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Citation

Schaick, G. van, Pot, S., Schouten, O., Hartog, J. den, Akeroyd, M., Hoeven, R. V., ... Dominguez-Vega, E. (2023). Evaluating the effect of glycation on lipase activity using boronate affinity chromatography and mass spectrometry. *Food Chemistry*, 421. doi:10.1016/j.foodchem.2023.136147

Version: Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).



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

Guusje van Schaick^{a,*}, Sanne Pot^a, Olaf Schouten^b, Joost den Hartog^b, Michiel Akeroyd^b, Rob van der Hoeven^b, Wim Bijleveld^b, Nicolas Abello^b, Manfred Wuhrer^a, Maurien Olsthoorn^b, Elena Dominguez-Vega^a

^a Leiden University Medical Center, Center for Proteomics and Metabolomics, Leiden, the Netherlands

^b DSM Science & Innovation, Biodata & Translation, Center for Analytical Innovation, Delft, the Netherlands

ARTICLE INFO

Keywords:

Boronate affinity chromatography
Mass spectrometry
Glycation
Lipase
Structure–function relationship

ABSTRACT

Protein glycation may occur naturally when reducing sugars and proteins coexist, which is often the case for industrial enzymes. The impact of post-translational modifications on enzyme performance (e.g., stability or function) is often not predictable, highlighting the importance of having appropriate analytical methodologies to monitor the influence of glycation on performance. Here, a boronate affinity chromatography method was developed to enrich glycated species followed by mass spectrometry for structural characterization and activity assays for functional assessment. This approach was applied to a (temperature-stressed) lipase used for food applications revealing that storage at $-20\text{ }^{\circ}\text{C}$ and $4\text{ }^{\circ}\text{C}$ resulted in minor glycation (below 9%), whereas storage at $25\text{ }^{\circ}\text{C}$ led to a higher glycation level with up to four sugars per lipase molecule. Remarkably, activity measurements revealed that glycation did not reduce lipase activity or stability. Altogether, this novel strategy is a helpful extension to the current analytical toolbox supporting development of enzyme products.

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 (van Schaick et al., 2022) and a glucoamylase for glucose syrup production (Sutthirak, Assavanig, Dharmstithi, & Lertsiri, 2010; Sutthirak, Dharmstithi, & Lertsiri, 2005). This PTM occurs via a non-enzymatic reaction between reducing sugars and primary amines of proteins, as originally described by Maillard (Maillard, 1912; Priego Capote & Sanchez, 2009). 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 (Cardoso, Wierenga, Gruppen, & Schols, 2018; Misset & van Dijk, 1998; Wei, Berning, Quan, & Zhang, 2017). Interestingly, extensive glycation can impact enzyme functionality, stability, and solubility (Liu, Ru, & Ding, 2002). For instance, in a recent study, glycated proteoforms of an endo-xylanase, induced by an accelerated stability study (storage at $40\text{ }^{\circ}\text{C}$), showed reduced activity compared to their non-glycated forms (van Schaick et al., 2022). Conversely, glycated glucoamylases, obtained after incubation at $60\text{ }^{\circ}\text{C}$ in the presence of

reducing carbohydrates, showed a more efficient conversion of maltose and maltodextrin into glucose (Sutthirak et al., 2005). Since glycation can influence 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 (Priego Capote et al., 2009). 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 (Gstöttner et al., 2020; Viski, Gengeliczki, Lenkey, & Baranyane Ganzler, 2016; Zhang, Liu, & Zhang, 2018). 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

* Corresponding author at: Leiden University Medical Center, Center for Proteomics and Metabolomics, Albinusdreef 2, 2333 ZA Leiden, the Netherlands.

E-mail address: g.van_schaick@lumc.nl (G. van Schaick).

proteoforms. For this, charge-based separation techniques, such as ion exchange chromatography and isoelectric focusing (Mo, Jin, Yan, Sokolowska, Lewis, & Hu, 2018; Quan et al., 2008) 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 (Duivelshof, Fekete, Guillard, & D'Atri, 2019; Koval, Kasicka, & Cottet, 2011).

Alternatively, boronate affinity chromatography (BAC) has emerged as a valuable tool for the 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 (Hall, 2011). Currently, BAC is mainly employed to monitor overall glycation levels for (therapeutic) proteins, mainly monoclonal antibodies (mAbs), or enrich glycated proteoforms for in-depth structural characterization of these proteins (Lhota, Sissolak, Striedner, Sommeregger, & Vorauer-Uhl, 2021; Quan et al., 2008). Moreover, various applications used BAC to analyze endogenous proteins related to diseases, including diabetes (Priego-Capote, Ramirez-Boo, Finamore, Gluck, & Sanchez, 2014; Viski et al., 2016; Zhang et al., 2018; Zhang & Zhang, 2020). Of note, only a 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 the antigen-binding affinity of the BAC-separated proteoforms (Mo et al., 2018; Quan et al., 2008). Recently, the suitability of BAC in conjunction with MS detection and functional assays was shown for monitoring the influence of glycation on the enzymatic activity of an endo-xylanase (van Schaick et al., 2022). Using this approach was found that 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 the application of this approach to larger and more heterogeneous proteins has not yet been demonstrated.

Lipases are complex glycoproteins extensively used in many different application areas, including food processing. Therefore, analytical tools permitting to study glycation of lipases are of great value to ensure efficient enzymatic products. Here, the influence of glycation on the performance of an industrial lipase used for food applications was investigated. Lipases are commonly used in food applications, including the baking industry, where the enzyme catalyzes lipid hydrolysis to improve dough tolerance and crumb structure (Gerits, Pareyt, Decamps, & Delcour, 2014; Melis, Meza Morales, & Delcour, 2019; Stemler & Scherf, 2022). Due to the presence of reducing sugars (remaining from the production process) in the final enzyme product, glycation can naturally occur during processing and storage. Since glycation may alter enzyme functionality (Misset et al., 1998), the current study aimed to determine whether glycation impacts the activity and stability of an industrial lipase. For this purpose, a workflow was developed to separate glycated from non-glycated species with BAC followed by structural and functional characterization with MS and activity assays, respectively. Combining all obtained information enabled evaluation of the effect of glycation on the enzyme performance. 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 number 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 lipase was subjected to different storage temperatures (-20 °C, 4 °C, and 25 °C) for a year. The recommended product storage temperature is 4 °C in order to guarantee prolonged shelf-life. In addition, the lipase sample was stored at 25 °C to increase the glycation level of the enzyme. The obtained results were compared to the lipase reference sample stored at -20 °C. Moreover, the influence of two often applied formulation matrices (wheat or maltodextrin) on the glycation levels after exposure was compared at the 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.

