

Thermostable β -cyclodextrin conjugates of two similar plant amine oxidases and their properties

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Syntheses of conjugates of garden pea (*Pisum sativum*) and grass pea (*Lathyrus sativus*) amine oxidases (PSAO and GPAO respectively) with BCD (β -cyclodextrin), performed to improve the thermostability of the enzymes, are described in the present study. Periodate-oxidized BCD reacted with the enzyme proteins via free primary amino groups in a buffered solution containing cyanoborohydride as a reductant. Although the specific activities of PSAO and GPAO partially decreased after modification, K_m values determined for the best diamine substrates remained almost unchanged. Both the BCD conjugates could be incubated at 65 °C for 30 min without considerable inactivation, and the residual activity remained detectable even after incubation at 75 °C. The conjugates contained approx. 30% of neutral sugars. Molecular masses of BCD–PSAO and BCD–GPAO (180 kDa), as estimated by gel-permeation chromatography, were higher compared with the value of 145 kDa for the native enzymes. This was in good correlation with the number of modified lysine residues determined by a spectrophotometric method. Peptide mass fingerprints of tryptic digests of BCD–PSAO and BCD–GPAO were less specific than those of the native enzymes when compared with the database sequence of PSAO. As a consequence of the modification, many unidentified peaks were observed in the digests of the studied conjugates that were not seen in the digests of native PSAO and GPAO. Only some of these peaks overlapped between BCD–PSAO and BCD–GPAO. The BCD conjugates described in the present study represent suitable candidates for biotechnological applications, e.g. in analyses using biosensors, which might benefit from increased storage stability and amine oxidation at high temperatures.

Introduction

Copper-containing amine oxidases (CAOs; EC 1.4.3.6) play a crucial role in the metabolism of primary amines. These

enzymes are widely distributed in nature [1]. In microorganisms, CAOs play a nutritional role. In mammals and plants, CAOs appear to be tissue-specific and are implicated in wound healing, detoxification, cell growth, signalling and apoptosis [1]. The oxidative deamination of amine substrates catalysed by CAOs yields the corresponding aldehydes with the concomitant production of H_2O_2 and ammonia [2].

The reaction proceeds through a transamination mechanism and is mediated by an active-site cofactor, TPQ (topaquinone). The cofactor is derived from the post-translational self-processing of a specific tyrosine residue that requires both active-site copper and molecular oxygen [2]. A key step in the oxidative deamination is the conversion of the initial substrate Schiff base (quinoimine) into a product Schiff base (quinoaldimine), facilitated by $C\alpha$ -proton abstraction through a conserved aspartate residue acting as a general base at the active site [3]. This step is followed by the hydrolytic release of the aldehyde product, and the reduced cofactor is finally reoxidized by molecular oxygen, accompanied by the release of H_2O_2 and NH_4^+ . The reduced TPQ occurs in two molecular forms. The first form is an aminoresorcinol derivative co-existing with Cu(II), which is in equilibrium with the second form, Cu(I)-semiquinolamine radical [3]. The role of active-site copper in the reoxidation step has not been sufficiently elucidated so far. Recently published evidence, however, suggests that it might be involved in electron transfer from substrate-reduced TPQ to oxygen that is bound at a site separate from copper [2].

CAOs are composed of two identical subunits [1]. The sequence of the PSAO [garden pea (*Pisum sativum*) amine

Key words: amine oxidase, cyclodextrin, matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF), mass spectrometry (MS), protein modification, thermostability.

Abbreviations used: BCD, β -cyclodextrin; CAO, copper-containing amine oxidase; DABY, but-2-yne-1,4-diamine; GPAO, grass pea (*Lathyrus sativus*) amine oxidase; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; MSDB, Mass Spectrometry protein sequence DataBase; PSAO, garden pea (*Pisum sativum*) amine oxidase; TNBS, 2,4,6-trinitrobenzenesulphonic acid; TPQ, topaquinone.

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oxidase] subunit has been published [4]. Only a partial sequence, which is derived from directly sequenced N-terminus and internal peptides, is known for the GPAO [grass pea (*Lathyrus sativus*) amine oxidase] subunit [5]. The crystal structure of PSAO was published in 1996 [6]. PSAO and GPAO show thermostability to some extent. Hence, a step of controlled heat denaturation at 60 °C is often involved in their purification [7,8]. Plant CAOs have been exploited as the biorecognition parts of biosensors for the detection of various amine compounds [9,10]. The enzymes could also be utilized in new approaches for controlling the cellular physiopathology [11].

