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The diabetogenic antibiotic streptozotocin modifies the tryptic digest pattern for peptides of the enzyme O-GlcNAc-selective N-acetyl-β-D-glucosaminidase that contain amino acid residues essential for enzymatic activity

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Abbreviations:
PBS, phosphate buffered saline
STZ, streptozotocin
O-GlcNAcase, O-GlcNAc-selective N-acetyl-β-D-glucosaminidase
OGT, O-GlcNAc transferase
GlcNAc, N-acetylglucosamine
MNU, methylnitrosourea
PUGNAc, O-(2-acetamido-2-deoxy-D-glucopyranosylide) amino-N-phenylcarbamate

ABSTRACT

Streptozotocin (STZ) inhibits O-GlcNAc-selective N-acetyl-β-D-glucosaminidase (O-GlcNAcase), the enzyme that removes O-GlcNAc from proteins. The active site of the enzyme was recently proposed to include aspartates 174, 175, and 177, with STZ inhibition via a transition state analog. We explored the effect of STZ on the tryptic peptide digest pattern of O-GlcNAcase. LC/MS/MS analysis demonstrated that STZ modified two areas of the enzyme. One peptide, LGCFEIAK (894–901), in a C-terminal region previously proposed to possess O-GlcNAcase activity, was methylated by STZ. Another peptide, EYEIEFIYIASPGLDITFSNPK (128–149), was detected only after treatment with STZ and was in an N-terminal region, overlapping a glutamate-rich area containing an adjacent phenylalanine residue. No covalent modification of this peptide could be demonstrated. Detection of this peptide after treatment with STZ was accompanied by the simultaneous inability to detect the nearby peptide KLDQVSQFGCR (157–167), which contains a cysteine residue recently shown to be essential for enzymatic activity. To determine which of the first two peptides might also be important for O-GlcNAcase activity, site-specific mutagenesis was performed. Mutation of the N-terminal phenylalanine and serine residues resulted in almost complete inhibition of activity. In contrast, mutation of conserved C-terminal glycine and cysteine residues caused little inhibition of enzymatic activity. Together, these data extend the region of the active site N-terminally and give independent evidence to support the idea that STZ inhibits O-GlcNAcase through formation of a transition state analog that resides in the active site of the enzyme and in doing so alters its conformation and ensuing tryptic digest pattern.

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1. Introduction

A number of nuclear and cytoplasmic proteins are covalently modified by the attachment of a single monosaccharide N-acetylglucosamine (GlcNAc) to either serine or threonine, a reaction catalyzed by the enzyme O-GlcNAc transferase (OGT) [1–6]. In the pancreatic β-cell, which is uniquely enriched in OGT [7,8], this enzyme links the extracellular

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glucose level to intracellular protein O-glycosylation [9–11]. The enzyme that removes O-GlcNAc from proteins, O-GlcNAc-selective N-acetyl-β-D-glucosaminidase (O-GlcNAcase) [12,13], is also present in β-cells [14]. We have previously shown that the diabetogenic toxin streptozotocin (STZ), an analog of N-acetylglucosamine (GlcNAc) [15], irreversibly inhibits the activity of O-GlcNAcase [7,14]. Interestingly, islet O-GlcNAcase was found to be particularly sensitive to STZ [14]. Thus, the high level of expression of OGT in pancreatic β-cells combined with increased sensitivity of islet O-GlcNAcase to STZ appears to explain the selective β-cell toxicity of STZ [16].

Subsequently, the gene coding for O-GlcNAcase has gained significant further attention as a candidate gene for type 2 diabetes [17]. A single nucleotide polymorphism (SNP) in this gene was recently shown to be highly correlated with type 2 diabetes in humans [17]. Very recently, the active site of the enzyme was proposed by two independent groups to include aspartate residues at positions 174, 175, and 177, and the mechanism of action of STZ proposed to be through formation of a transition state analog [18–21]. In light of these observations, we further explored the possible mechanism by which STZ inhibits O-GlcNAcase, focusing on the effects of STZ on the tryptic peptide digest of the protein and additional regions of the enzyme that might be important for enzymatic activity.

2. Materials and methods

2.1. Recombinant O-GlcNAcase cloning and expression

The entire gene for human O-GlcNAcase with a His_{10} epitope tag inserted upstream was subcloned into pCRScript, then pET16b (Novagen) using *Bam*HI and *Nde*I. The new construct, called pWA28, was used to transform *Escherichia coli* BL21 (DE3) pLysS (Novagen). After overnight incubation on selective agar at 37 °C, isolated colonies were picked and used as inoculum for Terrific Broth (12 g Bacto tryptone, 24 g Bacto yeast extract, 4 ml glycerol, 0.17 M KH_2PO_4, and 0.72 M K_2HPO_4 per liter), supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. After overnight incubation at 37 °C, 200 rpm, 1 ml of the culture was diluted 1:250 into fresh Terrific Broth supplemented with 100 μg/ml ampicillin and incubated at 37 °C with shaking at 200 rpm. When the OD_{600} reached 0.400, IPTG was added to a final concentration of 1.0 mM. After 3 h, cells were harvested, and the pellet was stored at −80 °C until needed.