2. Materials and methods

2.1. Materials and samples

Ammonium acetate ($\geq 98\%$), acetic acid ($\geq 99\%$), bovine serum albumin (BSA), D-sorbitol ($\geq 99.5\%$), hydrogen chloride (37%), magnesium chloride ($\geq 98\%$), p-nitrophenyl palmitate (Sigma N2752), L- α -phosphatidylcholine (Sigma 61755), and Tris(hydroxymethyl)aminomethane ($\geq 99.8\%$) were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands). Ammonium bicarbonate ($\geq 99.5\%$) 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). Digalactosyldiacylglycerol 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). Deionized water (18.2 m Ω) was used from a Purelab ultra (ELGA Labwater, Ede, the Netherlands) or Millipore system (Bedford, MA, USA).

The industrial lipase samples were provided by DSM (Delft, the Netherlands). All samples contained between 15 and 25% enzyme protein standardized on activity with a formulation matrix composed of either wheat flour or maltodextrin. Hence, the final enzyme activity was the same for both formulations. For the BAC method development, two lipase control samples with varying degrees 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 milli-Q water (300 mg/mL), followed by centrifugation at 14,140 rcf for 15 min. The supernatant was collected and directly injected into 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.

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 photodiode array detector. The fraction collection of the samples was done using an Agilent HPLC 1100 system (Waldbronn, Germany) with a degasser, autosampler, quaternary pump, column thermostat, and diode array detector. For the BAC separation, a TSKgel Boronate-5PW column (7.5 \times 75 mm, 10 μ m) 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 the first 15 min at 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 μ L sample was injected and fractions of the peaks were collected. Prior to MS analysis, the samples were deglycosylated by incubation with 10 μ L PNGaseF (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.

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 from Waters (Wilmslow, UK), composed of a binary pump, autosampler, and column thermostat. An Acquity UPLC Protein BEH300 C4 column (2.1 × 50 mm, 1.7 μm) from Waters was employed. The mobile phases were 0.1% formic acid in milli-Q (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 size exclusion chromatography (SEC)-UV-MS. These measurements were performed with a biocompatible Ultimate 3000 instrument from Dionex Corporation (Sunnyvale, USA) equipped with a quaternary pump, autosampler, column thermostat, and variable wavelength detector. A TSKgel UP-SW3000 (4.6 × 150 mm, 2 μm) was obtained from TOSOH (Griesheim, Germany). As a 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 to coupling to Impact qTOF-MS (Bruker, Daltonics, Bremen Germany), the flow rate was reduced with a factor of 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.

2.4. Protein dosage

The protein concentration in the samples was determined using a bicinchoninic acid (BCA) assay (Smith et al., 1985) purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). 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 H₂O (w/v) solution, Sigma C-2284) (v/v). Using a Konelab Arena 30 (Thermo Fisher, Manchester, UK) equipped with a sample disk, reagent disk, cuvette loader, incubator, and photometer, 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.

2.5. Activity assays

For the functional characterization, the activity of different samples was determined using a Konelab Arena 30 instrument before and after BAC separation (in triplicate). The general lipase activity was measured using the chromogenic substrate p-nitrophenyl palmitate (3 mg/mL in 2-propanol). Under vigorous 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 and 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 a 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.

In addition, the phospholipase and galactolipase activity of the start material and collected BAC fractions were also measured. First substrate solutions (0.7% L-α-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 were 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 activity between 0.015 and 0.075 U/mL). After 20 min incubation, 130 μL NEFA-R1 was added and incubated for 2 min followed by addition of 65 μL 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 × t) × 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.

2.6. Statistical analysis

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. For the calculations, in-house developed software was used. First, the differences in specific activity were calculated, either between samples with different storage temperatures or between different BAC peaks from the same sample. Thereafter, a *t*-test test for a significant difference between two independent samples was performed. The results of each step can be found in Table S7, where the significant differences (using a 95% confidence interval) are marked in green.

3. Results and discussion

In this study, the effect of glycation on the functionality of a commercially available lipase was investigated. 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 (Gargano et al., 2020). 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).

3.1. BAC method optimization

For the optimization of the BAC method, two lipase control samples

were used with different levels of glycation, namely low- and high-glycated control samples. The low-glycated control sample was stored at $-20\text{ }^{\circ}\text{C}$ and therefore, no to minor glycation was expected. The high-glycated control sample consisted of a lipase standard stored at room temperature for approximately 2 years to increase glycation levels. 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). Besides, the presence of the previously reported variants with different C-termini (i.e., M, M-W, and M-SW) was confirmed and also N-terminal truncated proteoforms were detected (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 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 (Lhota et al., 2021; Li, Larsson, Jungvid, Galaev, & Mattiasson, 2001; Quan et al., 2008). 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 ($\text{pH} > 8.2$) (Hall, 2011). 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, different concentrations of Tris (0–200 mM) were added 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, 120 mM Tris was selected 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. This optimal Tris concentration is relatively high compared to previously reported methods, where Tris concentrations below 30 mM were selected for enrichment for glycated mAbs (Quan et al., 2008; Viski et al., 2016) indicating that the lipase is more susceptible to non-specific interactions.

To determine the composition of the proteoforms present in the non-retained (or non-binding) peak and retained (or binding) peak, fractions were collected and analyzed 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, BAC enrichment allowed the detection of glycated proteoforms with one or two hexose residues attached to the protein comprising $\sim 6\%$ 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 influence BAC retention. The higher hydrophobicity of tryptophan on the C-terminus (M form) compared to 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 (Espina-Benitez, Randon, Demesmay, & Dugas, 2017). Unfortunately, these solvents can affect the nativity of protein hampering further functional characterization.