The PSAO subunit contains a number of lysine residues [12], which represent potential targets for chemical modification. To enhance PSAO and GPAO thermostability further, the enzymes were allowed to react with BCD (β -cyclodextrin), which had been activated by periodate oxidation. The BCD conjugates obtained were purified by gel chromatography and characterized by bioanalytical methods. We found that significant changes in the structure were accompanied by only minor changes in the catalytic activity. The thermostability and storage stability of these conjugates were higher compared with those of the native enzymes.

Materials and methods

Enzymes

PSAO and GPAO were isolated by a previously published method [8]. The enzymes were additionally repurified by ion-exchange chromatography on a Mono S HR 5/5 column (Amersham Biosciences, Uppsala, Sweden) connected to a BioLogic Duo Flow liquid chromatograph (Bio-Rad, Hercules, CA, U.S.A.). The following buffers were used: buffer A, 20 mM potassium phosphate buffer (pH 5.8); buffer B, 20 mM potassium phosphate buffer (pH 5.8) containing 2 M NaCl. Enzyme samples were loaded on to the column equilibrated with buffer A. The retained proteins were then eluted at a flow rate of 1 ml · min⁻¹ by a linear gradient from 0 to 100% of the buffer B in the time interval 1.5–17.0 min. Fractions showing activity were pooled, dialysed against 5 mM potassium phosphate buffer (pH 7.0) and concentrated in an ultrafiltration cell (Amicon, Danvers, CA, U.S.A.), equipped with an XM10 filter. The purified enzymes were stored frozen at –80 °C. Horseradish (*Armoracia rusticana*) peroxidase was obtained from Fluka (Buchs, Switzerland). Double-crystallized bovine (*Bos taurus*) pancreatic trypsin (catalogue no. 101179) was purchased from ICN Biomedicals (Aurora, OH, U.S.A.).

Chemicals

BCD was purchased from Fluka. Sodium cyanoborohydride, sodium periodate and 5% (w/v) TNBS (2,4,6-trinitroben-

zenesulphonic acid) solution were from Sigma–Aldrich Chemie (Steinheim, Germany). Barium chloride hydrate, phenol and sulphuric acid were obtained from Lachema (Neratovice, Czech Republic) and Tris base was from ICN Biomedicals. 1-Cyano-4-hydroxycinnamic acid [a matrix for preparing MALDI (matrix-assisted laser-desorption ionization) probes] was purchased from Bruker Daltonik (Bremen, Germany). DABY (but-2-yne-1,4-diamine) dihydrochloride was synthesized as described in [13]. All other chemicals and buffer components were of analytical-grade purity.

Activity and protein assay

Amine oxidase activity was determined using a coupled reaction with horseradish peroxidase and guaiacol [14], where putrescine served as a substrate. The reaction mixture in a spectrophotometric cuvette (1.7 ml) contained 0.1 M potassium phosphate buffer (pH 7.0), 0.5 mM guaiacol, horseradish peroxidase (10 nkat) and an appropriate amount of diluted enzyme solution. The reaction was carried out at 30 °C. After the addition of 50 μ l of 87.5 mM putrescine, the reaction was monitored by measuring the absorbance at 436 nm [ϵ (molar absorption coefficient) 4500 M⁻¹ · cm⁻¹]. For V and K_m determinations, the final amine concentrations in the cuvette varied between 0.1 and 2.5 mM. Thermostabilities of PSAO, GPAO and their BCD conjugates were evaluated by measuring the activities after incubation in 20 mM potassium phosphate buffer (pH 7.0) at temperatures from 45 °C up to 75 °C (in 5 °C steps) for 30 min. The residual activity was measured with putrescine after rapid cooling of sample aliquots in a water/ice bath. The protein content was determined by a colorimetric method using bicinchoninic acid (BCA) [15].

Glycosylation of amine oxidases

Glycosylation of PSAO or GPAO was performed by the method of Morand and Biellmann [16]. BCD–PSAO and BCD–GPAO were prepared in two steps. First, BCD was oxidized in a water solution by a 10% molar excess of sodium periodate (calculated to one constituent monosaccharide subunit) at 23 °C. After 24 h, the polyaldehyde formed was separated by the addition of a calculated amount of barium chloride. The excess reagent and iodate as reaction products were precipitated as barium salts (white precipitate) and removed by filtration [16]. If not used immediately, the polyaldehyde solution was stored frozen at –50 °C. PSAO or GPAO (20 mg) was dissolved in 10 ml of 0.25 M potassium phosphate buffer (pH 7.8). Then, 12 ml of BCD/polyaldehyde solution (10 mM) and 10 ml of a fresh, 160 mM solution of sodium cyanoborohydride were added, and the mixture was stirred for 30 min and then kept at 23 °C for 24 h. Finally, the mixture was dialysed against 20 mM potassium phosphate buffer (pH 7.0).

