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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Changes in tyrosinase specificity by ionic liquids and sodium dodecyl sulfate

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Abstract Tyrosinase is a member of the type 3 copper enzyme family involved in the production of melanin in a wide range of organisms. The ability of tyrosinases to convert monophenols into diphenols has stimulated studies regarding the production of substituted catechols, important intermediates for the synthesis of pharmaceuticals, agrochemicals, polymerization inhibitors, and antioxidants. Despite its enormous potential, the use of tyrosinases for catechol synthesis has been limited due to the low monophenolase/diphenolase activity ratio. In the presence of two water miscible ionic liquids, [BMIM][BF_4] and ethylammonium nitrate, the selectivity of a tyrosinase from *Bacillus megaterium* (TyrBm) was altered, and the ratio of monophenolase/diphenolase activity increased by up to 5-fold. Furthermore, the addition of sodium dodecyl sulphate (SDS) at levels of 2–50 mM increased the activity of TyrBm by 2-fold towards the natural substrates L-tyrosine and L-Dopa and 15- to 20-fold towards the non-native phenol and catechol. The R209H tyrosinase variant we previously identified as having a preferential ratio of monophenolase/diphenolase activity was shown to have a 45-fold increase in activity towards phenol in the presence of SDS. We propose that the effect of SDS on the ability of tyrosinase to convert non-natural substrates is due to the interaction of surfactant molecules with residues located at the entrance to the active site, as visualized by the newly determined crystal structure of TyrBm in the presence of SDS. The effect of SDS on R209 may enable less polar substrates such as phenol and catechol, to penetrate more efficiently into the enzyme catalytic pocket.

Keywords Tyrosinase · *Bacillus megaterium* · Ionic liquids · Sodium dodecyl sulphate · Diphenols

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Introduction

Tyrosinase (EC 1.14.18.1) is a type 3 copper protein, able to perform two successive reactions in the presence of molecular oxygen; the hydroxylation of phenols to form *ortho*-diphenols (monophenolase activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) (Fig. 1) (Burton 2003; Claus and Decker 2006). The formation of melanins, known as the cause for skin pigmentation and fruit and vegetable browning, is a consequence of further spontaneous polymerization of these reactive quinones. Tyrosinase is abundant in almost all domains of life where it is involved in various biological functions, and its active site is well conserved among the different species (Decker and Tuczek 2000; Martinez and Whitaker 1995; Olivares et al. 2002; Olivares and Solano 2009; Selinheimo et al. 2007). The active site comprises six histidine residues which coordinate the two copper ions CuA and CuB (Claus and Decker 2006; Decker et al. 2007). Along with tyrosinases, the type 3 copper protein family includes catechol oxidase which performs only the oxidation of diphenols, and hemocyanins, which are oxygen carriers from the hemolymph of many molluscs and arthropods (Itoh and Fukuzumi 2007; Klabunde et al. 1998; van Holde et al. 2001).

The ability of tyrosinases to convert monophenols into diphenols has stimulated studies regarding the production of

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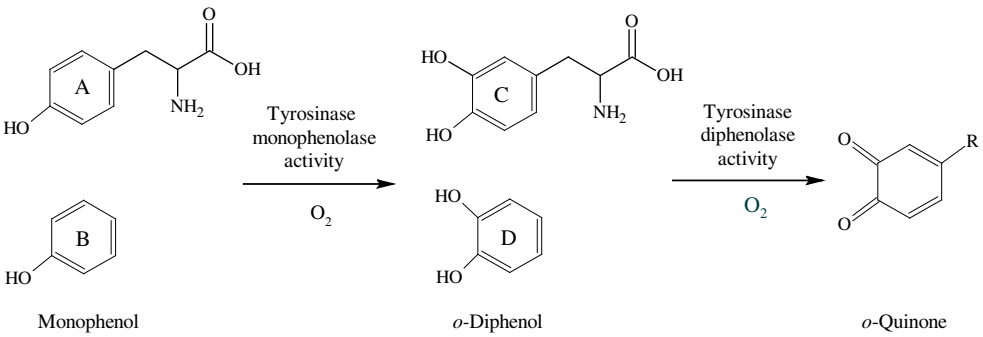