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 (Espina-Benitez et al., 2017). However, similar elution profiles were observed 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 to 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. Efficient enrichment of glycated species requires a separation temperature between 35 and $40\text{ }^{\circ}\text{C}$ for most proteins (e.g., endo-xylanases and various mAbs) (Fischer, Hoernschmeyer, & Mahler, 2008; Lhota et al., 2021; van Schaick et al., 2022). Surprisingly, this was not the case for the studied lipase, where the relative area of the binding peaks increased with increasing separation temperature (i.e., $20\text{ }^{\circ}\text{C}$, $25\text{ }^{\circ}\text{C}$, and $30\text{ }^{\circ}\text{C}$) (Fig. 2c). Specifically, an increase from 13% to 25% for the low-glycated control sample and from 78% to 88% for the high-glycated control samples was observed when changing the separation temperature from $20\text{ }^{\circ}\text{C}$ to $30\text{ }^{\circ}\text{C}$. An explanation could be that higher temperatures alter protein conformation, such as partly unfolding, resulting in additional nonspecific retention. Therefore, the

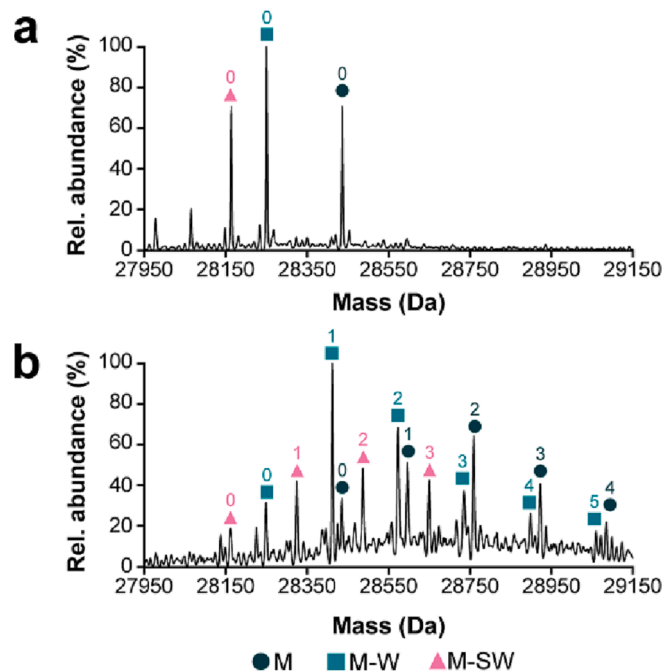


Fig. 1. Zero charge deconvoluted mass spectra of the two lipase standards measured with RPLC-MS, including low-glycated control sample (a) and high-glycated control sample (b). The symbols indicate different sequence variants (i.e., C-terminal or N-terminal truncation), where the circle corresponds to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

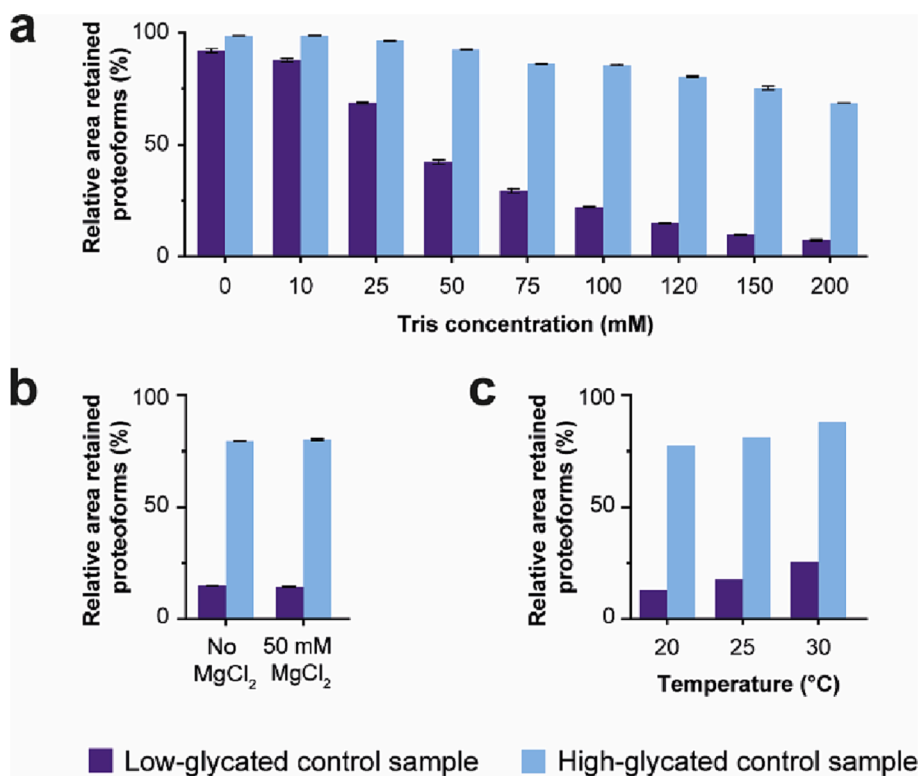


Fig. 2. Optimization of the BAC mobile phase composition and separation temperature. The effect of different parameters on the UV peak area (recorded at 280 nm) of the binding peak was assessed for the low-glycated control sample (dark blue) and the high-glycated control sample (light blue). In particular, the effect of different concentrations of shielding agent Tris (a), presence of MgCl₂ (b), and column temperature (c). The peak areas of the measurements evaluating the Tris and MgCl₂ concentration were measured in duplicate (error bars represent standard deviation) while the evaluation of temperature was performed by single measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