2.2. Purification of recombinant O-GlcNAcase

A 5 g cell pellet was resuspended in 12 ml B-PER (Bacterial Protein Extraction Reagent, Pierce Chemical Co.) supplemented with 10 μg/ml lysozyme and 10 μg/ml DNase. The cell suspension was sonicated for 2 min with interspersed cooling cycles and centrifuged at 12,000 rpm for 30 min at 4 °C to pellet unbroken cells and debris. The supernatant was mixed with an equal volume of 2× binding buffer (80 mM phosphate, 1.0 M NaCl, and 12 M urea, pH 7.40) and passed through a 0.45 μm filter. The lysate was added to a 5.0 ml Ni^{2+} column and eluted with a linear gradient of elution buffer (40 mM phosphate, 0.5 M NaCl, 6 M urea and 0.5 M imidazole) that increased from 0 to 100% over 60 min. Fractions were analyzed for O-GlcNAcase protein by SDS-PAGE, combined for dialysis overnight against 20 mM sodium phosphate, and after quantification, stored at −80 °C until needed.

2.3. Production and affinity purification of anti-O-GlcNAcase antibodies

Four peptides corresponding to the human O-GlcNAcase amino acid sequence were synthesized (Anaspec, Inc.). Terminal cysteine residues were added at the time of synthesis to allow directional conjugation to carrier proteins and chromatography beads. Chicken egg albumin (Sigma) was activated for conjugation with a 10-fold molar excess of Sulpho-SMCC (Pierce). Free SMCC was removed from Carrier-SMCC conjugates by chromatography through sephadex G-50 equilibrated in PBS. The first peak was collected, and protein concentration determined by measuring absorbance at 280 nm. Peptides were conjugated to activated carriers with a 10-fold molar excess of peptide by incubation at room temperature for 2 h. Four rabbits per antigen were immunized every 21 days with 100 μg peptide carrier conjugates. Animals were bled 10 days after booster injections beginning after the third injection. All antisera were tested via Western blotting against recombinant O-GlcNAcase protein and found to be approximately equally reactive. As a result, antisera were pooled. Afterward, anti-O-GlcNAcase polyclonal antibody was affinity purified. For affinity column preparation, 5 mg of peptide were conjugated to 5 ml of hydrated activated thiol sepharose 4b beads (Amersham) in a total volume of 10 ml 0.1 M Tris–HCl, 0.2 M NaCl, pH 7.50. The conjugation reaction was incubated overnight at 4 °C. The column was washed thoroughly before being loaded with sera. Approximately 10 ml of rabbit sera was mixed with an equal volume PBS, incubated with the affinity beads overnight at 4 °C, washed extensively, and eluted with low pH buffer (60% 0.6 M acetic acid, 40% 1× PBS). The eluate was immediately neutralized with 2 M Tris base and dialyzed four times with 1 l PBS. The purified antibody was then concentrated with an Amicon stir cell concentrator (YM30 membrane) and the yield determined by absorbance at 280 nm.

2.4. Western blotting

Samples were loaded onto SDS-polyacrylamide gels. Colored molecular weight markers were run on each gel. Proteins were separated for 1 h at 175 V at room temperature and transferred to ECL nitrocellulose paper (Amersham) for 1 h (100 V, 4 °C). Nitrocellulose blots were blocked for 1 h at room temperature in TBS-casein blocking buffer (Pierce) containing 0.1% Tween-20. After blocking, blots were probed with HRP-labelled anti-O-GlcNAcase antibody (1 μg/ml) in blocking buffer for 1 h at room temperature. Blots were washed three times (10 min each) with TBST (10 mM Tris pH 7.40, 150 mM NaCl, 0.1% Tween-20). After washing, blots were developed with ECL reagent (Amersham). After air-drying, blots were exposed to Bio-Max X-ray film (Kodak).

2.5. LC/MS/MS characterization of O-GlcNAcase

Two hundred microliters of purified recombinant O-GlcNacase was placed into a microfuge tube and incubated with STZ, methylnitrosourea (MNU), or O-(2-acetamido-2-deoxy-D-glucopyranosylide) amino-N-phenylcarbamate (PUGNAc) for 30 min. Ten microliters of each treated sample and one untreated control O-GlcNAcase sample were placed in a 200-μl tube, and 1 μl of 1 M ammonium bicarbonate was added. Sequencing grade-modified trypsin (Promega) was prepared by adding 1 ml of 50 mM ammonium bicarbonate to 1 vial (20 μg). Next, 10 μl of the trypsin solution were added to each tube and the samples were incubated at 37 °C for 6 h. The samples were spun at 15,000 rpm, and the supernatant was placed in a 200-μl auto-sampler vial.

