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Research Article

Connie Pontoppidan, Svend G. Kaasgaard, Carsten P. Sønksen, Carsten Andersen*, Birte Svensson*

Deamidation and glycation of a *Bacillus licheniformis* α -amylase during industrial fermentation can improve detergent wash performance

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Abstract: The industrial thermostable *Bacillus licheniformis* α -amylase (BLA) has wide applications, including in household detergents, and efforts to improve its performance are continuously ongoing. BLA during the industrial production is deamidated and glycated resulting in multiple forms with different isoelectric points. Forty modified positions were identified by tandem mass spectrometric peptide mapping of BLA forms separated by isoelectric focusing. These modified 12 asparagine, 9 glutamine, 8 arginine and 11 lysine residues are mostly situated on the enzyme surface and several belong to regions involved in stability, activity and carbohydrate binding. Eight residues presumed to interact with starch at the active site and surface binding sites (SBSs) were subjected to mutational analysis. Five mutants mimicking deamidation (N $\rightarrow$ D, Q $\rightarrow$ E) at the substrate binding cleft showed moderate to no effect on thermostability and k_{cat} and K_M for maltoheptaose and amylose. Notably, the mutations improved laundry wash efficiency in detergents at pH 8.5 and 10.0. Replacing three reducing sugar reactive side chains (K $\rightarrow$ M, R $\rightarrow$ L) at a distant substrate binding region and two SBSs enhanced wash performance especially in liquid detergent at pH 8.5, slightly improved

enzymatic activity and maintained thermostability. Wash performance was most improved (5-fold) for the N265D mutant near substrate binding subsite +3.

Keywords: mass spectrometric peptide mapping; site-directed mutagenesis; surface binding sites; thermostability; enzyme kinetics.

Abbreviations

AGC, automated gain control; AGE, advanced glycation end-product; BHA, *Bacillus halmapalus* α -amylase; BLA, *Bacillus licheniformis* α -amylase; DSC differential scanning calorimetry; ETD, electron transfer dissociation; GH, glycoside hydrolase; HCD, high-energy C-trap dissociation; IEF, isoelectric focusing; MG-HI, methylglyoxal-derived hydroimidazolone; SBS, surface binding site.

1 Introduction

α -Amylase (E.C. 3.2.1.1., 1,4- α -D-glucan glucanohydrolase) catalyses hydrolysis of α -D-(1,4) glucosidic linkages in starch and related oligo- and polysaccharides, releasing maltooligosaccharides, maltose and glucose [1-3]. Most α -amylases belong to the very large glycoside hydrolase family 13 (GH13) of the CAZy database ([4]; <http://www.cazy.org/>). Several α -amylases important for starch liquefaction and saccharification in starch and fuel alcohol industries, stain removal in household detergents, and in baking and brewing belong to the subfamily GH13_5 of bacterial α -amylases [5-8]. Its stability at alkaline pH and elevated temperature makes *Bacillus licheniformis* α -amylase (BLA) particularly suitable for application in laundry detergents, where α -amylases constitute the second most used enzyme group after proteases [6,7,9].

***Corresponding authors:** Birte Svensson, Enzyme and Protein Chemistry, Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, building 224, DK-2800, Kgs. Lyngby, Denmark, E-mail: bis@bio.dtu.dk; Carsten Andersen, Novozymes A/S, Biologiens Vej 2, DK-2800, Kgs. Lyngby, Denmark, E-mail: cara@novozymes.com
Connie Pontoppidan, Enzyme and Protein Chemistry, Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, building 224, DK-2800, Kgs. Lyngby, Denmark; Ascendis Pharma A/S, Tuborg Boulevard 12, DK-2900 Hellerup, Denmark
Svend G. Kaasgaard, Connie Pontoppidan, Carsten P. Sønksen, Carsten P. Sønksen, Novo Nordisk A/S, Novo Alle 1, DK-2880 Bagsvaerd, Denmark

