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Effect of tunicamycin on the activity and immunoreactivity of ascorbate oxidase (*Cucurbita pepo medullosa*) expressed in cultured green zucchini cells

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Ascorbate oxidase activity and immunoreactivity were evaluated in crude tissue extracts obtained from callus cell cultures induced by green zucchini sarcocarp and grown in the presence of tunicamycin, a powerful N-glycosylation inhibitor. Tunicamycin at 2 or 4 μg ml^{-1} blocked cell growth within a couple of weeks, although a sustained cell viability was observed in the same period. A significant inhibition of total protein synthesis was observed at 10 and 15 days of culture time, with a decrease of 30% and 43% respectively when cells were grown in the presence of 2 μg ml^{-1} tunicamycin, and of 48% and 57% respectively when the tunicamycin concentration was 4 μg ml^{-1}. After the same culture times ascorbate oxidase specific activity assayed in crude tissue extracts showed increases of about 1.9-fold and 3.5-fold (10 days) and 1.7-fold and 3.1-fold (15 days) at 2 and 4 μg ml^{-1} tunicamycin, respectively. Ascorbate oxidase mRNA levels, however, did not appreciably differ between control and treated samples, measured at the same growing times. Lectin-blot, based on the use of concanavalin A, indicated a marked decrease of glycosylated proteins in tunicamycin-treated cultures. As judged by immunoblot, anti-native ascorbate oxidase antibodies scarcely recognized the enzyme expressed in tunicamycin-treated cells; on the contrary, anti-deglycosylated ascorbate oxidase antibodies were more reactive to the enzyme expressed in tunicamycin-treated cultures.

***Keywords*: ascorbate oxidase, tunicamycin, cell culture**

***Abbreviations*: AO, ascorbate oxidase; Con A, concanavalin A; G-6-PDH, glucose-6-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TM, tunicamycin; TBS, Tris-buffered saline: 500 mM NaCl, 20 mM Tris-HCl, pH 7.4**

Introduction

Ascorbate oxidase (AO; L-ascorbate : oxygen oxidoreductase, EC 1.10.3.3) is a copper-containing glycoenzyme commonly found in higher plants, in the cell wall and the cytoplasm of both vegetative and reproductive organs [1]. AO catalyzes the *in vitro* oxidation of L-ascorbate and related compounds by molecular oxygen which, in turn, is reduced to water. Although the biological function of AO is still under debate, its role in a redox system involving ascorbic acid, in growth promotion by wall-loosening and in defence against diseases has been postulated [2, 3].

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The enzyme from green zucchini squash (*Cucurbita pepo medullosa*) is a homodimer of 70-kDa subunits, each containing four copper atoms of three different spectroscopic forms. The X-ray structure of AO at 1.9 Å resolution has been determined [4], as well as the primary structure of the N-linked carbohydrate side chain (N-glycan) [5]. An exhaustively deglycosylated active AO has also recently been obtained by peptide N-glycosidase F treatment [6].

Cell cultures developed from sarcocarp explant of pumpkin [7, 8], cucumber [9], and green zucchini [10] have been used often as models for studying the expression of AO. In cell suspension cultures, 50% of the enzyme is released into the medium [8, 10]. Furthermore, it has been shown that for the secretory pathway in cucumber [11] and pumpkin [12], AO mRNA codes for a signal sequence. As a follow-up

study of our previous findings [6, 13] related to the activity of AO after its *in vitro* deglycosylation, we now report the effect of tunicamycin (TM), which specifically inhibits the N-glycosylation step [14], on the enzymatic function and immunoreactivity of AO expressed in cultured cells derived from sarcoplasm explant of green zucchini. In our experimental conditions TM-treated cells showed fewer total proteins, but greater specific activity for AO. Furthermore, anti-native AO polyclonal antibodies formed weak bonds with the AO expressed in TM-treated samples; conversely, anti-deglycosylated AO polyclonal antibodies were more prone to react with AO expressed in TM-treated cultures than that expressed in control samples.