A Surveyor HPLC system (Thermo) was run at 50 μl/ml/min with Solvent A as 0.1% formic acid in water and Solvent B as 0.1% formic acid in acetonitrile. The flow was split with an LC Packings flow splitter to approximately 1.5–2 μl/min and directed into a LC Packings FAMOS auto-sampler. Five microliters of each sample was injected onto a PicoFrit, Aquasil C18, 75-μm × 5-cm column (New Objectives). The samples were loaded at 5% B. After 8 min, a splitting tee was opened between the auto-sampler and the column via a valve reducing the flow across the column to 200–300 nl/min. A linear gradient was run from 5% B to 45% B over 40 min. The outlet of the column was placed inside a polyethylene tube with a 15 psi flow of nitrogen directed at the inlet of the mass spectrometer. A LTQ-FT mass spectrometer (Thermo) was used for analysis. The electrospray voltage was set to 2500 V and was applied to a metal union in the flow path. The mass spectrometer was operated in a triple play mode where a parent mass scan was collected in the LTQ, ions were selected in a data-dependent manner and the most intense ion was selected for a FT SIM scan and MS/MS analysis on the LTQ. Analyzed ions were placed on the rejection list for 2 min and the cycle was repeated through the LC gradient. The data from the mass spectrometer was processed with Xcalibur 1.4 and Molecular Weight Calculator from PNNL. The data from the mass spectrometer were processed with Xcalibur 1.4, Sequest, and Molecular Weight Calculator from PNNL.

2.6. Site-specific mutagenesis

Site-specific mutagenesis was performed using Gene Tailor Site-Directed Mutagenesis system as directed by the manufacturer (Invitrogen, Carlsbad, CA). All mutant constructs were completely sequenced in order to verify error free mutagenesis (Agencourt Bioscience Corporation, Beverly, MA), and mutant enzymes were expressed as described above. Amino acid changes were made based on analysis of tryptic peptides identified using LC/MS/MS. The following amino acid changes were made within the O-GlcNAcase protein sequence: Y129F, E130A, F133V, S138I, G895A, and C896W. The amino acid changes Y129F, E130A, F133V, and S138I are contained in the tryptic peptide EYEIEFIYAISPGLDITFSNPK (128–149) identified by LC/MS/MS, while the amino acid changes G895A and C896W are contained in the tryptic peptide LGCFEIAK (894–901) identified by LC/MS/MS. Site directed mutagenesis was achieved by synthesizing primers which would incorporate nucleotide changes within the MGEA5 coding region. To maximize correct annealing, mutagenic primers were designed to change one nucleotide within the MGEA5 coding region. We chose to mutate one codon nucleotide which would subsequently lead to the most non-polar amino acid substitution available. The cysteine codon UGC was mutated to yield a tryptophan UGG codon, and the tyrosine UAU codon was mutated to yield a UUU phenylalanine codon. Likewise, G (GGC) to A (GCC), F (UUC) to V (GUC), E (GAA) to A (GCA), and S (AGC) to I (AUC) mutations were also made.

2.7. Measurement of O-GlcNAcase activity

For measurements of O-GlcNAcase activity, 0.2 μg of recombinant O-GlcNAcase was incubated directly with 20 mM STZ, MNU, or PUGNAc for 30 min in 20 mM Na_2HPO_4, pH 7.7, containing 10 mM $MgCl_2$, and supplemented with 0.1% BSA carrier protein, prior to a 1-h incubation with 50 mM sodium cacodylate, pH 7.0, supplemented with 2 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide. At the end of all incubations, the reaction was stopped with the addition of 0.5 M sodium carbonate. Samples were placed on ice, vortexed, and color was measured spectrophotometrically at 400 nm with non-specific blanks routinely subtracted. In each experiment performed, control samples were considered to have 100% activity, and all values were expressed as a percentage of the control.

2.8. Data analysis

Films were photographed using a digital camera. Results were expressed as the mean ± S.E.M., using the Windows-compatible version 2.98 of the program FigP (Biosoft, St. Louis, MO). Statistical analysis was performed using the same program. Data were analyzed by one-way analysis of variance followed by comparisons between the means using the least significant difference test. A probability of $p < 0.05$ was considered to indicate statistical significance.

3. Results

In light of recent data regarding the emerging importance of O-GlcNAcase in β-cells [17–21], we further investigated the effect of STZ on recombinant O-GlcNAcase activity. Recombinant O-GlcNAcase was first expressed, and its identity was confirmed by Coomassie blue staining following one-dimensional gel electrophoresis (Fig. 1A) as well as by Western blotting with anti-O-GlcNAcase antibody (Fig. 1B). As Fig. 1A and B demonstrate, recombinant O-GlcNAcase was visualized as an approximately 130 kDa protein consistent with previous observations that the protein runs significantly higher than its predicted molecular weight of 103 kDa [12,13].

Recombinant O-GlcNAcase was incubated in the absence or presence of STZ, methylnitrosourea (MNU, the nitric oxide-containing portion of STZ), or O-(2-acetamido-2-deoxy-D-glucopyranosylide) amino-N-phenylcarbamate (PUGNAc, a known inhibitor of O-GlcNAcase) prior to measurement of enzymatic activity. Fig. 2 shows the results of these experiments.