Fig 1 Tyrosinase reaction scheme presenting the monophenolase and diphenolase activities. The monophenolase activity in this study was tested with tyrosine (**A**) and phenol (**B**) and the diphenolase activity with L-Dopa (**C**) and catechol (**D**). *R* respective substituent on the benzene ring

various *ortho*-diphenols (also referred to as substituted catechols). Catechols, are important intermediates for the synthesis of pharmaceuticals, agrochemicals, flavors, polymerization inhibitors, and antioxidants (Halaouli et al. 2006; Kawamura-Konishi et al. 2007; Nolan and O'Connor 2007; Wang et al. 2000). Despite the great potential, the use of tyrosinases for catechol synthesis has been limited since their diphenolase activity is much greater than their monophenolase activity (Hernandez-Romero et al. 2006). In nature, there is one reported unique tyrosinase from *Ralstonia solanacearum* with an abnormally high tyrosine hydroxylation/DOPA oxidation ratio (Hernandez-Romero et al. 2006). Recently, we performed directed evolution of a tyrosinase from the soil bacterium *Bacillus megaterium* (TyrBm) in an attempt to alter the selectivity of the enzyme (Shuster Ben-Yosef et al. 2010; Shuster and Fishman 2009). Variant R209H exhibited a monophenolase/diphenolase activity ratio 2.6-fold higher than the wild-type enzyme. Such an increased monophenolase/diphenolse activity ratio will enable the use of tyrosinase for industrial bioproduction of substituted catechols. The crystal structures of both wild-type TyrBm and variant R209H have been recently determined (Sendovski et al. 2011), and it was discovered that arginine at position 209 exhibits alternative conformations and is a critical residue located in the entrance to the active site.

Ionic liquids (ILs) are novel nonaqueous solvents with great potential for biocatalysis (Yang and Pan 2005). Their unique property of negligible vapor pressure and the ability to "fine-tune" their chemical and physical properties by a combination of different anions and cations, has placed them as leading candidates in replacing conventional organic solvents. Prior work on tyrosinase activity in ILs was restricted to mushroom tyrosinase and examined the IL effect on the diphenolase activity only.

Sodium dodecyl sulphate (SDS) is an anionic detergent that inactivates most enzymes. In contrast, tyrosinase and other type 3 copper proteins have been shown to be activated by SDS. The enhanced activity in the presence of SDS has been reported in numerous studies and for tyrosinase from various species (Gandia-Herrero et al. 2005; Lopez-Serrano et al. 2002; Nillius et al. 2008). In all cases, the enhanced activity of tyrosinase was obtained at very low concentrations of under 1 mM SDS. The mechanism by which SDS facilitates this enhancement is still unclear but was found to involve changes in the protein tertiary structure.

We show here that both ILs and SDS have the ability to alter TyrBm selectivity. Furthermore, we provide crystallographic evidence for the association of SDS with the enzyme resulting in conformational changes which may explain the improvement in activity.

Materials and methods

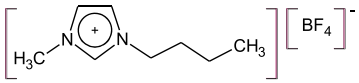
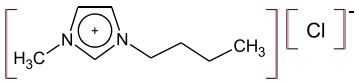
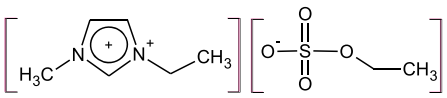
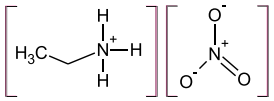
Materials

L-3,4-dihydroxyphenylalanine (L-Dopa) was purchased from Acros (Geel, Belgium). L-tyrosine (L-Tyr), catechol, kanamycin, sodium dodecyl sulfate (SDS), mushroom tyrosinase, imidazole, trizma base, sodium cacodilate trihydrate and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from Sigma-Aldrich Chemical Co. (Sigma-Aldrich, Rehovot, Israel). Phenol and dimethyl sulfoxide were purchased from Merck (Whitehouse Station, NJ, USA). All materials used were of the highest purity available and were used without further purification. The four ILs used in this research (Table 1) were purchased from Iolitec, Denzlingen, Germany.

Expression and purification of tyrosinase from *B. megaterium*

A tyrosinase producing *B. megaterium* (TyrBm) was isolated by our lab from soil samples and the gene encoding for the tyrosinase was cloned into *Escherichia coli* BL21. The enzyme was expressed and purified as previously described (Sendovski et al. 2010; Shuster and Fishman 2009). Variant R209H was obtained by directed evolution as described previously (Shuster Ben-Yosef et al. 2010). Briefly, mutagenesis was introduced using error-prone PCR and a high throughput assay for enhanced activity on tyrosinase substrates was used to locate mutants that have higher activity on L-tyrosine and lower

Table 1 ILs used in the research

IL	Structure
1-Butyl-3-methylimidazolium BF_4 ([BMIM][BF_4])	
1-Butyl-3-methylimidazolium Cl ([BMIM][Cl])	
1-Ethyl-3-methylimidazolium ethyl sulfate ([EMIM][$EtSO_4$])	
Ethylammonium nitrate (EAN)	

activity on L-Dopa compared with wild type. Purification steps were identical to those performed on the wild type.