Crystal structures are available for a number of industrially important α -amylases, including BLA [1]. It contains three domains (A, B and C) similarly to most of the GH13 α -amylases. The active site is situated on the $(\beta/\alpha)_8$ -barrel domain A, and the loop domain B, that is inserted between β -strand 3 and α -helix 3, also participates in substrate binding. By contrast, the domain C, which has an anti-parallel β -sheet structure is rarely involved in function, but required for the overall structural integrity of the enzyme [3]. BLA has in addition to the catalytic site nucleophile (D231), acid/base (E261) and a third acid residue (D328), involved in stabilizing substrate reaction intermediates at binding subsite -1 [10], five substrate-accommodating glycon-binding subsites (-1 through -5) and three aglycon-binding subsites (+1 through +3) [1,11] formed by domains A and B. A unique linearly arranged Ca^{2+} - Na^{+} - Ca^{2+} ion triad at the interface of domains A and B is characteristic of α -amylases in subfamily GH13_5 [1,10,12]. This triad stabilizes the structure around the active site and is important for the thermostability [11,13]. Similar to the related *Bacillus halmapalus* α -amylase (BHA) [14], domains A and C of BLA contain so-called surface binding sites (SBSs) or secondary carbohydrate binding sites, which are non-catalytic, but functionally important and situated at a certain distance from the active site. SBSs are particularly prominent in GH13, but have been reported in about 25 other GH and glycosyl transferase (GT) families [15-18]. SBSs typically contain aromatic residues engaged in the carbohydrate binding and can cooperate with the active site, for example, by anchoring polysaccharides undergoing processive degradation, serving as point of attachment onto starch granules and soluble polysaccharides or be involved in allosteric regulation of activity [15-18].

Industrial-scale production of α -amylases by heterologous expression in bacterial or fungal hosts has been found to result in modifications of protein side chains, typically deamidation, glycation and oxidation that can alter enzyme stability and activity [19,20]. Glycation involves reactions between lysine and arginine side chains and reducing sugars resulting in formation of ketoamines, which can rearrange to more reactive carbonyl compounds termed advanced glycation end-products (AGEs) [21,22]. Commonly reported AGEs include 3-deoxyglucosone, carboxymethyl, carboxyethyl, and methylglyoxal-derived hydroimidazolone (MG-HI) [23-26]. Industrial BLA as a consequence encompasses multiple protein forms presenting a pI range due to deamidation of asparagine and glutamine residues and glycation of lysine and arginine residues, which can be identified by mass spectrometry. The functional impact of these chemical

modifications of amino acid side chains is here assessed by mutational analysis in BLA, involving eight modified residues selected in binding regions including substrate aglycon and glycon interacting subsites at the active site and two SBSs, respectively. Remarkably, most of these BLA mutants acquired improved laundry wash performance.

2 Materials and methods

2.1 *Bacillus licheniformis* α -amylase (BLA)

Industrial BLA was produced recombinantly in *Bacillus licheniformis* and kindly provided by Novozymes A/S.

2.2 Production of BLA wild-type and mutant

BLA mutants were prepared by Novozymes A/S using site-directed mutagenesis according to described principles [27]. Wild-type and BLA mutant proteins were produced recombinantly with *B. subtilis* as host by fermentation at 37 °C for 96 h in a medium consisting of 100 g/L sucrose, 40 g/L soybean meal, 10 g/mL $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.01 % (v/v) Dowfax (Dow Chemicals) 63N10 and 5 g/L CaCO_3 .

2.3 Enzyme purification

Cultures (typically 100 mL) containing secreted BLA wild-type and mutants were centrifuged (5,400×g, 50 min). The supernatants were filtered (0.2 μm PES filters), added MOPS, CaCl_2 and $(\text{NH}_4)_2\text{SO}_4$ to 20 mM, 1 mM and 1 M, respectively, and adjusted to pH 7.5. BLA proteins were purified by hydrophobic interaction chromatography (Phenyl Sepharose 6 Fast Flow packed in a 50 mL XK 26/20 column; GE Healthcare) pre-equilibrated in the above buffer, washed with two column volumes of buffer and eluted by a linear $(\text{NH}_4)_2\text{SO}_4$ gradient (1-0 M) in the buffer. Amylase containing fractions were identified by using SDS-PAGE (NuPAGE 4-12% Bis-Tris gradient gel; Invitrogen) and enzyme activity analysis spectrophotometrically at 510 nm as described by the manufacturer (Amylzyme α -amylase assay; Megazyme). One Amylzyme tablet was suspended in 5 mL 25 mM HEPES, 1 mM CaCl_2 , pH 7.5 and 575 μL hereof was incubated 15 min at room temperature with 20 μL enzyme. Fractions containing activity were pooled, dialysed O/N against 10 mM Tris, 1 mM CaCl_2 , pH 8.9, and further purified by anion exchange

chromatography (50 mL Source 30Q packed in a XK26/20 column; GE Healthcare), washed with one column volume of buffer and eluted by a linear NaCl gradient (0–600 mM) in 20 mM Tris, 1 mM CaCl₂, pH 8.9 over 110 min at a flow rate of 8 mL/min. Fractions containing only BLA (verified by SDS-PAGE) were pooled, concentrated and buffer-exchanged to 20 mM HEPES, 1 mM CaCl₂, pH 7.0 (Vivaspin 20 10,000 MWCO PES spin columns; Sartorius) and stored frozen until use. Protein concentrations were determined by amino acid analysis [28,29].