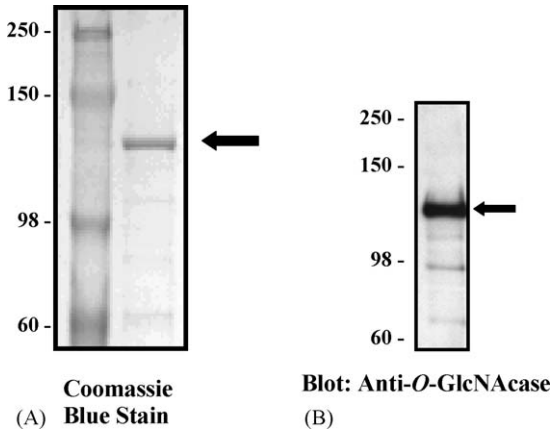

Fig. 1 – Expression of recombinant O-GlcNAcase. (A) Recombinant O-GlcNAcase was expressed and subjected to one-dimensional gel electrophoresis. Afterward, the gel was stained with Coomassie blue. (B) Recombinant O-GlcNAcase was expressed and analyzed by Western blotting with anti-O-GlcNAcase antibody.

Streptozotocin significantly dose-dependently inhibited recombinant O-GlcNAcase in a manner similar to what has been reported for purified rat spleen O-GlcNAcase [7]. In contrast, no significant inhibition of recombinant O-GlcNAcase activity was observed with MNU, indicating that nitric oxide generation alone or S-nitrosylation could not account for the inhibition of O-GlcNAcase. As expected, PUGNAc potently inhibited O-GlcNAcase activity ($3.8 \pm 0.3\%$ of control at 1.25 mM, $p < 0.05$ compared to control, data not shown).

In order to better understand the molecular mechanism by which STZ inhibits O-GlcNAcase, recombinant O-GlcNAcase was incubated in the absence or presence STZ, MNU, or PUGNAc. Afterward, recombinant O-GlcNAcase was digested with trypsin and subjected to LC/MS/MS analysis to search for any modified tryptic peptides. In the STZ-treated sample, a

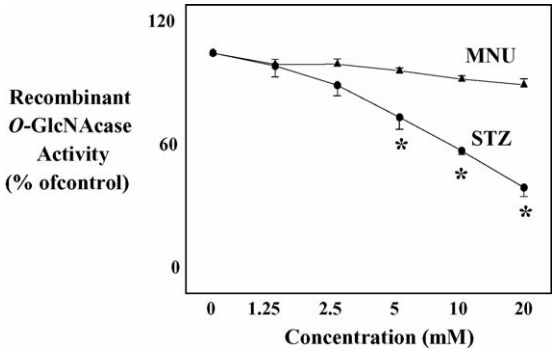

Fig. 2 – STZ inhibits recombinant O-GlcNAcase activity while MNU does not. Recombinant O-GlcNAcase protein was incubated for 30 min in the presence of 0–20 mM STZ or MNU. Afterward, O-GlcNAcase activity was measured enzymatically. Data shown are the mean ± S.E.M. from three observations from three independent experiments (*$p < 0.05$).

modified form of the peptide LGCFEIAK (894–901) was observed (Fig. 3A). This peptide demonstrated a shift in the y^6 and y^7 ions (710 and 767 *m/z*) by 14 Da but not the y^5 ion (607 *m/z*). This was confirmed by calculating the accurate mass difference from the FT SIM scan of this modified form and the unmodified peptide ($447.7420^{+2} - 440.7341^{+2} \times 2 = 14.0158$). Using the accurate difference in the formula finder in the Molecular Weight Calculator, the best match is to the molecular formula for the methyl adduct (CH_2) within 11 ppm. Fig. 3B shows the extracted ion chromatograms for all of the samples and the control for the methyl adduct (447–449 *m/z*). The only peak seen for this adduct is present in the STZ-treated sample. This modification was not observed with treatment with PUGNAc or MNU.

In addition to the LGCFEIAK (894–901) peptide, LC/MS/MS identified another peptide of potential importance. Interestingly, the tryptic peptide EYEIEFIYAISPGLDITFSNPK (128–149) was found to be detectable only in the sample treated with STZ. Fig. 4A shows the +3 extracted ion chromatogram for all of the samples and the control, with the peak of interest at around 44–46 min. This peak was seen only in the STZ-treated sample and was not present in any of the other samples, and MS/MS spectra of this peak in the STZ-treated sample confirmed this to be from the peptide of interest. In other samples, this peak was absent, but no additional MS/MS spectra were observed at later elution times. In order to confirm that this observation was not artifactual, LC/MS/MS data for the nearby glutamate-rich tryptic peptide EIPVESIEEVSK (258–269) were examined, confirming the presence of an appropriate peak at 35–37 min clearly present in all samples (Fig. 4B). None of the above peptides were noted to be covalently modified by STZ.

