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Studies on the glycosylation of L-amino acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*

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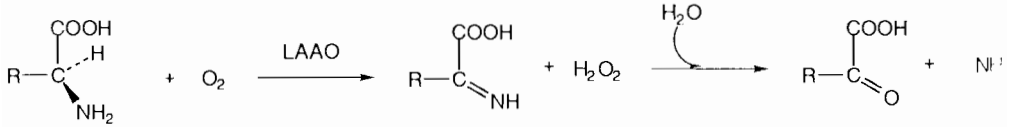
Introduction

Proteins constitute between 90 to 95% of snake venom dry weight and are responsible for many of its biological effects. The majority of these proteins are hydrolytic enzymes which unfold their tissue-destructive activity upon envenomization of the snake´s victim. The only oxidase known to occur in snake venom is L-amino acid oxidase (LAAO), an FAD-dependent enzyme catalyzing the oxidation of most L-amino acids to the corresponding α-imino acids as shown below:



L-amino acid α-imino acid α-keto acid

Although the role of LAAO in snake venom is not exactly known, the enzyme was reported to have antibacterial (*1, 2*) and apoptosis-inducing activities (*3-5*). These activities are attributed to the production of hydrogen peroxide during the course of the reaction (see Scheme above). However, other hydrogen peroxide producing flavoenzymes, such as D-amino acid oxidase (DAAO) lack these effects (*1*). A distinctive feature of snake venom LAAO is its glycosylation (*6*) which, for example, is not present in DAAO. In order to determine the importance of glycosylation for the reported effects of LAAO we set out to characterize the sugar moiety of LAAO from the Malayan pit viper *Calloselasma rhodostoma*.

Results and Discussion

Treatment of LAAO with neuraminidase produced a small electrophoretic shift of the protein band (Figure 1, panel A, compare lanes 2 and 3) indicating the presence of neuraminic acid. A much more pronounced shift was observed upon digestion of

denatured LAAO with peptide:N-glycosidase F (PNGase F) which cleaves N-linked oligosaccarides at the asparagine site (Figure 1, panel B). As judged from the mobility shift, the mass difference is approximately 4 kDa. This result is clear evidence for N-glycosylation of LAAO. Staining of the gel with the sugar specific Schiff's reagent showed that treatment with PNGaseF led to complete removal of the sugar moiety, i.e. no O-glycosylation is present in LAAO (Figure 1, panel C, lanes 3 and 4). On the other hand, when native LAAO was subjected to PNGaseF treatment, three bands were observed in a Coomassie and two with Schiff's reagent stained gel (Figure 1, compare lane 5 in panel B and C). This result indicates that the glycosylation site(s) are not readily accessible in the native protein. More importantly, this finding also suggests that LAAO may have two glycosylation sites.

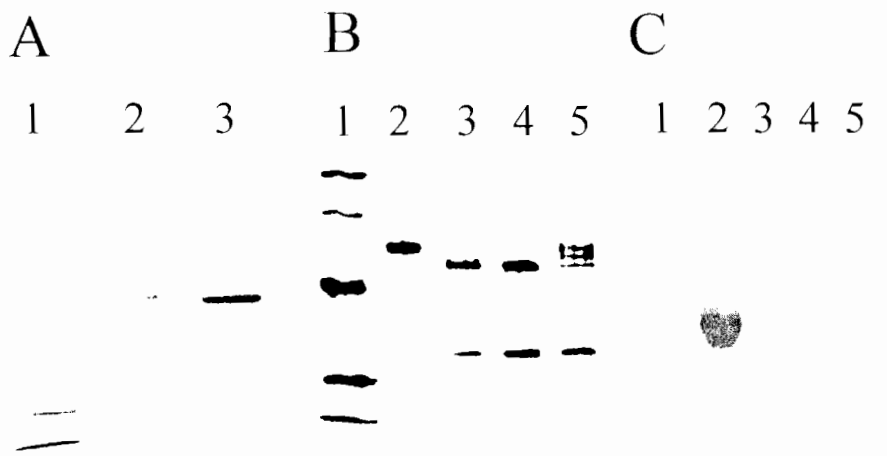


Figure 1: SDS-PAGE analysis of LAAO deglycosylation experiments
Panel A, lane 1: protein marker (from top to bottom: 97,4; 66; 45; 31 and 21.5 kDa), 2: LAAO before and 3 after neuraminidase treatment;
Panel B, lane 1: protein marker, 2: denatured LAAO before, 3 and 4: after PNGaseF treatment (1000 and 2500 units, respectively), lane 5: native LAAO after treatment with PNGaseF (2500 units).
Panel C, as above but stained with Schiff's reagent.