2.4 Isoelectric focusing (IEF)

BLA samples were buffer-exchanged to 25 mM HEPES, 1 mM mM CaCl₂, pH 7.5 and desalted (Centrifugal devices; Nanosep 10 K Omega, PALL life science) prior to IEF (FocusGel 3-7 with an Amersham Isoelectric Focusing Calibration Kit low range pH 2.5–6.5, GE Healthcare). IEF was run according to the manufacturer's protocol loading 60 µg protein samples. Gels were stained with Coomassie blue.

2.5 In-gel digestion in H₂¹⁸O

IEF protein bands were cut out, destained twice in 50% ethanol, dehydrated in 100% acetonitrile (15 min) and the gel fragments were dried in Speedvac (1 h, 50 °C). The following solutions were made with H₂¹⁸O (97 atom % ¹⁸O; Sigma-Aldrich) to distinguish deamidations present in purified BLA forms from those occurring during proteolysis [30,31]. The dried gel fragments were incubated in 15 µL of 27 ng/µL trypsin or 2.7 ng/µL Asp-N (both sequencing grade, Roche Diagnostics) in 50 mM NH₄HCO₃ for 45 min at 5 °C. After proteolysis the remaining protease solution was removed, and the gel samples were further incubated at 37 °C O/N in the presence of 50 µL 50 mM NH₄HCO₃. Peptides were extracted from gel pieces with 70% acetonitrile, 0.1% trifluoroacetic acid (thrice), dried in Speedvac (1 h, 50 °C) and redissolved in 80 µL 5% formic acid for MS analysis.

2.6 Tandem mass spectrometry

LC-MS/MS was performed with higher energy C-trap Dissociation (HCD) (LTQ Orbitrap Velos; Thermo Scientific) or electron transfer dissociation (ETD) fragmentation (LTQ Orbitrap XL; Thermo Scientific). ETD leaves labile modifications intact facilitating identification of glycations. Both instruments were equipped with a nano-

ESI source (Nanospray Flexion Source, Thermo Scientific) coupled to an EASY-nLC II (Thermo Scientific). Peptides were separated on Thermo Scientific EASY-Column (C18) 2 cm × 100 µm i.d. followed by a Thermo Scientific EASY-Column (C18) 10 cm × 75 µm i.d., eluting with a 5–50% acetonitrile gradient in 0.1% formic acid over 60 min at a flow rate of 250 nL/min.

Orbitrap Velos was run with automatic gain control (AGC) target of 1,000,000 with 30,000 resolution and 10 ms maximum ion injection time in full MS. For MS/MS the AGC target was 50,000 with 100 ms injection time and resolution of 7,500. The HCD normalized collision energy was 40% and the 10 most intense precursor ions in the range 350–1750 m/z were selected for MS/MS. Orbitrap XL MS¹ was performed with a resolution of 60,000 (at 400 m/z). AGC target was 500,000 with maximum ion injection time 300 ms for the orbitrap in full MS. MS/MS was performed in the ion trap with AGC target of 40,000. ETD reactions were performed for 85 ms detecting fragment ions in the ion trap with 200 ms maximum injection time using 3 microscans selecting the four most abundant precursor ions in the range 350–1200 m/z, rejecting charge state 1 and 2. Data analysis was performed with Mascot (Matrix Science). Searches were conducted against a custom-made database containing 14,100 sequences with decoy option enabled with trypsin or Asp-N as enzyme allowing 5 missed cleavages with 10 ppm precursor mass tolerance, 0.5 Da fragments mass tolerance for ETD and 0.02 Da mass tolerance for HCD. The following variable modifications were chosen: Deamidated (NQ), Deamidated ¹⁸O(1) (NQ), Label ¹⁸O(1)(C-term), Label ¹⁸O(2)(C-term), Hex (KR), 3-deoxyglucosone (KR), Carboxymethyl (KR), Carboxyethyl (KR), MG-HI (methylglyoxal-derived hydroimidazolone; KR), Oxidation (M), Carbamyl (KR), Acetyl (K).