Activity assay in the presence of ILs or SDS on L-tyrosine and L-Dopa

Tyrosinase monophenolase and diphenolase activity in the presence of ILs and SDS was determined by measuring the formation of L-dopachrome from 1 mM L-tyrosine or L-Dopa. The reaction was performed in 96-well plates for 10 min at 25 °C and monitored with a multi-plate reader (OPTImax tunable microplate reader; Molecular Devices, Sunnyvale, CA, USA) at 475 nm. Each reaction well consisted of Tris–HCl buffer (50 mM, pH=7.5), 0.01 mM $CuSO_4$, IL or SDS in a range of concentrations, and enzyme at a concentration of 0.006 mg/ml, either TyrBm or mushroom tyrosinase. Negative control experiments without the enzyme or without the substrate were performed. The rate of dopachrome formation was defined as the slope of the linear zone of absorbance versus the time plot. All measurements were carried out in duplicates. Specific activity was calculated using the absorption coefficient for dopachrome of 3,600 M^{-1} cm^{-1} (Rodriguez-Lopez et al. 1994).

Activity assay in the presence of SDS on phenol and catechol

The monophenolase and diphenolase activity on phenol and catechol was determined by monitoring the formation of an MBTH-quinone adduct from 1 mM phenol or catechol at a wavelength of 500 nm. MBTH is a potent nucleophile that traps *o*-quinones to form a soluble MBTH-quinone adduct with a high molar absorption coefficient (Rodriguez-Lopez et al. 1994). The reaction was performed in 96-well plates for 10 min at 25 °C. In addition to the reaction components mentioned earlier, MBTH was added at a concentration of 1.5 mM. The rate of MBTH-quinone adduct formation was defined as the slope of the linear zone of absorbance versus the time plot. All measurements were carried out in duplicates. Specific activity was calculated using the absorption coefficient for the MBTH-quinone adduct at 500 nm of 32,500 M^{-1} cm^{-1} (Rodriguez-Lopez et al. 1994).

Activity analysis using high-performance liquid chromatography

The conversion of L-tyrosine to L-Dopa was further determined by high-performance liquid chromatography (HPLC; Agilent 1100, Agilent Technologies, Santa Clara, CA, USA) by measuring the decrease in tyrosine concentration using an Eclipse XDB C18 column (5 μm, 4.6×150 mm; Agilent Technologies, Santa Clara, CA, USA). Two millimolars L-tyrosine was added to a 6 ml reaction volume containing 50 mM Tris–HCl at pH 7.5, 0.01 mM $CuSO_4$, 30 mM SDS or 10 % EAN, and 0.006 mg/ml purified enzyme. The reaction was stopped periodically by adding 0.5 ml of the reaction mixture to 0.1 ml of 2 M HCl. The samples were filtered using PVDF 0.45-μm filters (Millex HV, Millipore, Cork, Ireland) and analyzed with a method comprising 2 % acetonitrile in water (with 0.1 % formic acid) at a flow rate of 1 ml/min. A diode array detector was used at a fixed wavelength of 215 and 275 nm to monitor the reaction in the presence of SDS and EAN respectively. Twenty microliters of filtered samples were injected into the column and under these conditions L-tyrosine eluted at 3.3 min. A calibration curve was made with a commercial standard at 215 and 275 nm.

Crystallization, data collection, and structure determination

TyrBm was crystallized as previously described (Sendovski et al. 2010). Crystals were soaked in 10 mM SDS or 10–20 % IL for 1–5 min after which they were flash frozen in

liquid nitrogen. X-ray diffraction data were collected at the European Synchrotron Radiation Facility (ESRF), Grenoble, France, beamline ID 23–1. All data were indexed, integrated, scaled, and merged using Mosflm and Scala (Leslie 1992). The structure of TyrBm was solved by molecular replacement using Phaser (McCoy 2007) and the coordinates of earlier determined TyrBm structure (PDB code 3NM8). A single solution was obtained for two monomers in the asymmetric unit. Refinement was performed using Phenix (Adams et al. 2010) and Refmac5 (Murshudov et al. 1997; Skubak et al. 2004), coupled with rounds of manual model building, real-space refinement and structure validation, performed using COOT (Emsley and Cowtan 2004). The omit F_o-F_c electron density map was calculated using CNS (Brünger et al. 1998). Data collection, phasing, and refinement statistics are presented in Table 2.