Gel-permeation chromatography

The crude BCD conjugates were concentrated by ultrafiltration and purified by gel chromatography on a Superdex 200 HR 10/30 column (Amersham Biosciences) connected to BioLogic Duo-Flow liquid chromatograph. The column was equilibrated and run with 50 mM potassium phosphate buffer (pH 7.0), containing 0.15 M NaCl at a flow rate of 0.7 ml · min⁻¹. During separation, the A_{280} of the eluate was recorded. Fractions showing activity were pooled, dialysed against 5 mM potassium phosphate buffer (pH 7.0) and concentrated by ultrafiltration. The modified enzyme was stored frozen at -80 °C. Molecular masses of PSAO, GPAO and their BCD conjugates were evaluated after calibration of the Superdex column with protein standards (purchased from Bio-Rad) for gel chromatography (1.35, 17, 44, 158 and 670 kDa; catalogue no. 151–1901).

Electrophoretic separation of amine oxidases

PSAO, GPAO and their BCD conjugates were separated by SDS/PAGE as described by Laemmli [17] using 10% running and 4% stacking gels (thickness, 1 mm). After fixation, proteins in the gels were stained with Coomassie Brilliant Blue R250 (Serva Electrophoresis GmbH, Heidelberg, Germany). Protein standards for SDS/PAGE (14.2, 20, 24, 29, 36, 45 and 67 kDa, catalogue no. 69810; 29, 45, 66, 97.4, 116 and 205 kDa, catalogue no. 69811) were provided by Fluka.

Determination of neutral sugar content

The phenol/sulphuric acid method [18] was used for the determination of neutral sugar content in PSAO, GPAO and their BCD conjugates. Phenol (1 ml of a 5% solution in water) was added to an enzyme sample (0.1 ml of a solution containing 10–20 mg of protein · ml⁻¹; freshly prepared from a weighted lyophilisate) in a glass test tube and the solution was vortex-mixed. This was followed by careful addition of 5 ml of sulphuric acid and by repeated mixing. The resulting mixture was incubated at 23 °C for 30 min. Calibration was made with 10–50 μ g of glucose as a standard. After the incubation, all samples were measured at 490 nm against a blank containing water instead of the enzyme.

Determination of free amino groups

Primary amino groups were determined by a modification of the established spectrophotometric method with TNBS [19,20]. An enzyme sample (0.1 ml of a solution containing 10–20 mg of protein · ml⁻¹; freshly prepared from a weighted lyophilisate) was added to 0.9 ml of 4% (w/v) sodium bicarbonate (pH 8.5) in a glass test tube and the solution was vortex-mixed. Later on, 0.5 ml of 0.01% TNBS was added, the solution was mixed again and thermostatically maintained at 40 °C in the dark for 1 h. Calibration was made with 10–50 μ g of 6-aminohexanoic acid as a standard. After

the incubation, all samples were measured at 345 nm against a blank containing water instead of the enzyme.

Peptide mass fingerprinting by MALDI-TOF (time-of-flight)-MS

Native and modified PSAO and GPAO were first resolved by SDS/PAGE as described above. After the electrophoresis, protein bands were visualized by staining with Coomassie Brilliant Blue R250. The bands were excised from the gel slab, cut into pieces and placed in 0.65 ml microtubes (Roth, Karlsruhe, Germany). In-gel digestion was performed using a modified trypsin (details on its preparation from native bovine pancreatic trypsin are obtainable from M.S. on request) by an established digestion method [21] with a few modifications: the digestion proceeded for 16 h and the temperature was set to 55 °C. An aliquot (1 μ l) was withdrawn from the digest and MALDI probe was prepared as described in [22]. Analysis was performed on a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonik), equipped with a Scout 384 ion source. The spectra obtained were processed by XMass 5.1.1 and BioTools 2.1 software (Bruker Daltonik). Proteins were identified using the MASCOT 1.8 program (Matrix Science, London, U.K.) installed on a local server; database searches were performed against a non-redundant protein sequence database [MSDB (Mass Spectrometry protein sequence DataBase); downloaded on 12 December, 2003 from the European Bioinformatics Institute website].

Results

It has been shown that PSAO and GPAO represent abundant proteins in extracts of the respective plant seedlings [23]. Hence, they can be purified to homogeneity with a high yield of activity [8,23]. Preparations of PSAO and GPAO described in the present study had specific activities of 830 and 1050 nkat · mg⁻¹ respectively, determined using putrescine as a substrate. The enzymes were chemically modified with BCD by a classical approach of artificial protein glycosylation, involving the preparation of a BCD-polyaldehyde in the first step. This polyaldehyde then reacted with PSAO and GPAO, and intermolecular Schiff bases were formed with accessible lysine residues in the proteins. These Schiff bases were concurrently reduced by an excess of cyanoborohydride to render a covalent linkage between the proteins and the oligosaccharide.