2.7 Differential scanning calorimetry

Unfolding of BLA proteins (approx. 1 mg/mL in 25 mM HEPES, 1 mM CaCl₂, pH 7.0) was analysed by differential scanning calorimetry (DSC) (Microcal VP-capillary DSC; GE Healthcare) at a scan rate of 1.5 °C/min to a final temperature of 130 °C. T_{max} is the temperature at the peak maximum of the transition from folded to unfolded state. Data analysis was performed using the Origin software package (MicroCal; GE Healthcare).

2.8 α -Amylase activity assays

2.8.1 Maltoheptaose

The kit AMYL α -amylase liquid (Roche) containing a modified maltoheptaose substrate solution [4,6-ethylidene-(G7)-pNP] and an α -glucosidase solution was used to determine initial rates of hydrolysis at 11 substrate concentrations (0.025–3 mM) in 52.5 mM HEPES pH 7.0. The assay was run on a KONELAB ARENA 30 (Thermo Electron Corporation) at 30 °C mixing 147 μ L α -glucosidase with 83 μ L substrate and adding 20 μ L enzyme (3–15 nM in the above buffer). After 3 min pre-incubation, colour formation was measured 7 times over 2 min at 405 nm and quantified using 0–0.6 mM *p*-nitrophenol (Sigma). K_M and k_{cat} were obtained by fitting of the Michaelis-Menten equation (CurveExpert 1.3; <http://www.curveexpert.net/>).

2.8.2 Amylose DP440

Amylase (3–5 nM, 110 μ L in 20 mM HEPES, 5 mM CaCl_2 , pH 7.0, 0.005% w/v bovine serum albumin) was added to amylose DP440 (Amylose type III from potato; Sigma) at 10 different concentrations (0.1–2.5 mg/mL, 990 μ L) in 20 mM HEPES pH 7.0, 5 mM CaCl_2 , 4.44% v/v dimethyl sulfoxide, 0.005% w/v bovine serum albumin. Aliquots (200 μ L) were removed at 2.5 min intervals and initial rates of reducing power formation were determined at 30 °C using copper-bicinchoninate with maltose as standard and measuring colour formation spectrophotometrically at 540 nm [32,33]. K_M and k_{cat} were obtained as described above.

2.9 Wash performance assay

Wash performance was determined on coloured rice starch cotton textile, CS-28 (Center For Testmaterials BV), using a mini wash robot (Novozymes). CS-28 strips were mounted on racks, which moved up and down into wash beakers (50 times/min) during washing performed at 40 °C for 30 min at six enzyme concentrations (0–1.0 mg/L) using either a liquid detergent (pH 8.5) or a powder detergent (pH 10.0) followed by 5 min rinse in cold water.

The liquid detergent (0.8 g/L during the wash) was composed of 20% linear alkylbenzene sulphonate (Nacconol 90G), 10% alkyl ether sulphate (STEOL CS-370E), 10% alcohol ethoxylate (AEO, Bio-soft N25-7, Stepan Company), 4% oleic acid, 0.5% ethanol, 1.5%

sodium xylene sulfonate, 7% 1,2-propanediol, 8% tri-Na-citrate, 0.5% triethanolamine, 1.5% boric acid, pH 8.5. The powder detergent wash system contained 0.035 g/L AEO with 1.715 g/L powder detergent composed of 17% linear alkylbenzene sulphonate, 20% sodium carbonate, 12% sodium disilicate, 15% Zeolite A, 1% Sokalan CP5 (maleic acid/acrylic acid co-polymer), 34% sodium sulphate. Both detergent mixtures were prepared with water hardness of 15 °dH (Ca/Mg 4:1). All percentages are in w/w. After wash, rinse and drying, the colour remission of the CS-28 strips was measured at 460 nm (TIDAS; Zeiss spectrophotometer). Wash efficiency was given in delta remission units, i.e. the difference between the absorbance of the remission of a blank control (cotton patch washed in detergent without enzyme) and the enzyme sample. The higher the delta remission value the better was the wash performance of the enzyme.

