan residue compared to M form). Peak 1 mainly consisted of C-terminal truncated variants (M-W and M-SW) and a minor amount of mature lipase (**Fig. 4b**). Also, a portion of the proteoforms of these samples eluted in peak 3, where glycated proteoforms with one glycation were detected (7-9% of all identified proteoforms for wheat samples) (**Fig. 4c**). Interestingly, an additional peak around 14 min (peak 2) was observed (**Fig. 4a**), which was previously not detected. The lipase control samples used for method development were both centrifuged and filtered prior to BAC

separation, while the samples from the stability study were only centrifuged to avoid protein losses by filtration. To confirm whether the presence/absence of this peak was influenced by sample treatment, we measured the wheat-formulated sample (stored at -20 °C) also after centrifugation and filtration (**Fig. S4**). In the BAC chromatogram of the sample after filtration, peak 2 was lost indicating that these proteoforms were not recovered from the spin filter procedure. Further SEC-MS analysis of peak 2 revealed that the majority of the species elute at the retention time of the lipase suggesting that this peak was not composed of HMWS. Moreover, the mass spectrum this peak showed the presence of the (mainly) mature lipase (**Fig. 4b**). **Table S4** gives an overview of all species detected in the three peaks of the analyzed samples.

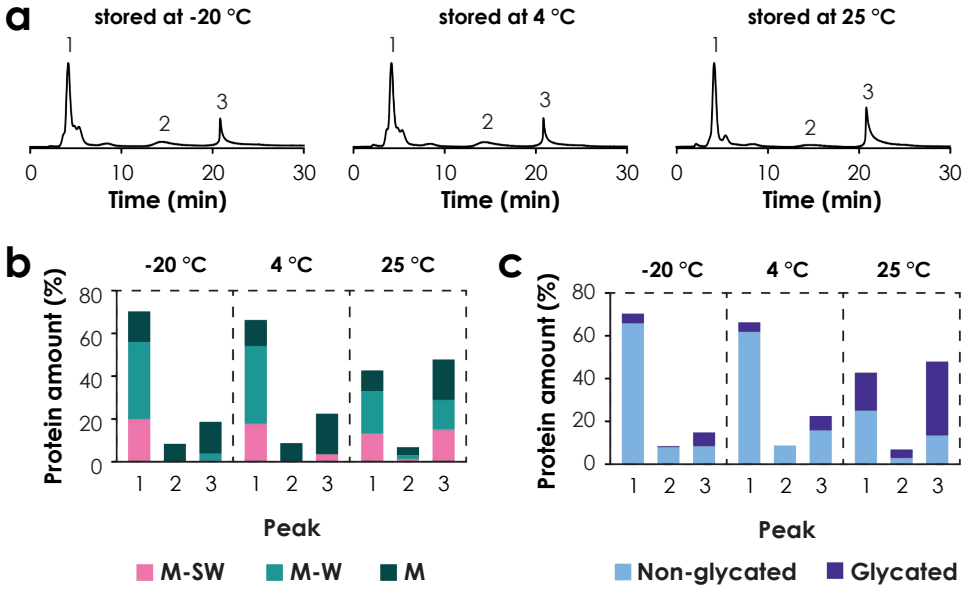



**Figure 4** | Analysis of the proteoforms present in the BAC peaks of the wheat four-formulated samples subjected to different storage temperatures (*i.e.*, -20 °C, 4 °C, and 25 °C). (**a**) The UV chromatograms of the optimized BAC method recorded at 280 nm of the lipase samples. The numbered peaks were collected, deglycosylated and measured with MS. The retention times and relative peak areas are provided in **Table S3**. (**b**) Representation of the C-terminal truncated variants (M, M-W, and M-SW) in the separated peaks, where the y-axis shows the protein amount measured with the BCA assay. (**c**) An overview of the total glycation level in each BAC peak. Details on the assigned (glycated) proteoforms can be found in **Table S4**.