Specific activities of the BCD-PSAO and BCD-GPAO conjugates were lower than those of the native enzymes (630 and 780 nkat · mg⁻¹ respectively). Table I presents V_{\max} and K_m values for PSAO, GPAO and their BCD conjugates measured using the diamines putrescine and cadaverine as substrates. BCD-PSAO and BCD-GPAO were both very sensitive to the mechanism-based inhibitor DABY. As shown

Table 1 Comparison of the kinetic parameters of PSAO, GPAO and their BCD conjugates for the best substrates putrescine and cadaverine

The V_{max} and K_m values for the substrates were measured by the guaiacol spectrophotometric method [14].

Enzyme	Putrescine		Cadaverine	
	V_{max} ($\text{nmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$)	K_m (mM)	V_{max} ($\text{nmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$)	K_m (mM)
PSAO	980	0.22	1240	0.10
BCD-PSAO	820	0.18	1000	0.08
GPAO	1220	0.30	1520	0.13
BCD-GPAO	1060	0.21	1250	0.10

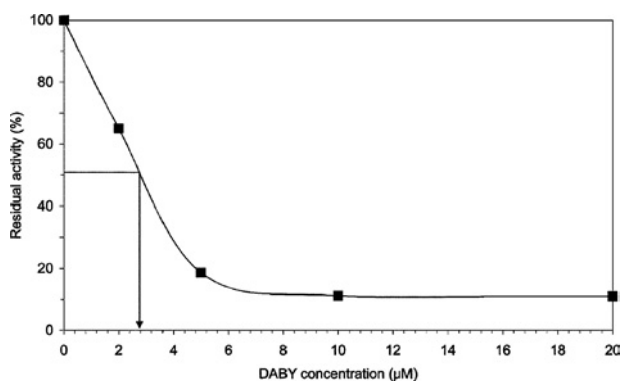


Figure 1 Time-dependent inactivation of BCD-GPAO by DABY

BCD-GPAO ($2 \mu\text{M}$) in 0.1 M potassium phosphate buffer ($\text{pH } 7.0$) was incubated with DABY ($2\text{--}20 \mu\text{M}$ final concentration) for 30 min and the corresponding residual activities were measured by the guaiacol spectrophotometric method [14]. The IC_{50} value subtraction is indicated by an arrow. A very similar curve obtained for native GPAO is not shown for clarity.

in Figure 1, the IC_{50} value for BCD-GPAO was $2.8 \mu\text{M}$. The same value was found for BCD-PSAO. For the unmodified enzymes, IC_{50} values were 2.5 and $3.0 \mu\text{M}$ respectively.

Plant CAOs are regarded as relatively thermostable proteins [7]. Nevertheless, when our PSAO and GPAO preparations were incubated at 60°C for a long time period, the catalytic activity gradually decreased. Samples of PSAO and GPAO ($10 \mu\text{M}$ solutions in 20 mM potassium phosphate buffer, $\text{pH } 7.0$) lost 36 and 41 % of activity respectively after incubation at 60°C for 6 h. Their BCD conjugates were substantially more stable and retained 90 % of the original activity in analogous experiments. GPAO and its BCD conjugate were incubated at various temperatures between 45 and 75°C for 30 min and the residual activity was then measured at 30°C . BCD-GPAO showed an increased thermostability in the temperature range $55\text{--}70^\circ\text{C}$ (Figure 2). The corresponding value of T_{50} (the temperature at which 50 % of the activity is retained after 30 min incubation) was shifted from 64 to 67°C . Maximum difference in thermostability between BCD-GPAO and GPAO was observed at approx. 65°C (Figure 2). A similar thermostability curve, providing

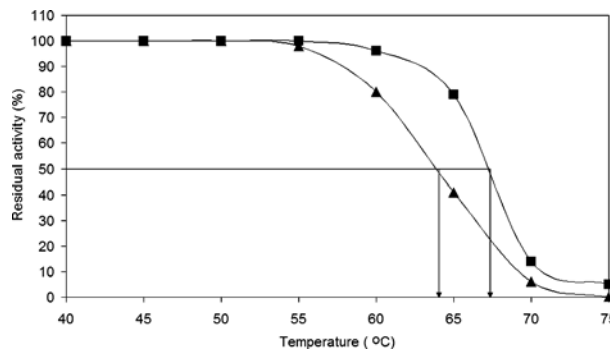


Figure 2 Comparison of the thermostabilities of GPAO and BCD-GPAO

GPAO (▲) and its BCD conjugate (■) were incubated at various temperatures between 45 and 75°C for 30 min and the residual activity was measured by the guaiacol spectrophotometric method [14] at 30°C . The corresponding T_{50} values are indicated by arrows.