Further analysis of LAAO was achieved by mass spectrometry (Figure 2). The native enzyme was desalted by reversed-phase chromatography and a mass of 59968 Da was obtained by electro-spray mass spectrometry (Figure 2, panel A). This value is in good

agreement with the mass estimated from SDS-gel electrophoresis (cf. Figure 1). The flavin containing fraction from reversed-phase chromatography was analyzed by MALDI-mass spectrometry (Figure 2, panel B). Three signals were observed with monoisotopic masses of 786.75 Da, 808.73 Da and 824.74 Da, corresponding to the $FADH^+$, $FADNa^+$ and $FADK^+$ ions. In a separate experiment, LAAO was first treated treated with neuraminidase and then with PNGase F to release the desialylated N-linked oligosaccharide. The oligosaccharide was purified by reversed-phase chromatography and MALDI-mass spectrometry revealed a single peak at 2014.9 Da (Figure 2, panel C).

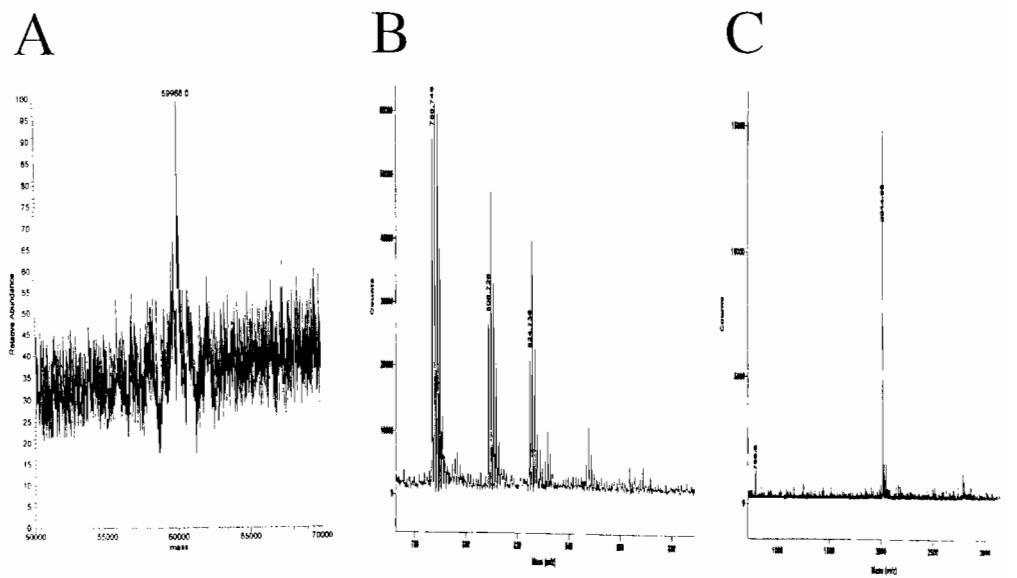


Figure 2
Panel A: Electrospray mass spectrum of native LAAO (59968 Da)
Panel B: MALDI-mass spectrum of the flavin isolated from LAAO ($FADH^+$ (found) = 786.75 Da, $FADH^+$ (calculated) = 786.56 Da).
Panel C:MALDI-mass spectrum of the isolated oligosaccharide from LAAO (2014.9 Da)

This mass fits to an oligosaccharide structure shown in Figure 3 consisting of one fucose, two galactose, three mannose and five *N*-acetylglucosamine residues (*7*). This sugar composition is similar to the one estimated for LAAO from *Crotalus adamanteus* (*8*).

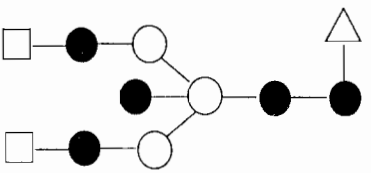


Figure 3
Putative structure of the oligosaccharide moiety of LAAO from *Calloselasma rhodostoma*. The symbols represent the following sugars: open triangle = fucose, full circles = *N*-acetylglucosamine, open circles = mannose and open squares = galactose.

Since the molecular mass of LAAO, calculated from the amino acid sequence of the protein is 56178 Da, the sugar moiety accounts for ca. 3800 Da in the native protein.This mass difference indicates the presence of one oligosaccharide structure (see Fig. 2, panel C) with several neuraminic acid units attached to the core sugar units. A possible site for attachment of the oligosaccharide is at asparagine 361, which is conserved in all three sequenced ophidian LAAOs. This study has paved the way to utilize exo- and endoglycosidases as tools to further investigate the importance of the oligosaccharide moiety for the physiological effects as well as the physical and biochemical properties of LAAO.

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