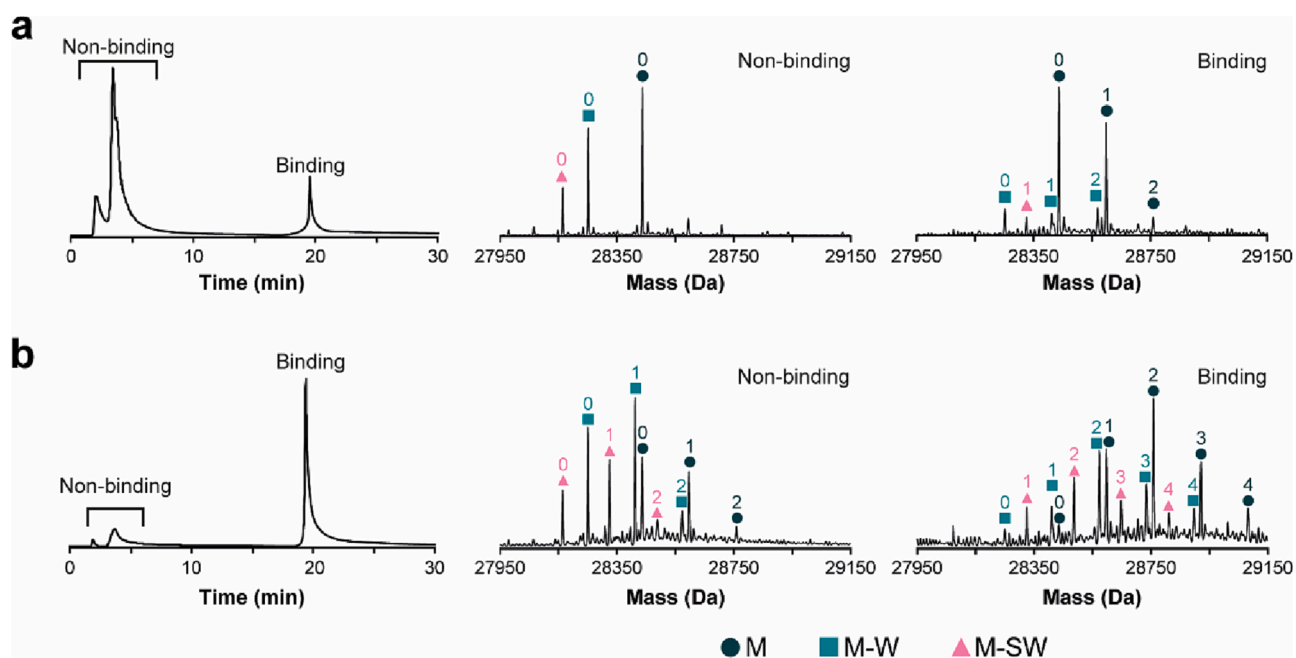


Fig. 3. BAC separation of the low-glycated control sample (a) and high-glycated control sample (b). For both samples, the UV chromatogram recorded at 280 nm and obtained mass spectra of the non-binding (spectrum on the left) and binding peak (spectrum on the right) are displayed. In the spectra, the three most abundant C-terminal truncated variants are assigned, that is M (represented as a circle), M-W (represented as a square), and M-SW (represented as a triangle). The number above the assigned masses represents the number of hexoses per protein. Details on the assigned proteoforms can be found in Table S2.

separation was performed at 20 °C 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). As above mentioned, these conditions are quite different compared to previously developed BAC methods for other glycosylated proteins highlighting that there is no universal BAC method and that

protein-specific tailoring of the BAC conditions is required.

3.2. Effect of different storage temperatures on lipase proteoforms activity

Since BAC is a native separation technique, the activity of resolved proteoforms could be assessed (in this case, glycosylated species). For this

purpose, the glycation levels were increased 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 first was used as a reference temperature and the latter was selected 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, the lipase activity of the BAC fractions on three substrates was determined, including p-nitrophenyl palmitate, phospholipid (PC), and galactolipid (DGDG). Where the p-nitrophenyl palmitate is a commonly employed substrate to measure general lipase activity (Hasan, Shah, & Hameed, 2009; Hernandez-Garcia, Garcia-Garcia, & Garcia-Carmona, 2017), the other two natural substrates are more specifically reflecting food application performance (Gerits et al., 2014; Liszka, Kutscher, Prashar, & Pop, 2021). The obtained lipase activity (in U/mL) was divided by the protein concentration determined with the BCA assay (Smith et al., 1985) to obtain the specific activity (U/mg protein), where the latter was used to compare different samples.

To allow comparison of proteoform function, it is important to first determine whether the experimental BAC conditions safeguard that the enzyme is still functional. While this might seem evident, the relatively high pH of the mobile phase can greatly reduce enzyme activity as was shown for a previously investigated endo-xylanase (van Schaick et al., 2022). In the case of this lipase, sufficient activity was observed for all substrates after dissolving the starting material in the BAC mobile phase (Fig. S4). To minimize interfering matrix compounds (e.g., salts, sugars, small peptides, or even insoluble particles and aggregates) during the separation, centrifugation to remove large particles followed by filtration using spin filters with 10 kDa cut-off was performed 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 glycosylated 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, the wheat-formulated sample (stored at -20 °C) was also measured 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 high molecular weight species (HMWS). Moreover, the mass spectrum of 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.

As expected, upon storage at 25 °C, the area of peak 3 increased indicating higher glycation levels (Table S3). MS analysis of this peak confirmed not only a higher level of glycation but also revealed glycosylated products with up to four hexoses (Table S4). Although the majority of proteoforms in peak 1 was non-glycated and in peak 3 was glycosylated,