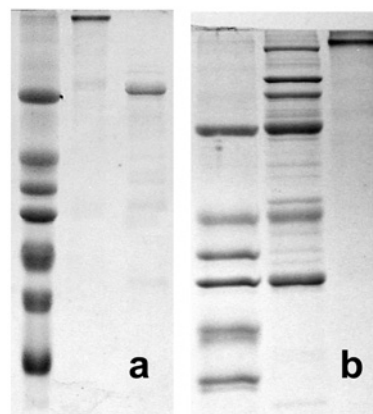


Figure 3 SDS/PAGE separation of BCD conjugates of PSAO and GPAO

(a) From the left, protein standards (\uparrow , 14.2, 20, 24, 29, 36, 45 and 67 kDa), BCD-PSAO ($10 \mu\text{g}$) and PSAO ($10 \mu\text{g}$). (b) From the left, protein standards (\uparrow , 20, 24, 29, 36, 45 and 67 kDa), another set of protein standards (\uparrow , 29, 45, 66, 97.4, 116 and 205 kDa) and BCD-GPAO ($10 \mu\text{g}$). Proteins were visualized by staining with Coomassie Brilliant Blue R250.

the same T_{50} , was obtained for BCD-PSAO (not shown). Under mild conditions (20 mM potassium phosphate buffer, $\text{pH } 7.0$ and 23°C), both BCD-PSAO and BCD-GPAO retained more than 90 % of the original activity after incubation for 14 days; however, the corresponding unmodified enzymes lost almost one-half.

Samples of purified PSAO and GPAO migrated as individual protein bands in SDS/polyacrylamide gels, providing an identical apparent molecular mass of 75 kDa (Figure 3). Unfortunately, SDS/PAGE could not provide us highly reliable molecular-mass estimates for the studied BCD conjugates. BCD-PSAO (Figure 3a) and BCD-GPAO (Figure 3b) migrated only very slowly and remained at the top of a 10 % running gel referring to estimates exceeding a value of 200 kDa . Molecular-mass estimations performed

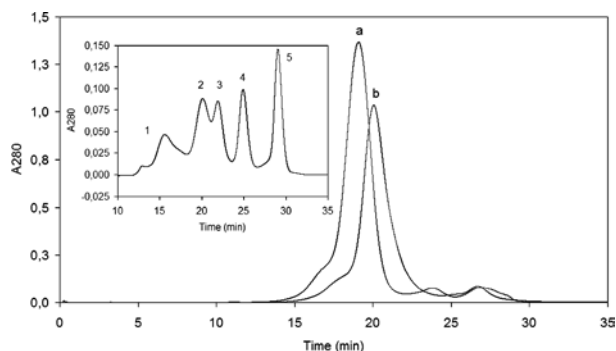


Figure 4 Chromatographic separation of PSAO and BCD-PSAO on a Superdex 200 HR column

Flow rate: $0.7 \text{ ml} \cdot \text{min}^{-1}$; buffer: 50 mM potassium phosphate (pH 7.0) containing 0.15 M NaCl. Approx. 5 mg each of BCD-PSAO (a) and PSAO (b) were loaded on to the column. The inset shows a separation of protein standards; from the left, 670 kDa (1), 158 kDa (2), 44 kDa (3), 17 kDa (4) and 1.35 kDa (5).

by gel permeation chromatography on Superdex were more accurate. Whereas native PSAO and GPAO were both eluted at a retention time corresponding to a molecular mass of 145 kDa, BCD-PSAO (Figure 4) and BCD-GPAO provided increased molecular-mass values of 179 and 182 kDa respectively.

Neutral-sugar contents in PSAO, GPAO and their BCD conjugates were determined by the colorimetric method using phenol and sulphuric acid [18]. We found that native PSAO and GPAO contained 12 and 13% of neutral sugars respectively. For both BCD-PSAO and BCD-GPAO conjugates, the sugar content increased to 31%. The number of free amino groups in PSAO and GPAO dimers (40 and 38 respectively) was determined spectrophotometrically using the TNBS reagent [19,20]. In both enzymes, the modification by BCD reduced the number of free amino groups by 22.