As expected, upon storage at 25 °C, peak 3 increased indicating a higher lipase glycation level (**Table S3**). MS analysis of this peak confirmed not only a higher level of glycation, but also revealed glycated products with up to four hexoses (**Table S4**). Although the majority of proteoforms in peak 1 was non-glycated and in peak 3 was glycated, some (non-)glycated species were detected in the other peaks. The presence of non-glycated species in peak 2 and 3 could be explained by the occurrence of non-specific interactions

between analytes and the column material. Moreover, the accessibility of glycations for interaction with the stationary phase could play a role, since buried glycations would not interact with the BAC column resulting in elution of glycated species in peak 1. Finally, the observed elution patterns could be related to the presence of aggregates (or high molecular weight species), which were detected with SEC-UV for all samples (**Fig. S5**). These aggregates may be composed of a mixture of glycated and non-glycated monomeric species, where glycation in one of these individual units could already cause binding to the column. Since our ESI-MS conditions did not allow monitoring of aggregates due to their dissociation during the ionization, detected non-glycated species could come from an aggregate where some units were glycated whilst others were not. For the samples stored at -20 °C and 4 °C the total amount of glycation was relatively low and therefore, the likelihood for glycated/non-glycated aggregates was also lower compared to the sample stored at 25 °C. Finally, results very similar to the ones observed for wheat formulation were obtained for the samples formulated with maltodextrin (**Fig. S6**; **Table S5**) indicating that there is no detectable influence of the formulation on (temperature-stressed) samples.

Despite the substantial increase in glycation levels after applying temperature stress, the activity measurements revealed only slightly reduced specific activity upon storage at higher temperatures for the three tested substrates (**Fig. 5**; **Table S6**). For instance, the wheat-formulated lipase (starting material) stored at -20 °C had a specific activity of 104 ± 2.1 U/mg protein (p-nitrophenyl palmitate assay) which decreased to 99 ± 1.3 U/mg protein for 4 °C and 80 ± 4.4 U/mg for 25 °C (**Fig 5a**, **Table S6** and **S7**). Similar results were found for the phospho- and galactolipids (**Fig. 5b** and **c**). After determining the activity of the starting material, the activity of the BAC peaks was evaluated to investigate whether differences in specific activity could be detected for the separated species. For all BAC peaks, between 54-74% of the activity was recovered compared to the starting material. Similar recoveries (between 50%-64%) were observed for the protein concentration indicating that the lipase remained active during separation and fraction collection, even though some protein losses were encountered (**Table S6**).

Both non-stressed lipase samples (stored at -20 °C or 4 °C) showed a significantly lower specific activity for peak 1 compared to peak 2 and 3 (**Fig. 5a**). Since the level of glycated proteoforms in these samples was only minor, we were able to assign the lower specific activity of peak 1 to the presence of C-terminal truncated variants. Peak 1 contained mostly the M-W and M-SW proteoforms, whereas the other two peaks contained (mainly) mature lipase

proteoforms. Besides altered activity of peak 1, also a difference in specificity towards the different substrates was observed. The ratios between specific activity of different assays indicated that the proteoforms in peak 1 have higher specificity towards phospholipids and galactolipids compared to the conventional p-nitrophenyl palmitate substrate (**Fig. 5; Table S6**). The specific activity of the sample stored at 25 °C was for all peaks significantly lower compared to the non-stressed samples. Similar observations were made for the maltodextrin-formulated samples (**Fig. S7**; **Table S6** and **S7**). Especially the specific activity of peak 2 was substantially reduced probably (partly) due to the presence of C-terminal truncated variants. Surprisingly, the decrease in specific activity found for peak 3 was minor, while the glycation level was greatly increased implying that glycation does not have a negative influence on the specific activity of these lipase samples.