Protein Data Bank accession numbers

Coordinates and structure factors of TyrBm in complex with SDS have been deposited in the RCSB Protein Data Bank under accession code 4D87.

Results

Effect of water miscible ILs on the activity of TyrBm

Monophenolase and diphenolase activities of TyrBm were measured in the presence of four water miscible ILs. Monophenolase activity was tested using 1 mM L-tyrosine and the diphenolase activity using 1 mM L-Dopa. In the presence of two ILs, [EMIM][$EtSO_4$] and [BMIM][Cl], both activities remained relatively unaffected at concentrations of less than 1 % (*v*/*v*) (Fig. 2a). In the presence of 10 or 20 % [EMIM][$EtSO_4$], the activity on both substrates decreased dramatically and became negligible. In the presence of [BMIM][Cl], the activity on L-tyrosine decreased gradually but slightly, while the activity on L-Dopa decreased more significantly to 0.6-fold in 20 % (*v*/*v*) (Fig. 2a). In the presence of the ILs [BMIM][BF_4] and EAN however, the monophenolase and diphenolase activities were affected differently, causing a significant change in the selectivity of TyrBm. The monophenolase activity of TyrBm on L-tyrosine improved by 1.7- to 2.6-fold, and the diphenolase activity on L-Dopa decreased by 0.9- to 0.6-fold, in the presence of 5–40 % EAN (Fig. 2b). Similar, though less pronounced results were obtained for [BMIM][BF_4]. The monophenolase activity of TyrBm on L-tyrosine improved by up to 1.4-fold in the presence of 5–20 % [BMIM][BF_4], whereas the diphenolase activity on L-Dopa decreased by 0.7- to 0.2-fold in the presence of 5–40 % [BMIM][BF_4], respectively (Fig. 2b). Therefore, the monophenolase/diphenolase activity ratio increased up to 5-fold with an increase in the IL concentration (Fig. 3).

In order to establish whether the IL effect was protein specific, the reactions were repeated for commercial mushroom tyrosinase from *Agaricus bisporus* (TyrAb). It is important to note that the commercial enzyme is a crude preparation which may consist of a number of different isomers or other proteins (Neeley et al. 2009). In the presence of [BMIM][BF_4], the diphenolase activity of TyrAb on L-Dopa was affected in a similar way to that of TyrBm; activity decreased with the increase in IL to 0.3-fold in the presence of 40 % [BMIM][BF_4] (results not presented). However, the monophenolase activity of TyrAb on L-

Table 2 Data collection and refinement statistics for structure *TyrBm_SDS* (PDB code 4D87)

X-ray data collection		Refinement	
Space group	$P2_1$	R (%)/R_{free} (%)[c]	25.9/29.3
Unit-cell parameters (Å, deg)		Amino acids	571
a	47.8	Total number of non-hydrogen atoms	4702
b	79.1	Number of copper ions	2
c	85.9		
α	90		
β	103.6	Average B factor (Å), protein atoms	10.4
γ	90	r.m.s.d.	
Resolution range	83-3.5	Bond length (Å)	0.015
Observed reflections	16950	Bond angle (°)	2.00
Unique reflections	5980	Ramachandran plot	
$I/\sigma(I)$[a]	8.5 (7.8)	Favored regions (%)	97.3
R_{merge}[a, b]	0.10 (0.16)	Outliers (%)	2.7
Completeness[a]	83 (73)		
Multiplicity[a]	2.6 (2.6)		

[a] Values in parentheses are for the last shell

[b] $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where I is the observed intensity, and $<I>$ is the mean value of I

[c] $R/R_{free} = \sum_{hkl}||F_{obs}|-|F_{calc}|| / \sum_{hkl}|F_{obs}|$ where R and R_{free} are calculated using the test reflections, respectively. The test reflections (10 %) were held aside and not used during the entire refinement process