MALDI-TOF peptide mass fingerprints of native PSAO and GPAO, together with those of the corresponding BCD conjugates, are presented in Figures 5 and 6. The PSAO digest provided a mass spectrum (Figure 5a), which allowed us to identify the enzyme in the MSDB protein database (accession no. JC7251). The identified peptides covered 37% of the sequence of the database entry and matched with an accuracy higher than 100 p.p.m. Slightly lower sequence coverage (22%) was achieved for BCD-PSAO (Figure 5b). Although the full-length sequence of GPAO is unknown, a few previously sequenced internal peptides and the N-terminal sequence indicate its high similarity to the sequence of PSAO [5]. A mass spectrum of the GPAO tryptic digest is presented in Figure 6(a). As expected, a major part of the peptides in the spectrum matched the PSAO sequence with a 25% coverage. A similar pattern of peptides, but with lower sequence coverage (15%), was observed in a peptide mass map of BCD-GPAO (Figure 6b).

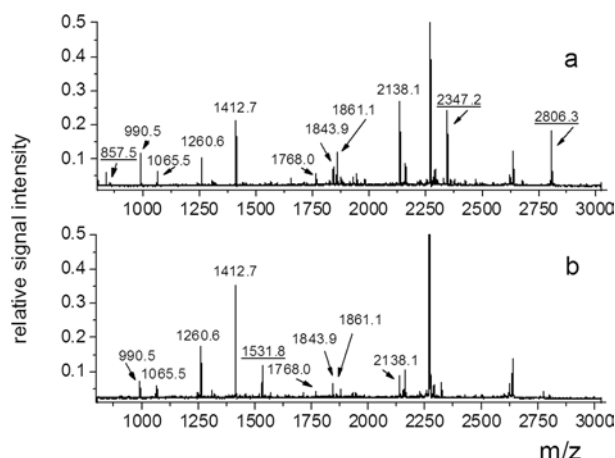


Figure 5 Peptide mass 'fingerprints' of PSAO and BCD-PSAO

The enzymes were separated by SDS/PAGE and then subjected to in-gel digestion by a modified trypsin. Peptide peaks that are unique in the MALDI-TOF mass spectrum of PSAO (a) or BCD-PSAO (b) are underlined. Both peptide maps matched the sequence of *P. sativum* amine oxidase (MSDB accession no. JC7251).

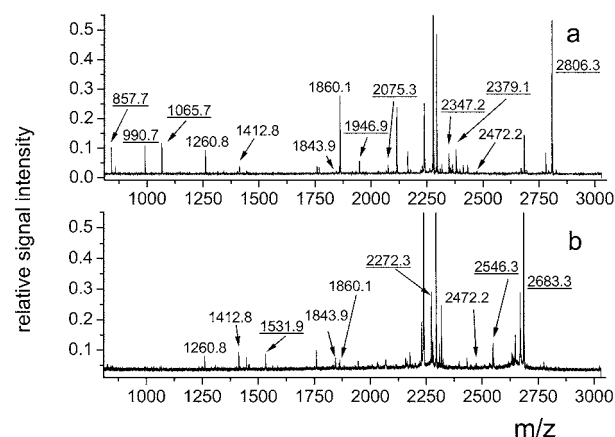


Figure 6 Peptide mass fingerprints of GPAO and BCD-GPAO

The enzymes were separated by SDS/PAGE and then subjected to in-gel digestion by a modified trypsin. Peptide peaks that are unique in the MALDI-TOF mass spectrum of GPAO (a) or BCD-GPAO (b) are underlined. Both peptide maps matched the sequence of *P. sativum* amine oxidase (MSDB accession no. JC7251).

Table 2 presents a list of peptide peaks observed in the above-mentioned peptide mass fingerprints that matched particular tryptic peptides from the PSAO sequence deposited in the MSDB database (accession no. JC7251). For both enzymes, the modification additionally brought a large number of peaks that did not match the sequence (20 peaks for BCD-PSAO and 16 peaks for BCD-GPAO), which probably resulted from the chemical modification or some concurrent side reactions.

Table 2 A list of peptide peaks observed in the MALDI-TOF mass spectra of tryptic digests of PSAO, GPAO and their BCD conjugates that matched predicted fragments of the PSAO sequence deposited in the MSDB database under the accession number Jc7251

(W, d.o.), double-oxidized tryptophan residue; (M, o.), oxidized methionine residue.