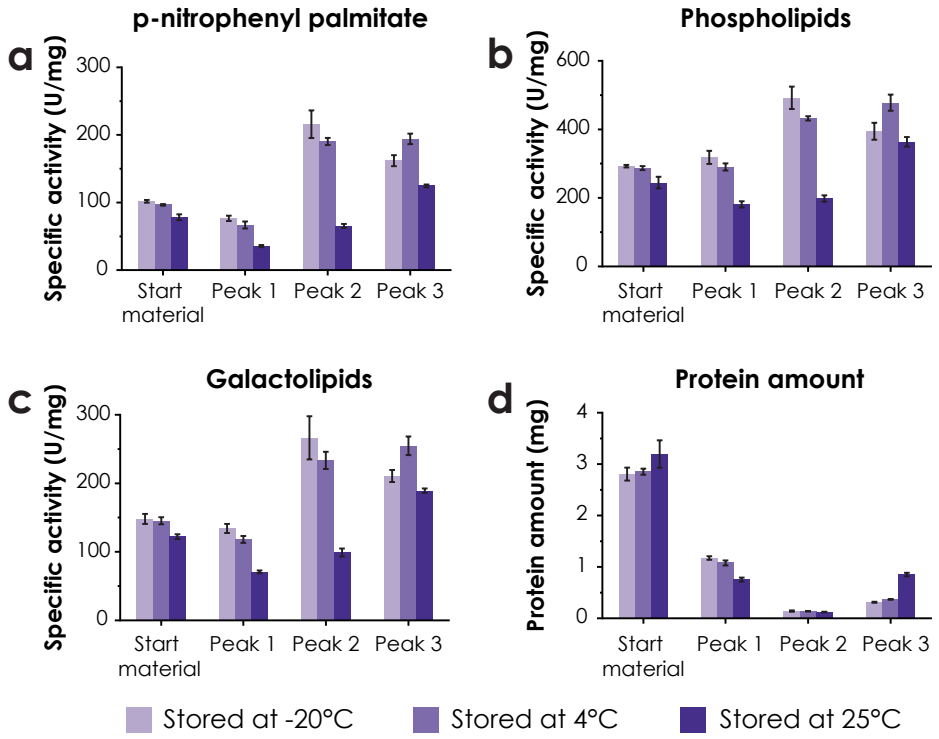



**Figure 5** | Specific activity (U/mg protein) and amount of protein of the non-separated lipase (start material) and the BAC fractions (Peak 1-3) formulated in wheat. The specific activity was calculated based on the measured activity and protein concentration. The activity was measured using different substrates, p-nitrophenyl palmitate (**a**), PC (**b**), DGDG (**c**), and the protein amount (**d**). The protein amount was calculated based on the protein concentration obtained with the BCA assay and the volume of the sample (injection volume for the starting material and volume of the fractions for the peaks). Measurements were performed in triplicate and error bars represent the standard deviation. A detailed overview on the obtained activity can be found in **Table S6** and the statistical analysis of the samples is presented in **Table S7**. Activity data of the maltodextrin-formulated samples can be found in **Figure S6**, **Table S6** and **Table S7**.

Since glycation was not assigned as cause for activity loss, lipase inactivation during storage at 25 °C must have another origin. As no mass change (other than glycation) was observed in the BAC fractions with lowered specific activity, non-covalent modifications (e.g., denaturation or aggregation) play probably a role [50]. To obtain information on possible protein denaturation, tryptophan fluorescence (excitation at 295 nm and emission at 350 nm) was measured for all maltodextrin-formulated samples as was described by [331]. Protein unfolding results in exposure of more tryptophan residues at the surface and thereby, increasing the fluorescence signal. Nevertheless, all non-separated samples showed similar signal intensities suggesting that denaturation was most likely not the main cause of lipase inactivation (**Table S8**). Regarding protein aggregation, similar levels of HMWS were detected for all samples in the SEC-UV chromatograms (**Fig. S5**). Nevertheless, the nature of these HMWS could be different upon storage at 25 °C and as a result negatively impact the lipase activity. For instance, temperature-stressed samples often contain so-called advanced glycated end products (AGEs), which can be described as glycated proteins that undergo rearrangement reactions and cross-linking to form large heterogenous species [306, 332]. These AGEs are known to cause changes in structural properties of proteins leading to impaired functionality [333]. To detect AGEs, fluorescence can be measured at characteristic wavelengths, specifically excitation wavelength between 340-370 nm and emission wavelength between 420-440 nm [334, 335]. Unfortunately, in our hands this experiment provided no conclusive results to unravel the relationship between lipase inactivation and AGE formation (data not shown). Altogether, the exact cause for lipase inactivation remained unknown, however, we could show that glycation was not responsible for the altered functionality using the newly-developed BAC method together with MS detection and activity assays.