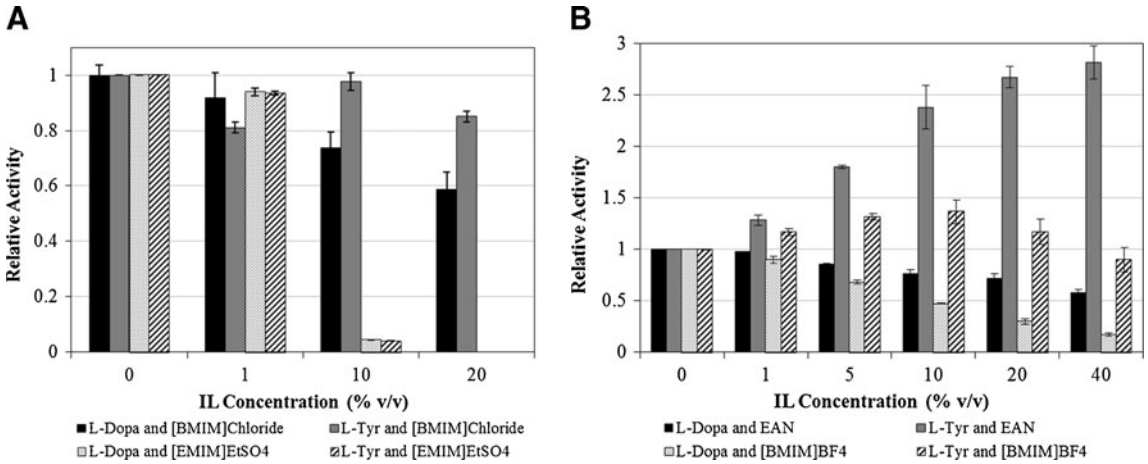


Fig 2 TyrBm relative activity in the presence of ILs. (**a**) Relative activity on L-Dopa and L-tyrosine (L-Tyr) in the presence of [BMIM][Cl] and [EMIM][$EtSO_4$] (At 20 % [EMIM][$EtSO_4$] no activity was obtained). (**b**) Relative activity on L-Dopa and L-Tyr in the presence of EAN and [BMIM][BF_4]. Tyrosinase activity was determined by monitoring the formation of L-dopachrome from 1 mM L-Tyr or L-Dopa at a wavelength of 475 nm

tyrosine was affected very negatively by the presence of [BMIM][BF_4], as opposed to the monophenolase activity of TyrBm which increased. The monophenolase activity decreased with the increase in IL to a negligible value in the presence of 40 % [BMIM][BF_4] (results not presented). In the presence of EAN both activities remained unchanged or decreased slightly. Therefore, the significant increase in monophenolase/diphenolase activity ratio seen in TyrBm is not characteristic of all tyrosinases, suggesting that the ILs affect the enzyme structure and not substrate solubility (Fig. 3).

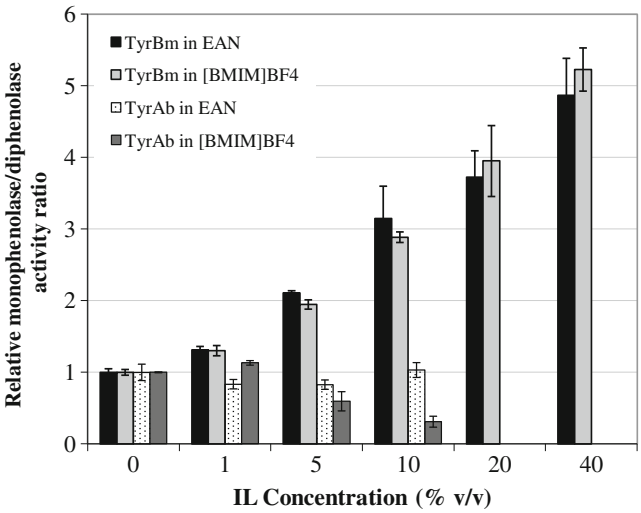


Fig 3 Relative monophenolase/diphenolase activity ratio of TyrBm and TyrAb in the presence of ILs. In the presence of both EAN and [BMIM][BF_4], the activity ratio of TyrBm is increased with the increase in IL concentration up to 40 %. However, the activity ratio of TyrAb remains unchanged or slightly decreases in the presence of ILs (above 10 % the activity is negligible and therefore the ratio is not presented). Tyrosinase activity was determined by monitoring the formation of L-dopachrome from 1 mM L-Tyr or L-Dopa at a wavelength of 475 nm