Interestingly, the appearance of the peptide EYEIEFIYAISPGLDITFSNPK (128–149) in the tryptic digest of STZ-treated enzyme was accompanied by the disappearance of the nearby peptide KLDQVSQFGCR (157–167) (Fig. 4C). This peptide is somewhat unusual in possessing an N-terminal lysine residue. A review of the protein sequence, however, indicated that this lysine residue is immediately preceeded by an arginine residue, thus allowing trypsin to cut between the two residues to generate the observed peptide. This peptide contains a cysteine residue, Cys_{166}, recently reported to be required for enzymatic activity [18]. Fig. 4C shows the +3 extracted ion chromatogram for all of the samples and the control, with the peak of interest at around 38–40 min. This peak was present in the other samples, and MS/MS spectra confirmed this to be from the peptide of interest. In the STZ-treated sample, this peak was absent, but no additional MS/MS spectra were observed at later elution times.

In light of these intriguing results, we hypothesized that one of the first two tryptic peptides identified might contain additional amino acids also important for O-GlcNAcase activity. To test this hypothesis, site-specific mutagenesis was performed. In the case of the first peptide, LGCFEIAK (894–901), the peptide was noted to lie in a C-terminal region of the enzyme that had previously been predicted to contain O-GlcNAcase activity [22]. In this region, the glycine residue was predicted to be essential for O-GlcNAcase catalytic activity [22], and our data above suggested that the adjacent cysteine residue might be the target of the methyl adduct. Accordingly, both of these residues were independently mutated (G895A