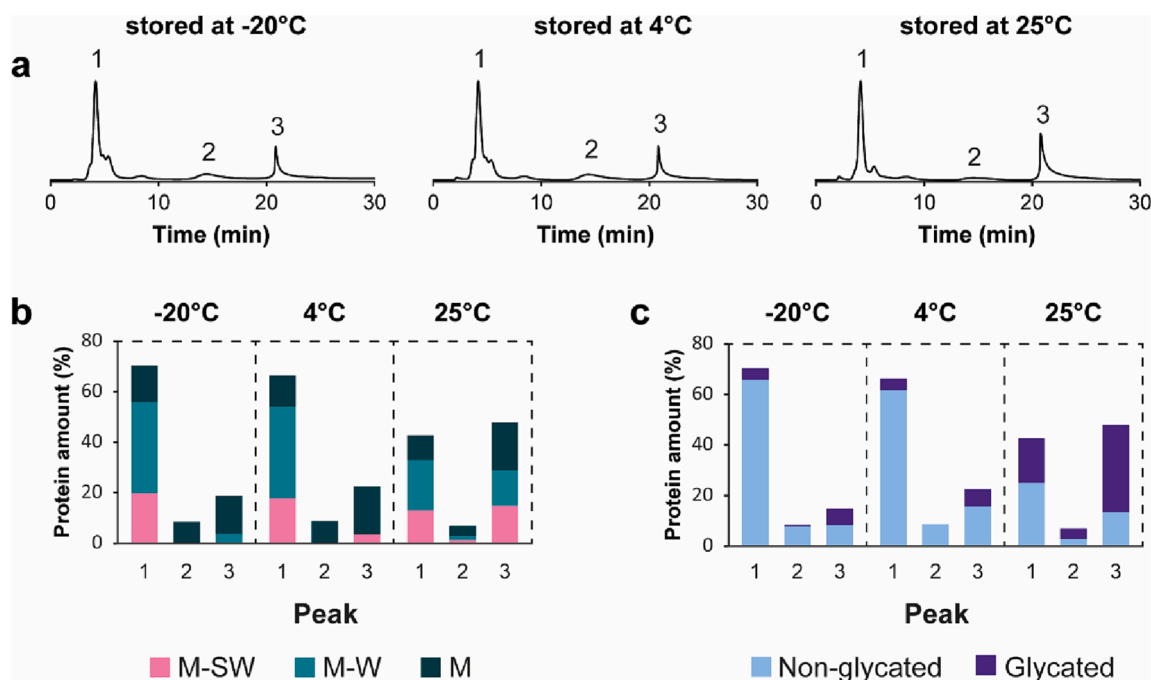


Fig. 4. Analysis of the proteoforms present in the BAC peaks of the wheat four-formulated samples subjected to different storage temperatures (i.e., -20 °C, 4 °C, and 25 °C). (a) The UV chromatograms of the optimized BAC method recorded at 280 nm of the lipase samples. The numbered peaks were collected, 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 in the separated peaks (M in pink, M-W in green, and M-SW in dark green), 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, where the non-glycated fraction is indicated in light blue and glycosylated proteoforms in purple. Details on the assigned (glycosylated) proteoforms can be found in Table S4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

some (non-)glycated species were detected in the other peaks. The presence of non-glycated species in peaks 2 and 3 could be explained by the occurrence of non-specific interactions between analytes and the column material, as has been described as a common challenge of BAC methods for proteins (Espina-Benitez et al., 2017). Moreover, the accessibility of glycation for interaction with the stationary phase could play a role, since buried glycation would not interact with the BAC column resulting in the elution of glycated species in peak 1. Finally, the observed elution patterns could be related to the presence of aggregates (high molecular weight species; HMWS), 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 $-20\text{ }^{\circ}\text{C}$ and $4\text{ }^{\circ}\text{C}$, 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 $25\text{ }^{\circ}\text{C}$. Non-specific elution due to the presence of aggregates was already proposed by Lhota et al. (2021) and could be a plausible explanation in the case of the samples stored at $25\text{ }^{\circ}\text{C}$. Finally, the overall results obtained for the samples formulated in

wheat flour were very similar to 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 $-20\text{ }^{\circ}\text{C}$ had a specific activity of $104 \pm 2.1\text{ U/mg}$ protein (p-nitrophenyl palmitate assay) which decreased to $99 \pm 1.3\text{ U/mg}$ protein for $4\text{ }^{\circ}\text{C}$ and $80 \pm 4.4\text{ U/mg}$ for $25\text{ }^{\circ}\text{C}$ (Fig. 5a, Table S6, and S7). Even though the decrease in activity was smaller than expected, the difference between the reference sample stored at $-20\text{ }^{\circ}\text{C}$ and lipase stored at $25\text{ }^{\circ}\text{C}$ was significant for all three substrates. When comparing storage at $4\text{ }^{\circ}\text{C}$ with the temperature-stressed sample stored at $25\text{ }^{\circ}\text{C}$, significant specific activity differences were also found. After determining the activity of the starting material, the activity of lipases in the different BAC peaks was evaluated to investigate whether differences in specific activity could be detected for the separated species. For all BAC peaks, between 54 and 74% of lipase activity was recovered compared to the starting material. Similar recoveries (between 50% and 64%) were observed for the protein concentration indicating that the lipase remained active during separation and fraction collection, even though some protein losses were