<i>m/z</i> of peptide peaks matching the PSAO sequence				
PSAO	BCD-PSAO	GPAO	BCD-GPAO	Sequence (MSDB Jc7251)
857.49	–	857.67	–	QSPFPGPK
990.53	990.56	990.68	–	FHIGFDVR
1065.40	1065.55	1065.68	–	TAVGNEVGYR
1260.58	1260.60	1260.70	1260.74	WAGGLYVDHSR
1315.57	–	–	–	SYWTTTETQTAK
1412.71	1412.73	1412.81	1412.84	YETHPTLVSIPIR
1443.66	–	1443.76	–	KSYWTTTETQTAK
1517.71	1517.75	–	–	LAFHYIGLDDPEK
1591.83	–	–	–	AGIVISLASIYDLEK
1768.03	1768.03	1768.09	–	ASGSIKPAIALSGILEIK
1843.93	1843.94	1843.95	1843.99	NAICVFEQYGNIMWVR
1857.02	–	1857.09	–	AGIVISLASIYDLEKHK
–	–	–	1860.06 (M, o.)	NAICVFEQYGNIMWVR
1861.09	1861.00	1861.14	–	ITIGLAPAEVLVVNPNIK
–	–	1946.93	–	SYWTTTETQTAKTESDAK
–	–	1978.94 (W, d.o.)	–	SYWTTTETQTAKTESDAK
–	–	2075.01	–	KSYWTTTETQTAKTESDAK
2138.11	2138.12	–	–	LAFHYIGLDDPEKDHVLR
–	–	–	2272.25	LIPAIPAHPLLTEDDYPQIR
2291.14	2291.15	–	–	TFFDSCGEFGFGLSTVSLIPNR
2296.30	–	–	–	KSFVVAIINSQTHEILIDLR
2347.15	–	2347.15	–	WAGGLYVDHSRGDDTLAVWTK
2379.14 (W, d.o.)	–	2379.14 (W, d.o.)	–	WAGGLYVDHSRGDDTLAVWTK
–	–	2411.11 (W, d.o.)	–	WAGGLYVDHSRGDDTLAVWTK
2472.16	2472.17	2472.13	2472.15	GYISELFVYPQDPTTEEFYFK
2492.17	–	–	–	TESDAKITIGLAPAEVLVVNPNIK
2637.31	2637.32	–	2637.28	DCPPHAQFIDTYVHSANGTPILLK
2806.33	–	2806.29	–	IVEYHRDRIEAVPTAENTEQVSK

Discussion

Chemical modifications of enzymes are performed to enhance or modify their functionality [24]. Our search for new approaches in this topic has been stimulated, among other things, by achievements such as converting a proteolytic enzyme into an oxidoreductase by a simple linkage of flavin [25]. One of the most attractive challenges on the way to practical applications of enzymes is to increase their overall stability, especially thermostability. This was achieved by coupling enzymes with various materials, including low-molecular-mass compounds [24], hydrophilic polymers such as methoxypoly(ethylene glycol) [26], linear oligosaccharides [27,28] or cyclodextrins [16,29]. The latter modifications are carried out via primary amino groups in enzymes after the sugar activation; they are frequent and most of them are not required for the catalytic activity. The stabilization occurs after both intramolecular cross-linking and reduction of the hydrophobic surface area [30].

In the present study, we report the chemical modification of two plant CAOs by a periodate-activated oligosaccharide. The PSAO subunit sequence determined by Koyanagi et al. [12] (MSDB accession number Jc7251)

partially differs from the sequence determined by Tipping and McPherson [4] (National Center for Biotechnology Information, NCBI Protein Database accession number AAA62490). The former sequence comprises 39 lysine residues, whereas the latter has only 38 lysine residues, as calculated for a mature form of the protein. Therefore native PSAO (a dimer) is expected to comprise 78 lysine residues, from which only 32 are solvent-accessible [5]. Our colorimetric determination of free primary amino groups in PSAO and GPAO dimers resulted in approximately one-half of the expected number. It is probable that mainly the solvent-accessible lysine residues were labelled by the TNBS reagent, and approximately one-half of them were chemically modified by periodate-oxidized BCD. Hence, we achieved only a partial modification of the solvent-accessible lysine residues.

Kinetic measurements performed with the best diamine substrates of PSAO and GPAO (putrescine and cadaverine) and with the mechanism-based inhibitor DABY suggested that the modification of the enzymes by BCD did not directly alter the active site. However, the observed decreases in both specific activity and V_{\max} values suggested that the catalytic activity was affected only remotely.