Materials and methods

Callus induction and cell culture

Callus was induced by sarcocarp explant from green zucchini (*Cucurbita pepo medullosa* var. Milano), as previously described [10]. Discs were prepared under aseptic conditions and cultured at 25 °C in the dark on Murashige and Skoog's agar medium (Mascia Brunelli, Monza, Italy) supplemented with sucrose (30 mg ml^{-1}), 2,4-dichlorophenoxyacetic acid (1 μg ml^{-1}), and kinetin (6-furfurylaminopurine; 0.1 μg ml^{-1}) (Fluka, Buchs, Switzerland).

Cell suspension cultures were initiated after 3–4 months of cultivation by transferring 1–2 g of callus to flasks containing 50 ml of liquid medium. Subcultures were incubated in darkness at 25 °C on an orbital shaker at 120 rpm, in the absence or presence of TM (0.5–8 μg ml^{-1}). Cell growth was evaluated by measuring fresh weight and total cell protein. The viability was quantified with 2,3,5-triphenyltetrazolium chloride and fluorescein diacetate using the procedure reported by Duncan and Widholm [15]; samples killed by ethanol were used as controls. Cells (1 g fresh weight per ml buffer) were usually harvested at 0, 5, 10, and 15 days, then resuspended in 50 mM Tris-HCl (pH 7.0) and homogenized. The homogenates were centrifuged at 10 000 rpm, dialyzed against 10 mM K-phosphate buffer (pH 6.0)/1 mM EDTA and used as crude extracts. The cell-free culture media were extensively dialyzed against phosphate buffer and the proteins were concentrated by Amicon Centricon 10.

Ascorbate oxidase purification and assay

AO was purified from green zucchini peelings according to Avigliano *et al.* [16]. AO enzymatic activity was measured at 25 °C by recording the decrease in absorbance at 265 nm due to ascorbate oxidation ($E_{265} = 15\ mM^{-1}\ cm^{-1}$). The reaction was primed by adding ascorbate (0.14 mM final concentration) to 1 ml of 0.2 M K-phosphate buffer (pH 6.0)/1 mM EDTA, containing 50–100 μl of sample. Total protein concentration was determined by Bio-Rad protein assay based on a dye binding procedure [17] using either bovine serum albumin or purified AO as standard. The concentration of purified AO was also determined by absorbance at 280 nm ($E_{280} = 240\ mM^{-1}\ cm^{-1}$).

Glucose-6-phosphate dehydrogenase assay

To ascertain cell damage, the glucose-6-phosphate dehydrogenase (G-6-PDH) assay was set up following the protocol of a Sigma Diagnostic kit (Sigma Chemical Co, St. Louis, MO, USA) and based on the reduction of $NADP^+$ to NADPH. Briefly, in the presence of phenazine methasulfate at pH 8.5, NADPH reduces the blue dye, dichlorophenolindophenol, to a colorless form. The rate at which the color disappears (at 37 °C) is proportional to the enzyme content. Yeast G-6-PDH (Sigma) and human red cell hemolysate were used as standards.

Antibody production

AO (1.5 mg in 20 mM phosphate buffer, pH 7.2), native or chemically deglycosylated according to Edge *et al.* [18], was emulsified with Freund's complete adjuvant and injected subcutaneously into New Zealand rabbits. Four boosters in Freund's incomplete adjuvant followed and rabbits were bled one week after the last injection. The immunoglobulin fraction was recovered from serum after three precipitations with ammonium sulfate at 33% final (w/v) [19].

Dot-blot

Total RNA (20 μg) purified from zucchini calluses as described by Logeman *et al.* [20], was spotted on a Hybond-N membrane (Amersham, Buckinghamshire, UK) and treated as recommended by the manufacturer. The probe was a 0.8-kb fragment of the zucchini AO gene [21] labeled with digoxigenin using the DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Under the conditions already reported [22], our probe hybridized only with AO mRNA. The hybridized probe was detected using the DIG Nucleic Acid Detection kit (Boehringer Mannheim) with either the alkaline phosphate substrate supplied in the kit or 3-(2′-spiro-adamantane)-4-methoxy-4-(3′-phosphoryl-oxy)-phenyl-1.2-dioxetane (Boehringer Mannheim).