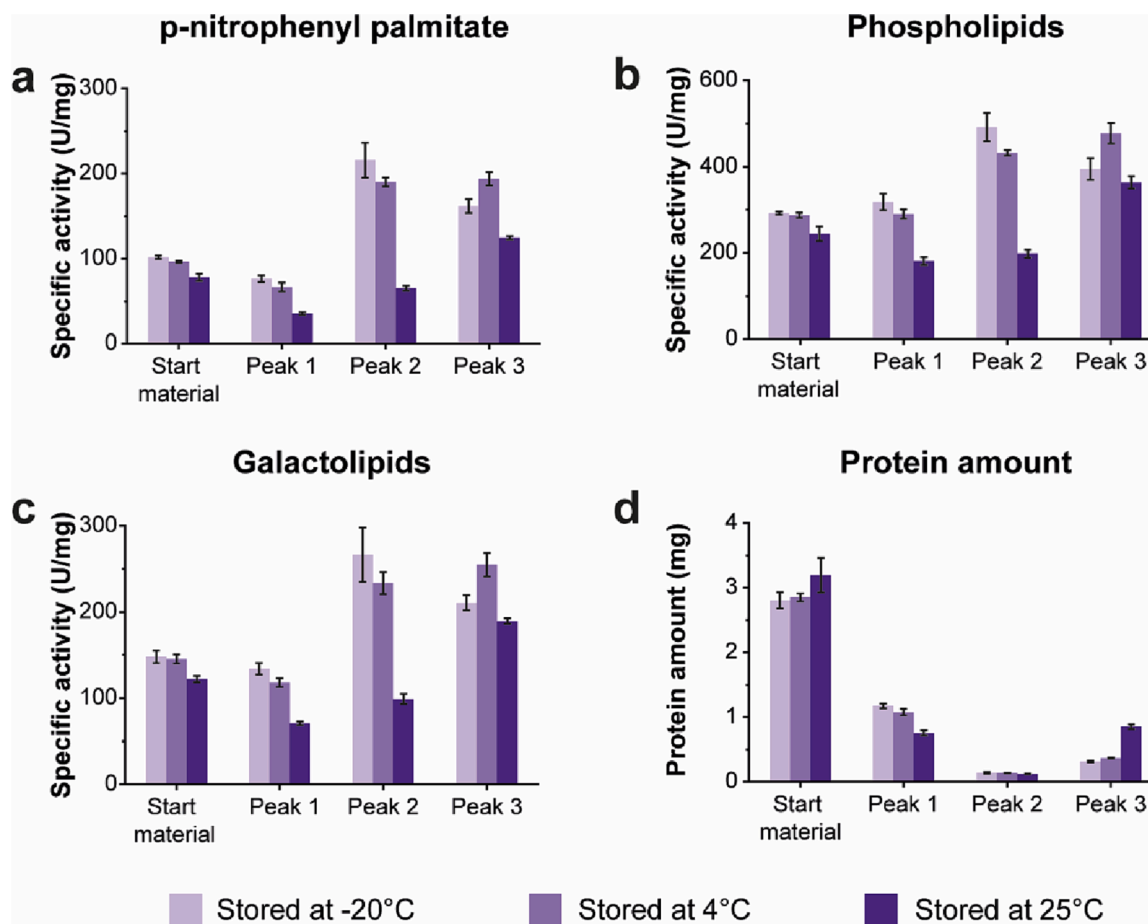


Fig. 5. Specific activity (U/mg protein) and amount of protein of the non-separated lipase (start material) and the BAC fractions (Peak 1–3) formulated in wheat. The results of the three samples with different storage temperatures, that is $-20\text{ }^{\circ}\text{C}$ (light purple), $4\text{ }^{\circ}\text{C}$ (purple), and $25\text{ }^{\circ}\text{C}$ (dark purple). 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 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 of 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 Fig. S6, Table S6, and Table S7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

encountered (Table S6).

Both non-stressed lipase samples (stored at -20 °C or 4 °C) showed a significantly lower specific activity of the lipase proteoforms in peak 1 compared to peaks 2 and 3 (Fig. 5a). Since the level of glycosylated proteoforms in these samples was only minor, the lower specific activity of lipase in peak 1 could be assigned 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 detected in the fraction of peak 1, also a difference in the specificity of the proteoforms present in this peak towards the different substrates was observed. The ratios between the 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 measured in 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 the lipase proteoforms in peak 2 was substantially reduced probably (partly) due to the presence of C-terminal truncated variants. Surprisingly, the decrease in specific activity found for the species present in peak 3 was minor, while the glycosylation level was greatly increased implying that glycosylation does not have a negative influence on the specific activity of these lipase samples.

Since glycosylation was not assigned as a cause for activity loss, lipase inactivation during storage at 25 °C must have another origin. As no mass change (other than glycosylation) was observed in the BAC fractions with lowered specific activity, non-covalent modifications (e.g., denaturation or aggregation) play probably a role (Misset et al., 1998). To obtain information on possible protein denaturation, tryptophan intrinsic fluorescence (excitation at 295 nm and emission at 350 nm) was measured for all maltodextrin-formulated samples as described by Togashi, Ryder, and O'Shaughnessy (2010). Protein unfolding results in the 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). Fluorescence data of the BAC fractions 1, 2, and 3 was also acquired, where more variations were observed (Table S8). This data was however deemed not conclusive as changes in truncation proteoform compositions (M, M-W, and M-SW) (see Fig. S6) significantly affected the tryptophan fluorescence response (independently of protein denaturation state) as well as the UV₂₈₀ response used for protein concentration normalization. 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 could contain so-called advanced glycosylated end products (AGEs), which can be described as glycosylated proteins that undergo rearrangement reactions and cross-linking to form large heterogenous species (Priego-Capote et al., 2014; Tessier, 2010). These AGEs are known to cause changes in structural properties of proteins leading to impaired functionality (Chen, Lin, Bu, & Zhang, 2018). Some AGEs are fluorescent at characteristic wavelengths, specifically excitation wavelength between 340 and 370 nm and emission wavelength between 420 and 440 nm (Bosch, Alegria, Farre, & Clemente, 2007; Matiacevich, Santagapita, & Buera, 2005). Unfortunately, in our hands, this experiment provided no conclusive results about possible AGE formation (data not shown).

Altogether, the exact cause of lipase inactivation remains unknown. However, the newly developed BAC method together with MS detection and activity assays revealed that initial glycosylation leading to a mass increment of 162 Da was not responsible for the altered functionality.

4. Conclusion

Glycosylation in industrial enzyme products can be increased upon storage at higher temperatures. In this study, the influence of glycosylation on a lipase used for food applications was monitored. To investigate glycosylation levels and the potential influence of glycosylation on activity, a BAC enrichment method coupled with MS was developed. 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 glycosylation, 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 glycosylated species were observed for the low-glycosylated control samples, while direct RPLC-MS analysis without BAC was unable to detect these species, herewith demonstrating the higher sensitivity of glycosylation detection thanks to BAC enrichment.

The applicability of the optimized BAC enrichment method was investigated by comparing glycosylation 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 glycosylation level increased after storage at 25 °C compared to -20 °C and 4 °C. The formulation of the enzyme product did not influence the glycosylation level as similar results were obtained for both wheat flour and maltodextrin-formulated. To assess influence of proteoforms on activity, it was first demonstrated that the BAC separation conditions were able to conserve lipase activity. Of note, the effect of sample preparation steps should be carefully 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 glycosylation levels in the non-stressed samples, lower specific activity and altered specificity were observed for the variants lacking the C-terminal tryptophan. Remarkably, extensive glycosylation of the temperature-stressed lipase was not linked to altered specific activity or substrate specificity. Nevertheless, the heterogeneity of the lipase notably due to glycosylation and N- and C-terminal truncation 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.