Effect of SDS on the activity and selectivity of TyrBm

The improvement in tyrosinase activity in the presence of SDS has been previously reported and studied by several research groups (Gandia-Herrero et al. 2005; Karbassi et al. 2004; Lopez-Serrano et al. 2002). The observed improvement in TyrBm activity on L-tyrosine and on L-Dopa was therefore expected. However, the increase in activity occurred at significantly higher concentrations of SDS than those previously reported. At concentrations of up to 1 mM SDS there was no significant effect on either activity, however at concentrations above 2 mM, both monophenolase and diphenolase activity improved by 1.5- to 2.0-fold (Fig. 4). In the presence of 50 mM SDS, improvement in the activity can still be observed. TyrAb also remains active at similar detergent concentrations however there is no improvement in its activity. The results obtained with TyrBm were further confirmed using HPLC analysis. A faster conversion of L-tyrosine to L-Dopa was measured in the presence of 30 mM SDS compared with the control (results not shown).

When the monophenol substrate, phenol, and the diphenol substrate, catechol, were assayed at similar conditions, surprising results were obtained. These substrates are used frequently in order to examine tyrosinase and related proteins' monophenolase and diphenolase activity. TyrBm activity on these substrates is very poor in comparison to L-tyrosine and L-Dopa (Shuster and Fishman 2009). In the presence of 5–50 mM SDS the activity on phenol improved by up to 13-fold and the activity on catechol by up to 18-fold (Fig. 5). Furthermore, the specific activity on phenol

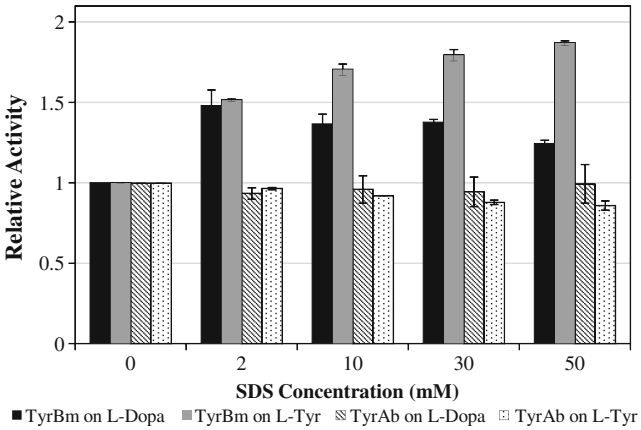


Fig. 4 TyrBm and TyrAb relative activity on L-Dopa and L-Tyr in the presence of SDS. The activity of TyrBm on L-Tyr and L-Dopa is increased in the presence of SDS. Activity on L-Tyr is improved to a greater extent than on L-Dopa. The activity of TyrAb on both substrates remains unchanged. Tyrosinase activity was determined by monitoring the formation of L-dopachrome from 1 mM L-Tyr or L-Dopa at a wavelength of 475 nm

was significantly higher than on L-tyrosine in the presence of 50 mM SDS, 8.7 versus 3.8 μmol mg protein^{-1} min^{-1}, respectively, a 2.3-fold increase. Thus, it is clearly evident that SDS caused a change in TyrBm substrate specificity towards monophenols. In the case of diphenols, despite the significant improvement in the presence of SDS, the specific activity on L-Dopa was still higher than on catechol. In the presence of 2 mM SDS, no significant change in the activity was observed. The critical micelle concentration (CMC) of SDS is between 1 and 7 mM, depending on the conditions (Cong et al. 2009; Moore and Flurkey 1990; Xiang et al. 2006; Zhou et al. 2011). Therefore, the effect of SDS on

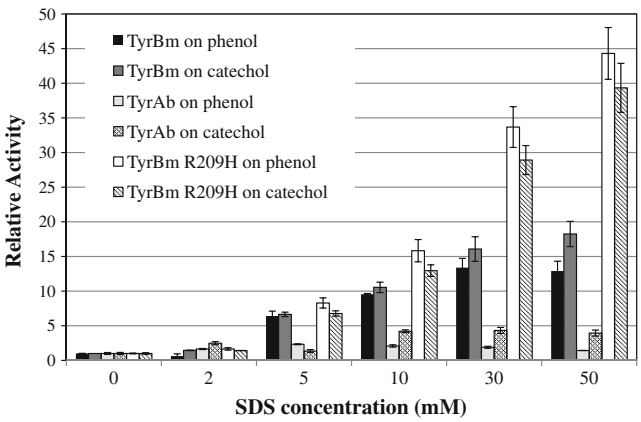


Fig. 5 WT TyrBm, variant R209H, and TyrAb relative activity on phenol and catechol in the presence of SDS. The activity of WT TyrBm and R209H on phenol and catechol increased significantly in the presence of high SDS concentrations. The activity of TyrAb on both substrates increased slightly, more so the activity on catechol. Tyrosinase activity was determined by monitoring the formation of an MBTH-quinone adduct from 1 mM phenol or catechol at a wavelength of 500 nm

TyrBm occurred at a concentration that is substantially above its CMC. TyrAb activity on these substrates was improved as well but much less profoundly; up to 4-fold on catechol and up to 2-fold on phenol (Fig. 5).