3 Results

3.1 Identification of BLA modifications by LC-MS/MS

Industrial BLA contained different forms of varying pI in the range pH 5.4–6.9, the most prominent component on the IEF gel having pI 6.7 (Fig. 1). Protein extracted from twelve bands was subjected to in-gel digestion with trypsin or Asp-N and the modified amino acid residues were identified in eleven of these bands by peptide mapping using LC-MS/MS. The predominant side chain modifications were deamidation and glycation identified for a total of 40 BLA residues (Table 1) together with oxidation of 6 methionine residues (not shown). From 2 to 16 modified positions were identified from the individual bands on the IEF gel. It is anticipated that protein forms contain more than a single chemical modification as supported by 21 of the 40 modified residues being identified in more than one of the 11 protein bands. Notably six bands contained modified R242, N27 and R437, while modified K237, N265 and N326 were found in 5 bands (Table 1). Overall, these identified modified positions comprised 21 deamidations (12 N→D and 9 Q→E of a total of 45 N and Q residues in BLA), and 19 glycations (8 of R and 11 of K of a total of 49 R and K residues in BLA) presenting different AGEs; i.e. 3-deoxyglucosone, carboxymethyl, carboxyethyl and MG-HI (Table 1). Deamidation and glycation occurred mostly on the BLA surface although a few deamidations took place in a buried region near the active site. Several regions involved in BLA stability, activity and substrate

Table 1: Chemical modifications identified from 11 bands of BLA in IEF (Fig. 1) by LC-MS/MS fragmenting with HCD and ETD.^a

Site	Modification	ID in band	Covered
Q9	Dea	1	+
R23	Cbm/Cbe	1, 7	+
R24	MG-HI	5	+
N27	Dea	1,2, 3, 5,6, 7	+
Q51	Dea	3	-
K88	Cbe	7	+
R127	MG-HI	2, 5, 9	+
K136	Cbm	7	+
K154	Cbe	5	+
N172	Dea	9	+
R173	3DG	2, 4, 5, 7	+
K234	Cbm	2	+
K237	Cbm/Cbe	5, 7, 8, 9, 10	+
R242	Cbe/MG-HI	4, 5, 7, 8, 9, 10	+
N246	Dea	5	+
Q264	Dea	1, 2	+
N265	Dea	2, 3, 4, 5, 7	-
N272	Dea	3	-
N278	Dea	1	+
N280	Dea	1	+
Q291	Dea	1, 4	+
Q298	Dea	1	+
K306	Cbm	2, 5, 7	+
N309	Dea	4, 6, 11	+
K315	Hex/Cbm	2, 4, 5, 7	+
N326	Dea	1, 2, 3, 8, 9	+
Q360	Dea	3	+
K381	Cbm	2	+
K383	Cbe	2	+
K389	Cbm/Cbe	4, 5, 9, 11	+
K392	Cbe	4	+
Q393	Dea	4	+
Q399	Dea	1, 2	+
R413	Cbe	2, 5, 7	+
N421	Dea	4	+
R437	MG-HI	2, 4, 5, 7, 9, 11	+
R442	MG-HI/Cbe/Hex	3, 7, 8, 9	+
Q443	Dea	3, 6, 8, 9	-
N444	Dea	3, 7, 8, 9	+
N463	Dea	5, 6, 7, 8	-

^a No modifications were identified from band 12. The identified types of glycation are indicated as Hex: glycation with hexose, 3DG: 3-deoxyglucosone, Cbm: carboxymethyl, Cbe: carboxyethyl, MG-HI: methylglyoxal-derived hydroimidazolone, Dea: deamidation. Covered indicates whether or not the modified residue site is directly mapped by the MS/MS results.

Figure 1: IEF of industrial BLA (Lane 2). Twelve bands were selected over the entire pI span for in-gel digestion by trypsin and Asp-N followed by LC-MS/MS identification of modified residues (see Table 1). Lane 1 is the pI marker.

binding were found to contain modifications and eight of these reacted positions were subjected to single-residue substitution (Q9E, N172D, Q264E, N265D, Q360E, K306M, K383M and R437L) to assess the impact of the modifications on the performance of BLA.

3.2 Physico-chemical and enzyme kinetic properties of BLA mutants

The produced eight BLA single mutants were made at positions either near the catalytic site, in the substrate binding site or at the two SBSs. While deamidated positions were directly substituted (Q→E, N→D), the glycations of lysine or arginine were mimicked by replacement with methionine and leucine, respectively, to contain comparable side chains that are inert to reactions with reducing sugars. All purified BLA mutants migrated in SDS-PAGE as single bands of about 55 kDa and for the majority pI decreased as expected by introduction of an acidic or removal of a basic side chain (Fig. 2). Surprisingly, however, BLA N172D and K306M essentially maintained wild-type pI, while pI of Q9E BLA decreased less than for the other Q→E/N→D mutants (Fig. 2). It is noted that the pattern of protein forms of different isoelectric points for the major bands 1 through 7 (Fig. 1) do recur with similar relative intensities in the recombinant BLA wild-type and mutants produced in *B. subtilis* (Fig. 2). The conformational

Figure 2: IEF of the eight BLA mutants and BLA wild-type produced in *Bacillus subtilis*.