In conclusion, the combination of BAC with MS detection and functional assays is powerful for detecting glycosylation and monitoring its effect on protein function. BAC followed by MS detection allowed the determination of the total glycosylation levels and provided an overview of the number of attached sugar moieties per enzyme. Furthermore, a major benefit of BAC separation is the ability to enrich glycosylated species without losing the enzyme functionality, thereby allowing to measure activity of collected BAC fractions. In future, this approach can be extended to other industrial enzymes by tailoring the BAC conditions. This novel strategy can be a valuable asset in the analytical toolbox for structure-function insights employed to support the development of enzyme products.

CRedit authorship contribution statement

Guusje van Schaick: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft. **Sanne Pot:** Conceptualization, Methodology, Investigation, Writing – original draft. **Olaf Schouten:** Investigation, Visualization, Writing – review & editing. **Joost den Hartog:** Investigation. **Michiel Akeroyd:** Conceptualization, Resources, Supervision. **Rob van der Hoeven:** Conceptualization, Writing – review & editing. **Wim Bijleveld:** Conceptualization, Resources, Supervision, Formal analysis, Writing – review & editing. **Nicolas Abello:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Manfred Wuhrer:** Conceptualization,

Funding acquisition, Supervision, Writing – review & editing. **Maurien Olsthoorn**: Conceptualization, Funding acquisition, Resources, Writing – review & editing, Project administration. **Elena Dominguez-Vega**: Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Co-authors Olaf Schouten, Joost den Hartog, Michiel Akeroyd, Rob van der Hoeven, Wim Bijleveld, Nicolas Abello, and Maurien Olsthoorn are affiliated with Royal DSM, a global company active in health, nutrition, and bioscience.

Data availability

The authors do not have permission to share data.

Acknowledgements

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2023.136147>.