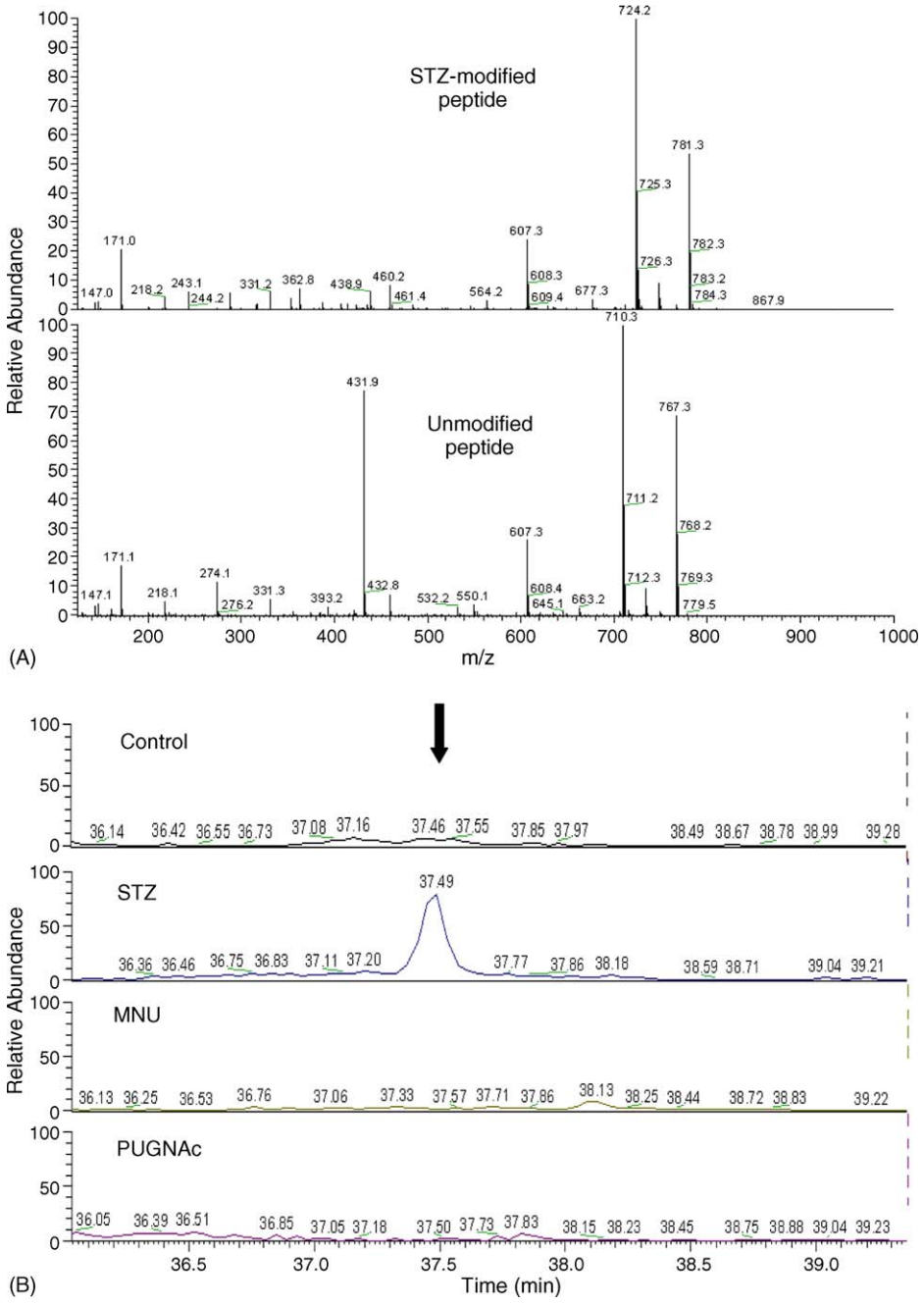

Fig. 3 – Comparison of the MS/MS spectrum of the methyl adduct (447.7420^{+2}) from STZ treatment to the MS/MS spectrum of the control peptide LGCFEIAK (894–901). (A) The MS/MS spectrum of the adduct +14 Da from STZ treatment (447.7420^{+2}) is shown in comparison to the MS/MS spectrum of the control, unmodified peptide LGCFEIAK (894–901). (B) Extracted ion chromatograms of the doubly charged ion of the methyl adduct (447–449 *m/z*) from all samples and the control.

and C896W). In the case of the second peptide, EYEIEFIYAISPGLDITFSNPK (128–149), the peptide was noted to lie in the N-terminal region of the enzyme, and to overlap a highly glutamate-rich area of the enzyme that contained an adjacent phenylalanine residue, with a serine residue lying farther downstream. Accordingly, both of these amino acids were independently mutated (F133V and S138I), Upstream tyrosine (Y129F) and glutamate residues (E130A) were also independently mutated to serve as controls.

After this site-specific mutagenesis was performed, mutants were assayed for *O*-GlcNAcase activity. Fig. 5 shows the results from this series of experiments. Somewhat surprisingly, site-specific mutagenesis of the C-terminal glycine and cysteine residues (G895A and C896W) present in the tryptic peptide LGCFEIAK (894–901) caused little or no inhibition of *O*-GlcNAcase activity (72.3 ± 1.6% of control and 129.9 ± 13.3% of control, respectively). In comparison, the phenylalanine mutant F133V identified from peptide

EYEIEFIYAISPGLDITFSNPK (128–149) was virtually devoid of O-GlcNAcase activity (3.3 ± 0.5% of control, $p < 0.05$), while the serine mutant S138I (identified from the same peptide) exhibited greatly reduced activity (15.4 ± 1.2% of control, $p < 0.05$). In contrast, the control upstream tyrosine and glutamate mutants (Y129F and E130A) retained almost full enzymatic activity (90.5 ± 9.5% of control and 97.3 ± 7.6% of control, respectively).

4. Discussion

The recognized importance of O-linked protein glycosylation continues to grow [23–27]. For proteins such as RNA polymerase II, Sp1, and probably other modified proteins, the cyclical addition and removal of O-GlcNAc is critical for biological function, with evidence suggesting that O-glycosylation may link the nutritional status of the cell to the stability