Table 2: $T_{\max}$ values for the BLA wild-type and mutants determined by DSC.

	WT	Q9E	N172D	Q264E	N265D	K306M	Q360E	K383M	R437L
$T_{\max}$ (°C)	103.2	100.6	98.9	99.2	102.9	103.4	102.7	103.2	103.1

integrity seemed barely affected by the mutations as DSC analysis confirmed the thermostability since the $T_{\max}$ of BLA wild-type determined to 103.2 °C was largely retained, although $T_{\max}$ values of Q9E, N172D and Q264E decreased by 2.6, 4.3 and 4.0 °C, respectively (Table 2).

Some mutations caused up to almost 3-fold and in other cases no changes in the enzyme activity occurred. Thus while the kinetic parameters (k_{cat} and K_M) for a maltoheptaose derivative and amylose of DP 440 were very similar to those of BLA wild-type, for N172D, K306M and K383M, Q264E and R437L significantly improved activity on both substrates essentially due to reduced K_M and increased k_{cat} , respectively (Table 3). Furthermore, Q360E showed 2-3 fold increase of both k_{cat} and K_M on both substrates, while N265D slightly improved k_{cat} and the Q9E mutation increased K_M on the oligosaccharide and reduced k_{cat} on the polysaccharide (Table 3).

3.3 Effect of mutations on wash performance of BLA

Laundry wash performance was assayed at 40 °C, a standard wash temperature in Europe, on cotton swatches with pink coloured starch using either a liquid detergent system at pH 8.5 or a so-called powder

detergent at pH 10. The wash efficiency was determined spectrophotometrically by exposing the cotton swatch to light and measuring the reflected light (referred to as the remission) and given in delta remission units. The higher the value of delta remission the better is the removal of the coloured starch and thus the wash performance.

In liquid detergent at pH 8.5 all eight BLA mutants showed superior wash performance compared to that of wild-type (Fig. 3A), N265D having 5-fold increased and the highest efficiency of all. The other mutants enhanced washing performance by >1.5 delta remission units (at 0.5 and 1.0 mg/L enzyme), considered a threshold for indicating a significant improvement. Q360E, however, only raised the value by about 1 delta remission unit over wild-type BLA. N265D also showed best wash performance in powder detergent at pH 10, where as well the K306M, Q360E, Q264E and K383M mutants were significantly improved over wild-type BLA, while N172D and R437L performed similarly to and Q9E worse than BLA wild-type (Fig. 3B).

Table 3: The kinetic parameters k_{cat} , K_M and k_{cat}/K_M for the BLA mutants and BLA determined at 30 °C pH 7.0 on the maltoheptaose derivative and amylose DP 440 substrates.^a

BLA	Maltoheptaose			Amylose		
	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat}/K_M (s ⁻¹ mM ⁻¹)	k_{cat} (s ⁻¹)	K_M (mg/mL)	k_{cat}/K_M (s ⁻¹ mg ⁻¹ mL)
WT	65 ± 12	0.011 ± 0.003	5922 (100)	200 ± 8	0.140 ± 0.03	1455 (100)
Q9E	63 ± 14	0.027 ± 0.008	2394 (40)	117 ± 12	0.112 ± 0.04	1099 (76)
N172D	70 ± 9	0.013 ± 0.005	5462 (92)	201 ± 6	0.129 ± 0.01	1555 (107)
Q264E	60 ± 5	0.005 ± 0.001	13013 (220)	228 ± 15	0.089 ± 0.02	2632 (180)
N265D	83 ± 5	0.011 ± 0.002	7277 (123)	232 ± 28	0.128 ± 0.03	1852 (127)
K306M	70 ± 4	0.010 ± 0.001	7335 (124)	213 ± 38	0.125 ± 0.04	1740 (120)
Q360E	155 ± 24	0.026 ± 0.005	5996 (101)	416 ± 17	0.261 ± 0.06	1630 (112)
K383M	69 ± 10	0.010 ± 0.002	7133 (120)	241 ± 33	0.139 ± 0.02	1743 (120)
R437L	81 ± 18	0.010 ± 0.003	8153 (138)	307 ± 67	0.137 ± 0.02	2249 (155)

^a k_{cat}/K_M values relative to wild-type are given in parenthesis.