Gel electrophoresis and blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [23], using 10% polyacrylamide gels. For protein detection, SDS-PAGE gels were silver stained following the protocol reported by Merril *et al.* [24]. Alternatively, proteins were transferred onto nitrocellulose sheets as already described [25]. After this transfer, the nitrocellulose sheets were fixed with acetic acid/isopropanol/H_2O (10/25/65, v/v/v) and rinsed several times with distilled H_2O and then with Tris-buffered saline (TBS; 500 mM NaCl, 20 mM Tris-HCl, pH 7.4). The nitrocellulose sheets were incubated for 1 h at

room temperature in blocking solution (3% w/v gelatin in TBS) and either stored at 4 °C overnight or immediately processed.

Immunoblot analyses

Immunoblots were performed according to a standard procedure [25] using anti-native or anti-deglycosylated AO polyclonal antibodies as a first antibody, then goat anti-rabbit antibodies as a second antibody and rabbit peroxidase anti-peroxidase complex as a third antibody. The staining solution was made as described by Young [26]. Briefly, 10 mg 3,3′-diaminobenzidine tetrahydrochloride were dissolved in 5 ml methanol and 30 mg 4-chloro-1-naphthol were dissolved in additional 5 ml of methanol. Both solutions were then mixed with 40 ml phosphate buffered saline (500 mM NaCl, 20 mM K-phosphate, pH 7.4) together with 10 µl 30% H_2O_2 just prior to use.

Glycoprotein visualization

Glycoprotein visualization was conducted following the protocol already reported [6], based on the use of concanavalin A (Con A), a lectin which recognizes both branched mannose and *β-N*-acetylglucosamine residues, and horseradish peroxidase. Staining was as for immunoblot analyses.

Results

Cell growth and viability

When sarcocarp explant of green zucchini was cultured, the fresh weight of the suspension culture doubled in 10 days in control samples. The presence of TM at 2 or 4 µg ml^{-1} blocked the growth but ensured cell viability for up to 10–15 days. However, treatment for longer periods (30 days for cultures treated with 2 µg ml^{-1} TM, and 22 days for those treated with 4 µg ml^{-1}) caused complete cell death; lower TM concentrations (0.5–1 µg ml^{-1}) affected neither cell growth and viability nor the glycosylation process, as revealed by blot analyses; conversely, 8 µg ml^{-1} TM killed the cells within one week (data not shown). We therefore used TM at 2 and 4 µg ml^{-1} and a maximum of 15 days of culturing. To monitor cell damage, we measured the activity of G-6-PDH, a cytosolic enzyme which is quickly released from damaged cells, in the culture media of control or treated samples, but found no such activity.

AO expression in TM treated cells

Total cell protein content was evaluated at 0, 5, 10, and 15 days of culturing. The ratio of total cell proteins to fresh cell weight did not change appreciably in the controls, but started to decrease both after 5 days for cultures treated with 4 µg ml^{-1} TM and after 10 days for cultures treated with 2 µg ml^{-1} TM (Figure 1a). In particular, decreases of about 30% and 43% were observed after 10 and 15 days' culturing, respectively, with 2 µg ml^{-1} TM; at the same culturing intervals, decreases of 48% and 57% were detected in the presence of 4 µg ml^{-1} TM. Furthermore, the content of proteins 'secreted' into the media was also measured at the same intervals. At 5 and 10 days of culturing, both the amount of 'secreted' proteins (60 µg per g fresh weight) and AO specific activity (30 nkat per mg of secreted proteins) were essentially the same in the media of both TM-treated and untreated samples; at 15 days of culturing a smaller amount of proteins (50 µg per g fresh weight) and a lower AO specific activity (20 nkat per mg of secreted proteins) were detected in all samples (data not shown).