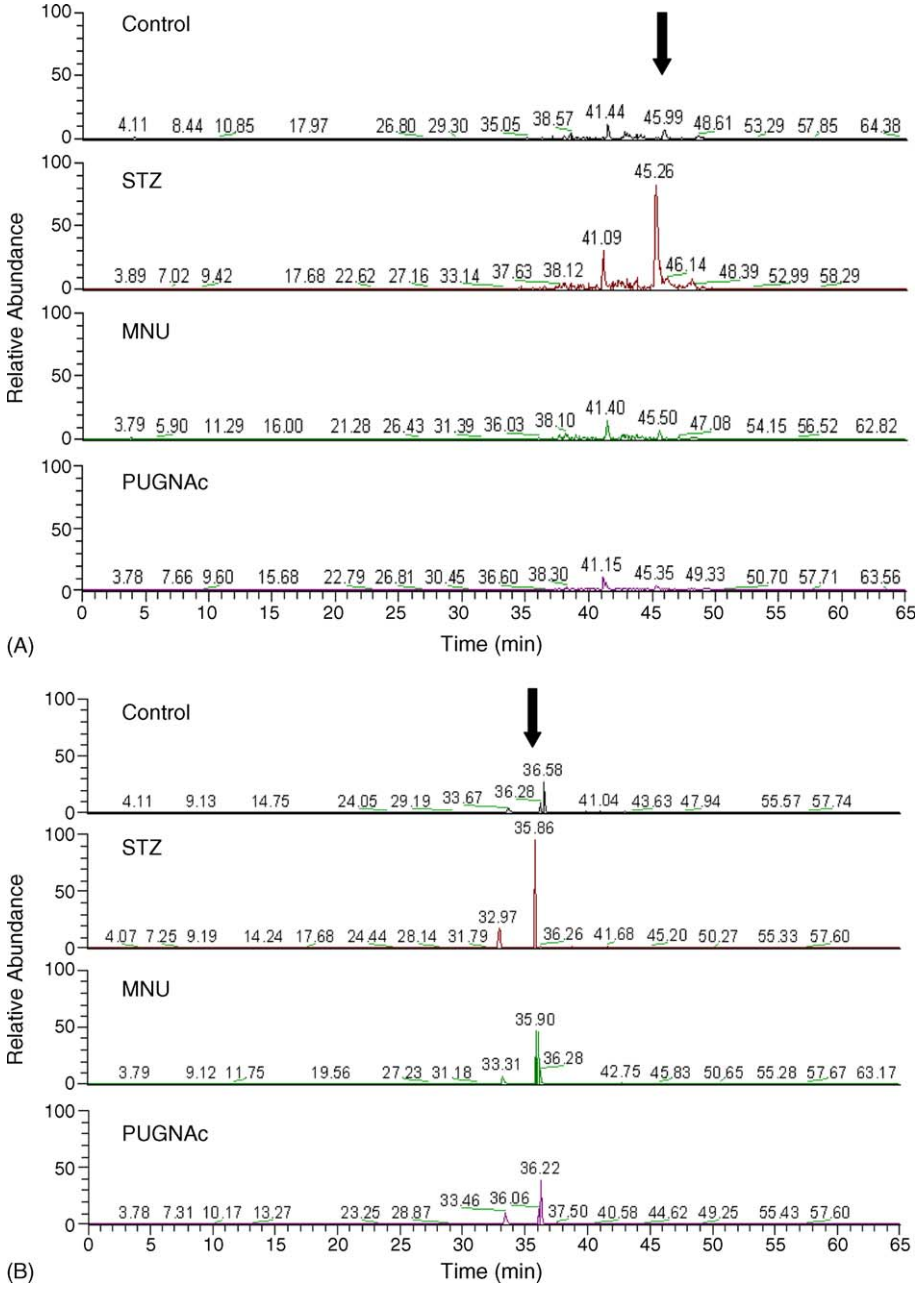

Fig. 4 – Extracted ion chromatogram of the O-GlcNAcase tryptic peptide EYEIEFIYAISPGLDITFSNPK (128–149) following treatment with STZ. (A) Recombinant O-GlcNAcase protein was incubated for 30 min in the absence or presence of STZ, MNU, or PUGNAc. Afterward, samples were subjected to tryptic digest and analyzed via LC/MS/MS to search for modified peptides. The region of the extracted ion chromatogram for the double charged ion of peptide EYEIEFIYAISPGLDITFSNPK (128–149) from each sample is shown. (B) The region of the extracted ion chromatogram for the double charged ion of the peptide EIPVESIEEVSK (258–269) is shown for each sample. (C) The region of the extracted ion chromatogram for the double charged ion of the nearby peptide KLDQVSQFGCR (157–167) is shown for each sample.

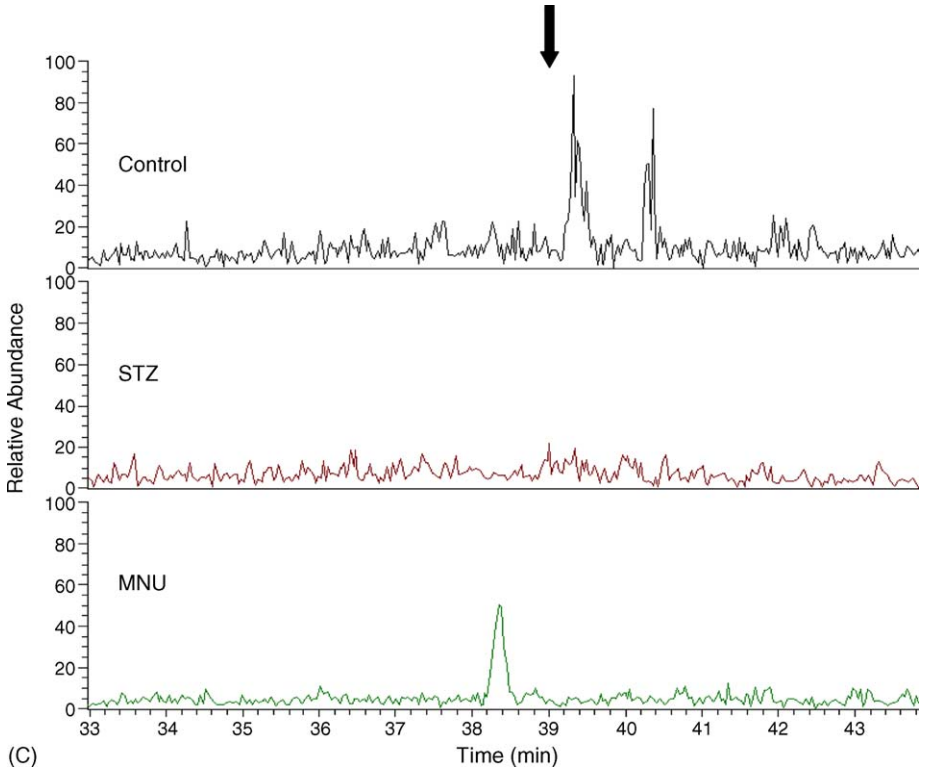

Fig. 4. (Continued).

of intracellular proteins normally degraded by the proteasome [28–35]. In addition, it has recently been shown that *O*-glycosylation regulates phosphorylation of the protein tau and that this could be an important mechanism involved in Alzheimer's disease [36]. *O*-glycosylation may also be involved in neuronal apoptosis [37].