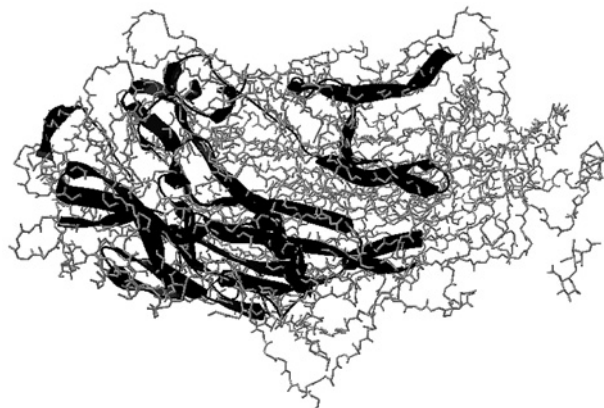


Figure 7 Location of the matched peptides from Table 2 in the crystal structure of PSAO

The structure [6] was obtained as a PDB formatted file from the RCSB (Research Collaboratory for Structural Bioinformatics, Rutgers University, Piscataway, NJ, U.S.A.) Protein Data Bank on the internet (PDB identification code 1KSI) and adapted using the computer program DeepView/Swiss-PdbViewer v3.7 (www.expasy.org/spdbv/). For clarity, the respective peptides are highlighted as ribbons in one subunit only.

Plant CAOs are natural glycoproteins [1]. The PSAO subunit contains several potential glycosylation sites, where sugars may be attached by N-glycosidic bonds via asparagine residues [6]. The determined sugar content in our PSAO and GPAO preparations matched previously published results [8,31]. The modification of the enzymes with BCD increased the sugar content. Considering a molecular mass of 1.135 kDa for BCD, this increase shows a good correlation with the observed decrease in the number of free amino groups. It also correlates well with the apparent molecular mass value of both the BCD conjugates (180 kDa), which was determined by gel-permeation chromatography.

PSAO and GPAO can be considered as homologous enzymes. Although the full-length sequence of GPAO is not yet available, partial sequencing by Edman degradation covered approx. 15% of the expected GPAO subunit sequence [5]. Peptide mass fingerprinting analyses using MALDI-TOF-MS further confirmed the anticipated strong homology. On the basis of the respective mass spectrum, PSAO was unambiguously identified by searching in the MSDB database. Peptide peaks of GPAO digests were compared with peptides from the sequence of the same database entry, but with lower sequence coverage. The PSAO crystal structure [6] is available in the RCSB (Research Collaboratory for Structural Bioinformatics) Protein Data Bank under the identification code 1KSI. When the matched peptides from Table 2 were highlighted in the structure (Figure 7), it became apparent that they originate from the surface regions in the PSAO subunit. BCD conjugates of PSAO and GPAO provided mass spectra that were very similar to the spectra of the native enzymes. However, the number of matched peptides was

reduced in both cases because of the attachment of BCD residues to lysine residues residing at the surface of the molecule. The chemical modification of these lysine residues probably occurred at random, since substantial differences were observed between the spectra of BCD-PSAO and BCD-GPAO digests, although the spectra of the digests of native enzymes seemed to be similar. Many unidentified peaks were observed in the digests of the BCD conjugates studied that were not seen in the digests of native PSAO and GPAO, and only some of these peaks overlapped.

Furthermore, we demonstrated increased thermostability of BCD conjugates of PSAO and GPAO when compared with the native enzymes. Although their T_{50} values were only slightly different, the difference in their thermostabilities at 65 °C was significant. Furthermore, the described BCD conjugates demonstrated higher storage stability. They also preserved the original enzymic activity and kept almost unchanged kinetic properties for the best substrates of plant CAOs. Hence, the BCD conjugates represent good candidates for biotechnological applications, where amine oxidation should be performed at high temperatures.

As already mentioned in the Introduction, plant CAOs have been employed in various amperometric biosensors for the determination of amines in biological samples. Carbon electrodes are now preferred for making such biosensors, where CAO is bound either through an affinity interaction within a modified carbon paste [9] or by a simple surface coating of a polished graphite rod [10]. There is no need for a covalent linkage and, thus, the absence of free surface lysine residues from BCD-CAOs do not imply an obstacle for immobilization. Anyway, plant CAO molecules contain other accessible amino acid residues (aspartic acid, glutamic acid and glutamine) that might be used for coupling the BCD conjugates with various polymers or active surfaces. Similarly, the presence of sugar moieties in BCD-CAOs could be utilized for both simple hydrophilic adhesion or coupling via an additional chemical reaction. Biosensors based on BCD-CAOs could significantly benefit from the main advantages of the conjugates, i.e. preserved functionality, increased thermostability and storage stability. In the free form, BCD-CAOs might be employed as a stable part of diagnostic sets for the spectrophotometric determination of polyamine compounds (depending on substrate specificity) as well as for the *in vitro* synthesis of various compounds by oxidative deamination [32]. Possible applications of CAOs for medical purposes still remain rather theoretical. It has been demonstrated that CAOs may participate in protection of cells against the damage induced by free radicals and in leucocyte-endothelia interactions [33]. Interestingly, GPAO was found to exert significant cardioprotection against postischaemic reperfusion damage [11]. Surface-bound cyclodextrin moieties would increase the biological lifetime of the enzyme in such processes.

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