The effect of SDS on tyrosinase and related proteins has been suggested to be structural (Cong et al. 2009). The crystal structure of native TyrBm was recently determined by our group (Sendovski et al. 2011), and to obtain insight on the structural effect of SDS on TyrBm, a crystal structure in the presence of SDS was sought. Introduction of SDS was attempted by both co-crystallization and crystal soaking methods. Co-crystallization was unsuccessful and crystals of diffraction quality could not be obtained. Soaking of SDS into pre-existing TyrBm crystals was performed by addition of 10 mM of SDS for 1–5 min. In most cases, this treatment caused a disruption in the crystal lattice order as seen by poor to non-existent diffraction. Nevertheless, one crystal, soaked for 2 min, diffracted to about 2.8 Å and a complete data set to 3.5 Å was collected (*TyrBm_SDS*), and enabled structure determination (Fig. 6). The overall structure of *TyrBm_SDS* is very similar to that previously reported (RMS=0.29 Å^2) (Sendovski et al. 2011). A large unoccupied patch of difference electron density was immediately identifiable in the difference maps in the entrance to the active site of one monomer, which could be fitted with an SDS molecule. The SDS molecule is in proximity (3.0 Å) to residue R209. Residue R209 has been observed in a number of different conformations in TyrBm structures, and was found in two alternate conformations in one of the subunits of TyrBm structure 3NM8. The SDS molecule stabilizes the arginine side chain in the least common conformation. In Fig. 6b, the more common conformation of R209 can be observed in comparison to that obtained in structure *TyrBm_SDS*. Furthermore, residue E158, which has also been observed in a number of conformations, seems to be affected by the movement of R209 or by the presence of the SDS molecule. The preference of a specific conformation of one or more residues in the structure in the vicinity of the active site can explain the change in substrate selectivity which was obtained in the presence of SDS.

The effect of SDS on the specific residue R209 has been tested further using the TyrBm variant R209H, previously reported as having a 2.6-fold higher monophenolase/diphenolase activity ratio (Shuster Ben-Yosef et al. 2010). In the presence of SDS, the relative activity of variant R209H on L-tyrosine and L-Dopa was similar to that of the WT (results not presented). However, when examining the activity on phenol and catechol the results were significantly higher than observed for the wild type; the activity on phenol improved by 45-fold and the activity on catechol by 40-fold (Fig. 5). Thus, the improved activity on phenol and catechol in the presence of SDS was further enhanced when the smaller histidine side chain replaced arginine.