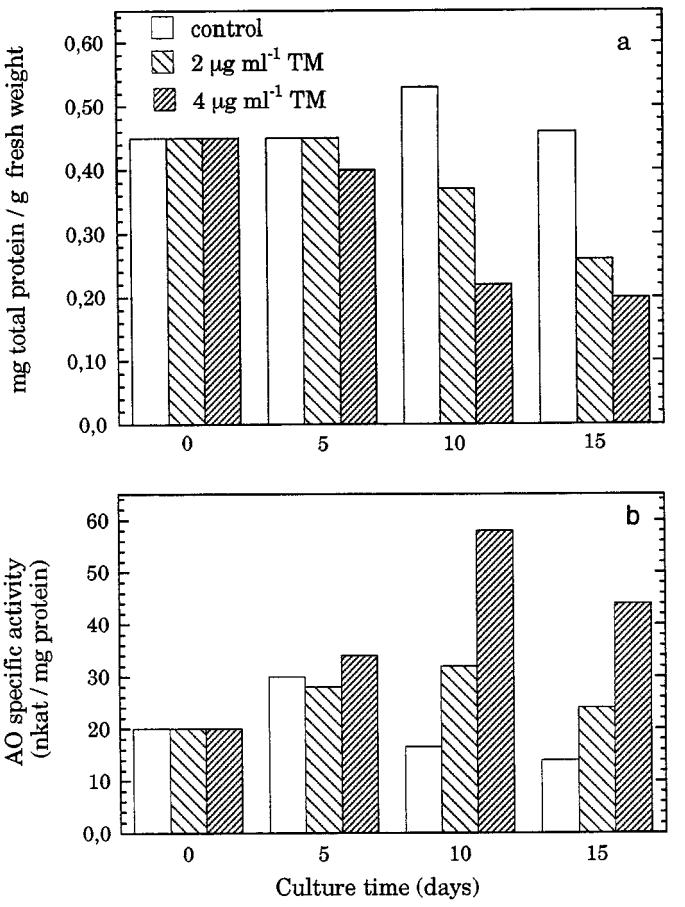

Figure 1. *Protein content (panel a) and AO specific activity (panel b) determined in cultured green zucchini cells.* Cells were harvested at the indicated times. Results are means of three separate determinations with standard deviations $< 2\%$.

In control cells, AO specific activity reached its maximum within 5 days after transfer to fresh standard liquid medium and then declined (Figure 1b). Different results were observed in TM treated samples. In fact, AO activity was maximal after 10 days of culturing and was 1.9-fold higher for cells treated with 2 µg ml^{-1} TM and 3.5-fold higher for cells treated with 4 µg ml^{-1} TM (Figure 1b). Determination of K_m and V_{max} for AO revealed that control samples

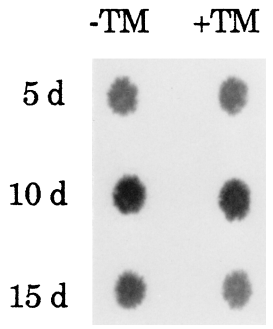

Figure 2. *Dot-blot analysis of AO mRNA.* AO mRNA was detected from zucchini calluses grown for 5 (5d), 10 (10d) or 15 days (15d) in the absence (−TM) or presence (+TM) of TM (4 μg ml^{-1}).

consistently showed larger values of K_m and lower values of V_{max} than samples treated with TM. In particular, after 10 days of culturing, K_m was 1.47×10^{-4} M for control samples, and 1.32×10^{-4} M for treated samples; V_{max} (expressed as nkat/mg of protein) was 14.6, 28.8, and 52.5 for control, 2 μg ml^{-1} TM-treated cells, and 4 μg ml^{-1} TM-treated cells, respectively.

Dot blot analyses (Figure 2) indicated that although the amount of AO mRNA (per g of fresh weight), in the absence or in the presence of TM, varied over time, mRNA levels measured at the same incubation times did not differ significantly, although a very slight decrease was observed in 4 μg ml^{-1} TM-treated sample at 15 days; identical results were obtained with 2 μg ml^{-1} TM-treated cells (data not shown). The same trend was observed for total RNA (data not shown). SDS-PAGE and blot analyses were performed on samples at 10 days of culturing. Although SDS-PAGE showed similar protein patterns for both control homogenates and those derived from TM-treated cells (Figure 3a), lectin-blot, based on the use of Con A/peroxidase system, revealed that the content of glycosylated proteins significantly decreased in TM-treated samples (Figure 3b). Immunoblot using anti-native AO polyclonal antibodies revealed that cultures treated with TM showed a very faint band corresponding to AO mobility, whereas the control sample showed a strong positive band (Figure 3c). Immunoblot performed using anti-deglycosylated AO polyclonal antibodies revealed the strongest positivity for AO expressed in cultures treated with 4 μg ml^{-1} TM (Figure 3d), and in cultures treated with 2 μg ml^{-1} TM (data not shown).