O-linked protein glycosylation appears to be a particularly critical process in the pancreatic β-cell, which is enriched in OGT and is able to dynamically couple the extracellular glucose concentration to increased intracellular *O*-linked protein glycosylation [9–11]. Furthermore, we previously demonstrated that the diabetogenic toxin streptozotocin irreversibly inhibits islet *O*-GlcNAcase, and that when this inhibition of *O*-GlcNAcase activity is blocked, streptozotocin is not able to cause β-cell death [14]. *O*-GlcNAcase itself is turning out to be an increasingly interesting protein and has recently been demonstrated to contain both histone acetyltransferase and *O*-GlcNAcase activities [38]. Importantly, a polymorphism in the MGEA5 gene which codes for *O*-GlcNAcase is highly correlated with type 2 diabetes in Mexican-Americans and even more highly correlated with age of onset of type 2 diabetes in this same population [17].

We observed that STZ was less potent at inhibiting purified recombinant *O*-GlcNAcase compared to islet *O*-GlcNAcase. Although STZ can act as a NO donor, it is unlikely that this mechanism can account for its inhibition of *O*-GlcNAcase due to the fact that no significant inhibition of enzymatic activity was observed with MNU, the nitric oxide-containing portion of STZ. This observation confirmed that the ability of STZ to inhibit *O*-GlcNAcase is due to its unique structure and not the mere production of nitric oxide.

Based on our LC/MS/MS data obtained after treatment of recombinant *O*-GlcNAcase with STZ, we were able to identify two areas of interest. One peptide, LGCFEIAK (894–901), was previously suggested to correspond to the active site of the enzyme, with the idea that this active site was identical to that of a family of acetyltransferases [22]. Subsequently, *O*-GlcNAcase was also shown to possess acetyltransferase activity [38,39]. Site-specific mutagenesis in this region, however, did not result in any large decrease in *O*-GlcNAcase enzymatic activity. In contrast, site-specific mutagenesis in the second region identified, EYEIEFIYAISPGLDITFSNPK (128–149), resulted in almost complete loss of enzymatic activity. Importantly, these data extend the *O*-GlcNAcase N-terminal active site of the protein, and confirm that the N-terminal domain is important for *O*-GlcNAcase activity. The appearance of the EYEIEFIYAISPGLDITFSNPK (128–149) peptide after STZ treatment, coupled with the inability to detect the nearby peptide KLDQVSQFGCR (157–167) after STZ treatment is consistent with a very recent suggestion that STZ may be metabolized by the enzyme to act as a stable transition state analog [18]. Our data suggest that such an analog likely occupies the active site of the enzyme and may change its conformation in such a way that the tryptic digest pattern is altered. Further evidence in favor of this idea includes the fact that no covalent modification of either N-terminal peptide could be detected following STZ treatment.

Taken together, these data extend the region of the *O*-GlcNAcase active site N-terminally and give independent evidence to support the idea that STZ inhibits *O*-GlcNAcase through formation of a transition state analog that resides in

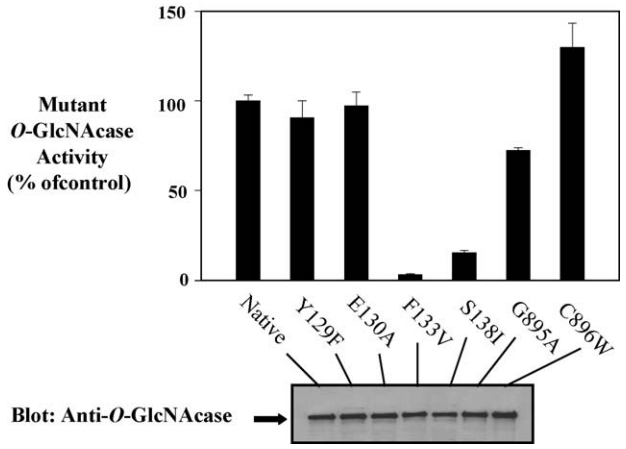

Fig. 5 – Site-specific mutagenesis of O-GlcNAcase. Site-specific mutagenesis was performed on recombinant O-GlcNAcase protein to create six mutant enzymes. The following amino acid changes were made within the O-GlcNAcase protein sequence: Y129F, E130A, F133V, S138I, G895A, and C896W. The amino acid changes Y129F, E130A, F133V, and S138I corresponded to the tryptic peptide EYEIEFIYAISPGLDITFSNPK (128–149), while the amino acid changes G895A and C896W corresponded to the tryptic peptide LGCFEIAK (894–901). All mutations were confirmed by complete DNA sequencing of the clones. Afterward, O-GlcNAcase activity of the mutant enzymes as well as the native enzyme was measured enzymatically. In each case, equal protein loading of the mutant O-GlcNAcase's was confirmed by Western blotting with anti-O-GlcNAcase antibody. Data shown are the mean ± S.E.M. from four observations from two independent experiments.

the active site of the enzyme. In doing so, STZ alters the conformation of the enzyme and thus its tryptic digest pattern.

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