Figure 3: Wash performance of the BLA wild-type and mutants on rice starch cotton patches. (A) Wash performance in liquid detergent (pH 8.5). (B) Wash performance in powder detergent (pH 10.0). The bars show standard deviation.

4 Discussion

4.1 Identification of deamidated and glycated residues in BLA

In industrial-scale production of BLA, sucrose used as carbon source in *B. licheniformis* fermentation can get hydrolysed during heat-sterilisation [34] causing protein glycation by reaction with glucose and fructose. Lysine glycation and also deamidation of BLA were noticed previously to reduce stability and affect activity and substrate specificity [35-40]. In the present work

altogether 40 asparagine, glutamine, lysine and arginine residues of BLA have been identified as modified during the fermentation, which resulted in multiple BLA protein forms of varying pI. As expected, generally the reacting side chains are exposed on the BLA surface, although a few less accessible, albeit still deamidated residues were situated near the active site. Twenty modified residues are marked at important sites on the BLA structural model (Fig. 4). Amongst these positions, R127, N172, R173, K237 and N272 are located at the interface between domains A and B that harbours the Ca^{2+} - Na^{+} - Ca^{2+} triad proposed to be vital for BLA thermostability [1,10,12,13,41]. The residues N172

Figure 4: Surface representation of BLA (PDB code: 1BLI) indicating 20 of the identified deamidated Asn and Gln residues (in grey) and glycosylated Lys and Arg residues (in black). PyMOL was used for molecular rendering (The PyMOL Molecular Graphics System, version 1.3, Schrödinger, LCC).

and R173 from domain B probably belong to an extended substrate binding site. The precise role of these residues has not been elucidated although the “170-loop” in BLA is important in substrate interactions involving D164, W165 and F167 [1]. Additionally, deamidation of Q9, N326 and Q360 situated in the substrate binding cleft as well as Q264 and the glycosylated K234, contributing to subsites +3 and +1, respectively, may affect enzyme activity [1,7,10]. Moreover, while the mascot score [42,43] for N265 was low and also the deamidation product D265 was not identified by MS/MS, E264, the deamidated Q264, was robustly identified and found in the most prominent BLA form seen in IEF (band 1, Fig. 1; Table 1). However, since K_m values of the N265D BLA mutant for maltoheptaose and amylose were the same as for BLA wild-type and k_{cat} increased modestly by 15–20 %, the deamidation of N265 situated adjacent to Q264, is not likely to influence activity. By contrast, the BLA Q264E mutation improved substrate affinity by 2–3 fold for both the oligosaccharide and polysaccharide substrates.

Sequence alignment and structural analysis suggest that BLA has counterparts to two SBSs, documented in the structure of the close homologues BHA and *Bacillus paralicheniformis* α -amylase containing W342, P432

and D430 bound to maltose, R437 and W467 binding glucose and L318, K319 and Y358 binding different maltooligosaccharides [14,44]. Indeed, BLA R437 is glycosylated and the structurally adjacent N463 is deamidated. K383 situated in the vicinity of W342 is also glycosylated. The modifications likely stem from the ability of the SBSs to bind carbohydrates thus promoting glycation of the arginine and lysine residues, which in turn may interfere with substrate binding functions of the SBSs. Finally, glycation occurred of K306 situated in an area extending beyond subsite +3 of the substrate binding cleft. While BLA R437L showed about 50% elevated activity towards the maltoheptaose and amylose substrates, the mutants K306M beyond substrate binding subsite +3 and K383M in the vicinity of an SBS, respectively, had slightly elevated activity on both substrates suggesting that glycation at these side chains reduces the wild-type BLA activity.

4.2 Properties of the individual BLA mutants

Q264E at subsite +3 showed the best catalytic efficiency corresponding to 2–3 fold larger than wild-type BLA due to higher substrate affinity and also clearly improved

Figure 5: Close-up views of the mutated amino acid residues in the crystal structure of BLA in complex with acarbose (unpublished structure of BLA in complex acarbose, courtesy of Novozymes). (A) Possible substrate interactions with Q264 and N265 (in green) as well as the Glu and Asp mutant residues (in grey). Subsites +2 and +3 are labelled as S+2 and S+3. (B) The active site showing the three catalytic site residues D231, E261 and D328 along with Q9, Q360 and H327. The original BLA residues (in green) and the two Glu mutant residues (in grey) are shown. The nitrogen between the two non-reducing end rings in acarbose is shown in blue. (C) BLA in complex with acarbose at the substrate binding cleft showing the residue N172 (in green) and the Asp mutant residue (in grey). The subsites of the binding site are numbered and indicated by S. (D) Acarbose in the active site and K306 shown in a putative extended substrate binding region. Substrate binding subsites are numbered and indicated by S. (E) K383 and W342 at one of the SBS. (F) R437 and W467 at another SBS with bound glucose. The position of R437 is assessed by modelling guided by the position of W439 in BHA solved with bound glucose [14]. (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LCC).