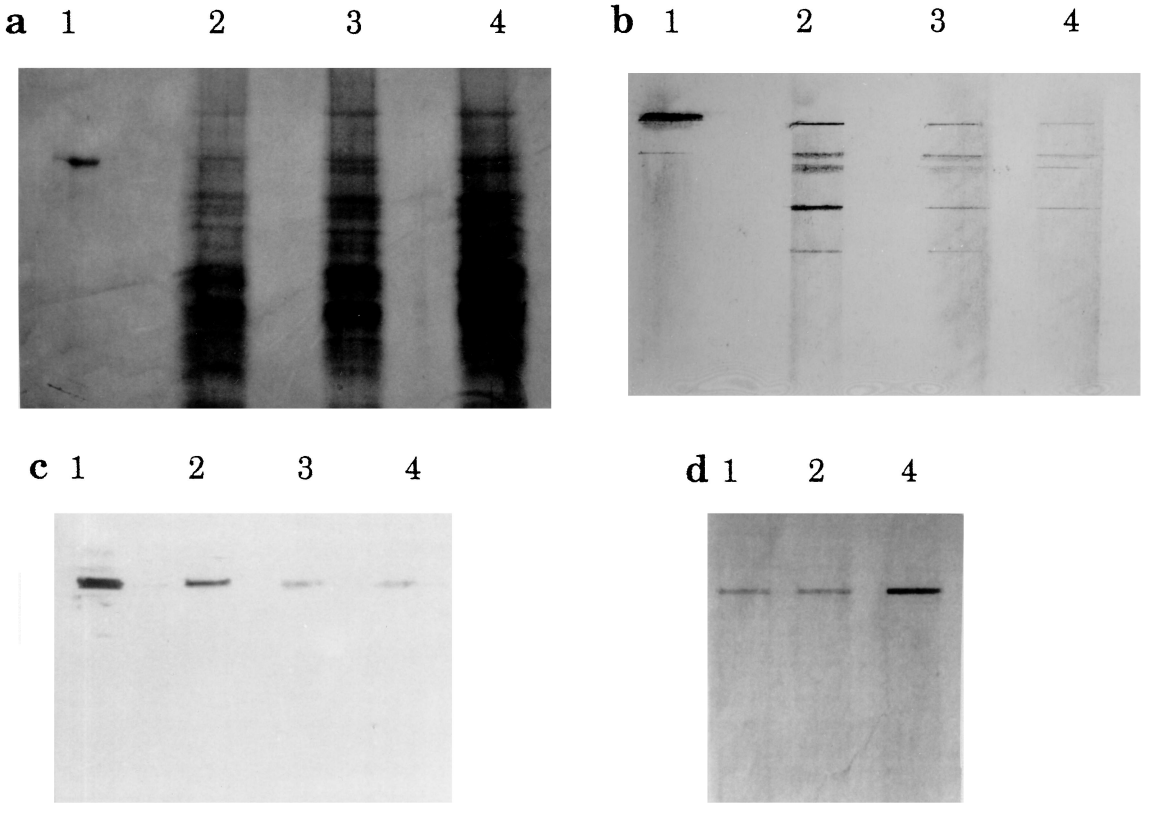

Figure 3. *SDS-PAGE, Con A blot and immunoblots of crude extracts after 10 days of cell growth.* Panel a, silver stain; panel b, Con A blot; panel c, immunoblot using anti-native AO polyclonal antibodies; panel d, immunoblot using anti-deglycosylated AO polyclonal antibodies. Lane 1, AO purified from green zucchini squash; lane 2, crude extract of control cells; lane 3, crude extract of cells treated with 2 μg ml^{-1} TM; lane 4, crude extract of cells treated with 4 μg ml^{-1} TM. AO = 1.5 μg; protein extract = 30 μg.