## 8.4 Conclusion

Glycation in industrial enzyme products can be increased upon storage at higher temperatures. In this study, we monitored glycation of a formulated lipase used for food applications. To investigate glycation levels and the potential influence of glycation on activity, we developed a BAC enrichment method coupled to MS. Extensive method optimization was important to minimize non-specific interactions between the enzyme and stationary phase, including the optimization of the Tris buffer concentration. Next to glycation, the C-terminal amino acids of the lipase were shown to play a role in BAC retention. Moreover, the native conformation of the lipase could only be maintained when performing BAC at 20 °C due to (partial) unfolding at

higher temperatures. Using the BAC enrichment followed by MS detection, low abundant glycated species were observed for the low-glycated control samples, while direct RPLC-MS analysis without BAC was unable to detect these species, herewith demonstrating the higher sensitivity of glycation detection thanks to BAC enrichment.

The applicability of the optimized BAC enrichment method was investigated by comparing glycation levels of lipase samples after temperature stress (storage at 25 °C) with non-stressed samples (stored at -20 °C and 4 °C). While the ratio of detected C-terminal truncated variants remained constant, the glycation level increased after storage at 25 °C compared to -20 °C and 4 °C. To assess influence of proteoforms on activity, we first demonstrated that the BAC separation conditions were able to conserve lipase activity. Of note, the effect of sample preparation steps should be careful evaluated, since sufficient protein losses could be encountered as was the case for this particular lipase during filtration. Due to the separation of truncated variants and the low glycation levels in the non-stressed samples, we could observe lower specific activity and altered specificity for the variants lacking the C-terminal tryptophan. Remarkably, extensive glycation of the temperature-stressed lipase was not linked to altered specific activity or substrate specificity. Moreover, formulation appeared to have minor impact on glycation levels and lipase activity, since similar results were obtained for both wheat flour and maltodextrin formulated samples.

Altogether, native separation technologies, such as BAC, together with MS detection and functional assays are powerful to monitor the effect of elevated storage temperatures on protein structure and function. The heterogeneity of the investigated lipase samples due to glycation, N- and C-terminal truncation and aggregation made it challenging to untangle the effect of each individual modification on activity. Even though this complexity hampered drawing unambiguous conclusions, valuable indications of structure-function relationships were obtained that should be confirmed with further research.

## Acknowledgments

This work was supported by the Dutch Research Council (NWO) (SATIN project, 731.017.202).

## Supplementary information

The following supplementary files are available on request:

Sequence of the investigated lipase (**Fig. S1**), Deconvoluted mass spectra glycosylated lipase control samples (**Fig. S2**), Evaluation of sorbitol concentration for elution in BAC (**Fig. S3**), Effect of sample preparation on recovered lipase activity (**Fig. S4**), SEC-UV chromatograms of wheat samples (**Fig. S5**), Structural characterization BAC peaks of maltodextrin samples (**Fig. S6**), Specific activity and protein concentration of maltodextrin samples (**Fig. S7**), Assigned proteoforms in control samples (**Table S1**), Assigned proteoforms BAC peaks of control samples (**Table S2**), Retention times and relative peak areas of lipase samples from the stability study (**Table S3**), Assigned proteoforms in BAC fractions of wheat-formulated samples stored at different temperatures (**Table S4**), Assigned proteoforms in BAC fractions of maltodextrin-formulated samples stored at different temperatures (**Table S5**), Activity, protein concentration, specific activity and recovery of the lipase samples prior and after BAC separation (**Table S6**), Statistical analysis of specific activity differences between samples (**Table S7**), Tryptophan fluorescence measurements of the lipases and BAC fractions (**Table S8**).