the wash efficiency at both pH 8.5 and pH 10. Probably the glutamic acid forms a stronger hydrogen bond with substrate than glutamine in BLA wild-type. Indeed, in the acarbose complex of a different BLA variant (unpublished) the side chain of Q264 points away from the inhibitor, whereas the flexible side chain of Q264E can form hydrogen bonds with glucose residues at subsites +2 and +3 (Fig. 5A). Even though mutation of the adjacent N265 to N265D caused only marginally improved k_{cat} and catalytic efficiency towards maltoheptaose and amylose, N265D was the best of all mutants in wash performance. N265 is within hydrogen bonding distance of acarbose at subsite +3 in the BLA complex (Fig. 5A) and the exceptionally large 5-fold increase in wash performance suggests reinforced interaction of N265D with the rice starch patch on the textile perhaps also facilitated by favourable binding of BLA N265D to the fabric.

Q9E, located behind the highly conserved H327 that is involved in transition state stabilisation at subsite -1 (Fig. 5B), had lowest catalytic efficiency for both maltoheptaose and amylose of all mutants, the loss of activity being largest on the oligosaccharide. The Q9E mutant is able to interact electrostatically with H327 and thus to weaken transition state stabilisation, possibly explaining the substantial decreases in activity by 60 and 24%. Still wash performance of Q9E was improved in liquid detergent at pH 8.5, but was in powder detergent wash at pH 10 reduced importantly compared to wild-type BLA and the seven other mutants. This pH dependency perhaps reflects that Q9E perturbs pK_a of amino acid side chains at or near the catalytic site [40] although it cannot be excluded that the poor wash performance stems from loss of in-wash stability caused by one or more detergent components [39]. Q360 is situated very close to Q9 and 5.9

Å from the catalytic acid/base E261. The Q360E mutation may have shifted the BLA pH activity profile. Still, the catalytic efficiency on maltoheptaose and amylose was maintained as a >2-fold increase in k_{cat} was accompanied by a similar increase in K_M and the wash performance of Q360E was slightly improved in powder detergent but unchanged in liquid detergent. The N172D belonging to domain B and situated in the near vicinity of the substrate binding cleft about 5 Å from the bound acarbose (Fig. 5C) showed a modest improvement only in liquid detergent.

K306M at a putative binding area extending beyond subsite +3 (Fig. 5D) maintained kinetic parameters for amylose and improved the wash performance. K383M close to W342 at a proposed SBS (Fig. 5E) showed marginal effect on kinetic parameters, but was clearly superior to wild-type in wash performance in both detergent systems and possibly weakened the interaction with substrate on the fabric. Noticeably, this is a mutation for which a possible loss of glycation of K383 during BLA production can allow substrate interaction with the SBS. R437 and W467 are at another SBS, found on the surface of domain C, and R437 hydrogen bonds with O-6 of glucose modelled (Fig. 5F) as guided by glucose binding to W439 in BHA [14]. R437L improved k_{cat} and catalytic efficiency towards amylose DP400 along with improved wash performance in liquid detergent in agreement with SBSs playing roles in activity.

5 Conclusion

During enzyme production by industrial fermentation deamidation and glycation generated multiple forms of BLA having different pI values. While the various modified enzyme forms were not characterized individually, a large number of chemically modified positions could be identified by mass spectrometry, of which some are situated at or near sites known to be important for enzyme stability, activity and substrate binding. Remarkably, single-site mutants of BLA mimicking some of these chemical modifications had improved textile wash performance and, except for a few, mostly influenced activity and substrate binding positively. Substitution in K306M, K383M and R437L of residues susceptible to glycation at a distance from the active site, including two SBSs, thus modestly improved enzyme activity and enhanced the wash performance, especially in liquid detergent. Among five mutants mimicking deamidation Q264E and N265D, both probably engaged at subsite +3, showed the highest activity towards substrates in solution

and remarkably improved wash performance removing rice starch on fabric.

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