Discussion

It is well known that sugar chains may contribute to the structural and physiological properties of glycoproteins [27]. Proposed roles for sugar moieties include participation in protein folding, stability, transport to the cell surface, and secretion. Thus, as a follow-up to our previous studies in which a fully active *in vitro* exhaustively deglycosylated AO was produced under mild-denaturing conditions [6], and a partly deglycosylated but still active AO was obtained by *in vitro* sequential exoglycosidases treatment [13], we attempted to produce an *in vivo* form of non-glycosylated AO, verifying its activity and immunoreactivity. Cell cultures of green zucchini were harvested in the presence of TM to inhibit the N-glycosylation process *in vivo* [14]. As already reported [28], the *in vivo* treatment with TM also results in the inhibition of protein synthesis. Thus, the cell culturing conditions were set up at a preliminary phase. TM at 2 and 4 $\mu g\ ml^{-1}$ and culture time of 10 days were chosen, since these conditions maximized the inhibitory effect on N-glycosylation and minimized that on the protein synthesis. When homogenates were run on SDS-PAGE and silver stained, small differences were observed between the protein patterns derived from control and TM-treated samples (Figure 3a); these differences, however, were uninterpretable. After 5 days of culturing, only the cells treated with 4 $\mu g\ ml^{-1}$ TM began to show a decrease in protein content (Figure 1a). For cells treated with 2 $\mu g\ ml^{-1}$ TM a (similar) decrease was apparent only after 10 days of culturing (Figure 1a). These decreases were not parallelled by a corresponding decrease in AO mRNA levels, as judged by dot-blot analysis (Figure 2). Dot-blot analysis was used because under our experimental conditions [22], only one band is produced in Northern blots. The use of ^{32}P to label the probe (necessary to achieve a useful sensitivity) and the presence of poly-A tails of various length do not permit the detection of small differences in the size of mRNA transcripts. However, no cell death was detected by a viability test and the secretion processes did not appear to be drastically affected since the production of extracellular proteins was similar in TM-treated and untreated samples (data not shown).

Moreover, as reported in our previous study [6], the decrease in molecular weight upon de-N-glycosylation of AO was too small to be detected by SDS-PAGE (Figure 3). However, the lectin-blot based on the use of Con A showed a decrease in total N-glycosylation as the TM concentration increased (Figure 3b). In particular, samples treated with 4 $\mu g\ ml^{-1}$ TM (Figure 3b, lane 4) gave the lowest positive staining glycoprotein pattern, indicating extensive N-glycosylation inhibition. When the blot was developed using anti-native AO polyclonal antibodies, a single band with a mobility close to that of reference AO appeared in both the control homogenate (Figure 3c, lane 2) and the TM-treated samples (Figure 3c, lane 3 and lane 4) although in the latter case the band was weak (Figure 3c, lane 3 and 4) denoting poor antibody recognition of scarcely glycosylated AO. Thus, as already reported [6], we can hypothesize that the anti-native AO antibodies were mostly directed against the N-glycans present in AO. Anti-deglycosylated AO antibodies better recognized AO expressed in the presence of TM at 4 $\mu g\ ml^{-1}$ (Figure 3d, lane 4), indicating a stronger reactivity toward amino acidic antigenic determinants uncovered in the absence of AO N-glycans; in fact, both native AO (used as control) and AO present in the crude extract produced faint bands (Figure 3d, lane 1 and lane 2).

Our observations confirm that a form of poorly glycosylated AO was produced when cells where treated with TM. Furthermore, as already reported for glycoproteins of cultured sycamore cells [29], N-linked oligosaccharide moiety appears unnecessary for AO secretion. Since activity persisted in the poorly glycosylated enzyme, it may be argued that glycosylation in AO is not a prerequisite for the acquisition of enzyme structure and catalytic function.

In fact, AO specific activity at 10–15 days of culturing was about 2–3-fold higher in TM treated cells than in control cells (Figure 1b). The increased affinity for the substrate may derive from a better exposure of the catalytic site residues upon de-N-glycosylation, as previously stated [13], or from a different folding of the protein.

In conclusion, although the inhibitory effect of TM on N-glycosylation is well documented [14], our study shows that it is possible to obtain an *in vivo* scarcely glycosylated AO which is more active than the native enzyme. This is in line with our previous findings [13] in which AO partly deglycosylated by sequential *in vitro* treatment with exoglycosidases in non-denaturing conditions, is more active than the native form. Moreover, as already reported for the *in vitro* exhaustively deglycosylated AO [6], the *in vivo* scarcely glycosylated AO is poorly recognized by anti-native AO antibodies, although it is better recognized by anti-deglycosylated AO antibodies than is the native enzyme. Thus, we may conclude that N-glycans in AO play an important role as antigenic determinants.

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