References

- Bosch, L., Alegria, A., Farre, R., & Clemente, G. (2007). Fluorescence and color as markers for the Maillard reaction in milk–cereal based infant foods during storage. *Food Chemistry*, *105*(3), 1135–1143. <https://doi.org/10.1016/j.foodchem.2007.02.016>
- Cardoso, H. B., Wierenga, P. A., Gruppen, H., & Schols, H. A. (2018). Maillard induced glycation behaviour of individual milk proteins. *Food Chemistry*, *252*, 311–317. <https://doi.org/10.1016/j.foodchem.2018.01.106>
- Chen, J.-H., Lin, X., Bu, C., & Zhang, X. (2018). Role of advanced glycation end products in mobility and considerations in possible dietary and nutritional intervention strategies. *Nutrition and Metabolism*, *15*(1), 72. <https://doi.org/10.1186/s12986-018-0306-7>
- Duivelshof, B. L., Fekete, S., Guilleme, D., & D'Atri, V. (2019). A generic workflow for the characterization of therapeutic monoclonal antibodies-application to daratumumab. *Analytical and Bioanalytical Chemistry*, *411*(19), 4615–4627. <https://doi.org/10.1007/s00216-018-1561-1>
- Espina-Benitez, M. B., Randon, J., Demesmay, C., & Dugas, V. (2017). Back to BAC: Insights into Boronate Affinity Chromatography Interaction Mechanisms. *Separation and Purification Reviews*, *47*(3), 214–228. <https://doi.org/10.1080/15422119.2017.1365085>
- Fischer, S., Hoenschmeyer, J., & Mahler, H. C. (2008). Glycation during storage and administration of monoclonal antibody formulations. *European Journal of Pharmaceutics and Biopharmaceutics*, *70*(1), 42–50. <https://doi.org/10.1016/j.ejpb.2008.04.021>
- Gargano, A. F. G., Schouten, O., van Schaick, G., Roca, L. S., van den Berg-Verleg, J. H., Haselberg, R., ... Somsen, G. W. (2020). Profiling of a high mannose-type N-glycosylated lipase using hydrophilic interaction chromatography-mass spectrometry. *Analytica Chimica Acta*, *1109*, 69–77. <https://doi.org/10.1016/j.aca.2020.02.042>
- Gerits, L. R., Pareyt, B., Decamps, K., & Delcour, J. A. (2014). Lipases and Their Functionality in the Production of Wheat-Based Food Systems. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 978–989. <https://doi.org/10.1111/1541-4337.12085>
- Gstöttner, C., Reusch, D., Habeger, M., Dragan, I., Van Veelen, P., Kilgour, D. P. A., ... Nicolardi, S. (2020). Monitoring glycation levels of a bispecific monoclonal antibody at subunit level by ultrahigh-resolution MALDI FT-ICR mass spectrometry. *MAbs*, *12*(1), 1682403. <https://doi.org/10.1080/19420862.2019.1682403>
- Hall, D. G. (2011). Structure, properties, and preparation of boronic acid derivatives. *Boronic acids: Preparation and applications in organic synthesis, medicine and materials*. Wiley-VCH Verlag GmbH & Co.
- Hasan, F., Shah, A. A., & Hameed, A. (2009). Methods for detection and characterization of lipases: a comprehensive review. *Biotechnology Advances*, *27*(6), 782–798. <https://doi.org/10.1016/j.biotechadv.2009.06.001>
- Hernandez-García, S., Garcia-Garcia, M. I., & Garcia-Carmona, F. (2017). An improved method to measure lipase activity in aqueous media. *Analytical Biochemistry*, *530*, 104–106. <https://doi.org/10.1016/j.ab.2017.05.012>
- Koval, D., Kasicka, V., & Cottet, H. (2011). Analysis of glycated hemoglobin A1c by capillary electrophoresis and capillary isoelectric focusing. *Analytical Biochemistry*, *413*(1), 8–15. <https://doi.org/10.1016/j.ab.2011.01.048>
- Lhota, G., Sissolak, B., Striedner, G., Sommeregger, W., & Vorauer-Uhl, K. (2021). Quantification of glycated IgG in CHO supernatants: A practical approach. *Biotechnology Progress*, *37*, e3124.
- Li, Y., Larsson, E. L., Jungvid, H., Galaev, I. Y., & Mattiasson, B. (2001). Shielding of protein-boronate interactions during boronate chromatography of neoglycoproteins. *Journal of Chromatography. A*, *909*, 137–145.
- Liszka, M., Kutscher, J., Prashar, A., & Pop, C. (2021). *Lipase enzymes*. United States: BASF SE.
- Liu, J., Ru, Q., & Ding, Y. (2002). Glycation a promising method for food protein modification: Physicochemical properties and structure, a review. *Food Research International*, *49*, 170–183.
- Maillard, L. C. (1912). Action des acides amines sur le sucres: Formation des melanoidines per voie methodique. *Comptes Rendus de l'Académie des Sciences*, *154*, 66–68.
- Matiacevich, S. B., Santagapita, P. R., & Buera, M. P. (2005). Fluorescence from the maillard reaction and its potential applications in food science. *Critical Reviews in Food Science and Nutrition*, *45*(6), 483–495. <https://doi.org/10.1080/10408390591034472>
- Melis, S., Meza Morales, W. R., & Delcour, J. A. (2019). Lipases in wheat flour bread making: Importance of an appropriate balance between wheat endogenous lipids and their enzymatically released hydrolysis products. *Food Chemistry*, *298*, Article 125002. <https://doi.org/10.1016/j.foodchem.2019.125002>
- Misset, O., & van Dijk, A. (1998). Diagnosing the inactivating process of enzymes. In A. Ballesteros (Ed.), *Stability and stabilization of biocatalysts*. Elsevier Science.
- Mo, J., Jin, R., Yan, Q., Sokolowska, I., Lewis, M. J., & Hu, P. (2018). Quantitative analysis of glycation and its impact on antigen binding. *MAbs*, *10*(3), 406–415. <https://doi.org/10.1080/19420862.2018.1438796>
- Priego-Capote, F., Ramirez-Boo, M., Finamore, F., Gluck, F., & Sanchez, J. C. (2014). Quantitative analysis of glycated proteins. *Journal of Proteome Research*, *13*(2), 336–347. <https://doi.org/10.1021/pr4000398>
- Priego Capote, F., & Sanchez, J. C. (2009). Strategies for proteomic analysis of non-enzymatically glycated proteins. *Mass Spectrometry Reviews*, *28*(1), 135–146. <https://doi.org/10.1002/mas.20187>
- Quan, C., Alcalá, E., Petkovska, I., Matthews, D., Canova-Davis, E., Taticek, R., & Ma, S. (2008). A study in glycation of a therapeutic recombinant humanized monoclonal antibody: Where it is, how it got there, and how it affects charge-based behavior. *Analytical Biochemistry*, *373*(2), 179–191. <https://doi.org/10.1016/j.ab.2007.09.027>
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., ... Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, *150*, 76–85.
- Stemler, C. D., & Scherf, K. A. (2022). Improvement of cake baking properties by lipases compared to a traditional emulsifier. *Food Chemistry: X*, *15*, Article 100442. <https://doi.org/10.1016/j.fochx.2022.100442>
- Sutthirak, P., Assavanig, A., Dharmthiti, S., & Lertsiri, S. (2010). Changes in the Stability and Kinetic Parameters up on Glycation of Thermostable α -Amylase from *Bacillus Subtilis*. *Journal of Food Biochemistry*, *34*(6), 1157–1171. <https://doi.org/10.1111/j.1745-4514.2010.00356.x>
- Sutthirak, P., Dharmthiti, S., & Lertsiri, S. (2005). Effect of glycation on stability and kinetic parameters of thermostable glucoamylase from *Aspergillus niger*. *Process Biochemistry*, *40*(8), 2821–2826. <https://doi.org/10.1016/j.procbio.2004.12.008>
- Tessier, F. J. (2010). The Maillard reaction in the human body. The main discoveries and factors that affect glycation. *Pathologie et Biologie*, *58*(3), 214–219. <https://doi.org/10.1016/j.patbio.2009.09.014>
- Togashi, D. M., Ryder, A. G., & O'Shaughnessy, D. (2010). Monitoring local unfolding of bovine serum albumin during denaturation using steady-state and time-resolved fluorescence spectroscopy. *Journal of Fluorescence*, *20*(2), 441–452. <https://doi.org/10.1007/s10895-009-0566-8>
- van Schaick, G., el Hajjouti, N., Nicolardi, S., den Hartog, J., Jansen, R., van der Hoeven, R., ... Dominguez-Vega, E. (2022). Native liquid chromatography and mass spectrometry to structurally and functionally characterize endo-xylanase proteoforms. *International Journal of Molecular Sciences*, *23*, 1307.
- Viski, K., Gengeliczki, Z., Lenkey, K., & Baranyane Ganzler, K. (2016). Parallel development of chromatographic and mass-spectrometric methods for quantitative analysis of glycation on an IgG1 monoclonal antibody. *Journal of Chromatography B*, *1032*, 198–204. <https://doi.org/10.1016/j.jchromb.2016.04.043>
- Wei, B., Berning, K., Quan, C., & Zhang, Y. T. (2017). Glycation of antibodies: Modification, methods and potential effects on biological functions. *MAbs*, *9*(4), 586–594. <https://doi.org/10.1080/19420862.2017.1300214>
- Zhang, L., Liu, C. W., & Zhang, Q. (2018). Online 2D-LC-MS/MS platform for analysis of glycated proteome. *Analytical Chemistry*, *90*(2), 1081–1086. <https://doi.org/10.1021/acs.analchem.7b03342>
- Zhang, L., & Zhang, Q. (2020). Glycated plasma proteins as more sensitive markers for glycemic control in type 1 diabetes. *Proteomics. Clinical Applications*, *14*(2), e1900104.