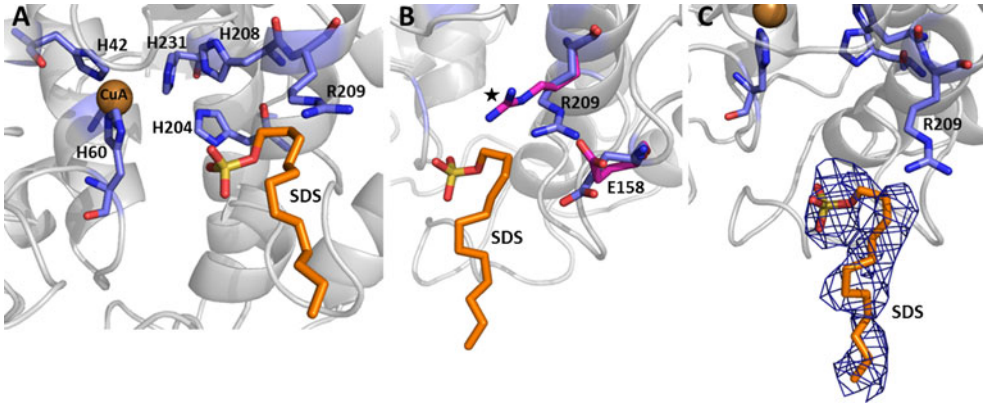


Fig. 6 Structure of TyrBm following a short soak in SDS (*TyrBm_SDS*). **a** The active site residues and R209 of *TyrBm_SDS*—subunit A, determined to 3.5 Å, are shown in stick representation colored in *light blue* and nitrogen atoms in *dark blue*. CuA is shown as a brown sphere, while CuB was not present in the crystal. The bound SDS molecule is shown in stick representation, colored in *orange* (carbon atoms), *yellow* (sulfur), and *red* (oxygen atoms). **b** Different conformations of residues R209 and E158 of *TyrBm_SDS* (colored as above) and of TyrBm structure 3NM8 (colored in *magenta*) (Sendovski et al. 2011). The *star* highlights the more common conformation of R209 in TyrBm structures. The bound SDS molecule is colored as above. **c** The SDS molecule, colored as above, is overlayed with an omit F_o-F_c electron density map (*blue wire*; 1.5σ)

The results obtained for R209H in the presence of SDS led to the investigation of this variant with ILs that were shown to affect the WT monophenolase/diphenolase activity ratio (Fig. 2b). Experiments showed that the influence of BMIM[BF_4] and EAN on the activity of R209H was similar to that of the WT, i.e., the activity ratio was increased. In fact, since the monophenolase/diphenolase activity ratio of R209H is higher than WT by 2.6, the total increase in activity ratio of R209H was higher, in most cases, in an additive manner. For example, in the presence of 40 % EAN the activity ratio of R209H increased by 7-fold in comparison to a 5-fold increase for the WT.

Discussion

TyrBm, has been shown to be highly active in water miscible organic solvents (Shuster and Fishman 2009). The goal of the present work was to examine the influence of water miscible ILs on activity and selectivity and to further investigate the influence of SDS, known to activate TyrBM and other tyrosinases.

The results of this study show that in the presence of water miscible ILs, [BMIM][BF_4] and EAN, the selectivity of TyrBm towards its substrates is altered, thus increasing the monophenolase/diphenolase activity ratio by 5-fold. This is the first investigation of the IL effect on the monophenolase/diphenolase activity ratio of tyrosinase. Tyrosinase from mushroom was previously tested for its diphenolase activity and stability in the presence of various ILs, and not surprisingly, the results were IL dependant (Yang et al. 2009; Yang et al. 2008). Yang et al. tested the activity and stability in the presence of 2–20 % (*v*/*v*) [BMIM][BF4] and [BMIM][MeSO4] and in saturating concentrations of [BMIM][PF6]. The effect on the activity of mushroom tyrosinase was in the following order: [BMIM][PF6]>[BMIM][BF4]>[BMIM][MeSO4], the latter having the most destructive effect on the enzyme. However, it must be highlighted that [BMIM][PF6] is an immiscible IL and was therefore probably present at low concentrations. The enzyme was nearly inactive and unstable in the presence of [BMIM][MeSO4]. The influence of ILs on enzymes has been often elucidated using the Hofmeister series, which is used to explain the effect of salts on enzymes and is related to their kosmotropic/chaotropic properties. The suggested hypothesis is that in aqueous solutions kosmotropic anions and chaotropic cations stabilize the enzyme, while chaotropic anions and kosmotropic cations destabilize it (Yang et al. 2009). This effect is actually caused by the interactions of the ions with the water molecules leading to changes in the interaction between the enzyme and the water molecules. However, the Hofmeister series was shown to be occasionally reversed depending on the enzyme, and more importantly on the anion and the cation which comprise the IL tested (Yang 2009). Our study adds a second complexity to the issue of IL effect, since it presents different results depending on the substrate, tyrosine vs. L-Dopa. The influence of IL on TyrBm activity is first of all IL dependent since two ILs, [BMIM][Cl] and [EMIM][$EtSO_4$], decreased the monophenolase and diphenolase activity while two others, [BMIM][BF_4] and EAN, improved the activity. For [EMIM][$EtSO_4$], a complete inhibition was observed above 10 % IL, consistent with Yang and co-workers' results on mushroom tyrosinase. [BMIM][BF_4] and EAN influenced the activity in a substrate-dependent manner enabling a change in the monophenolase/diphenolase activity ratio. Possible interactions or solvation effects of the IL on the substrates should have affected any

tyrosinase in the same way. However, results with the commercial enzyme, TyrAb, showed that the activity was decreased or unaffected for both tyrosine and L-Dopa simultaneously with these ILs, once again in concomitance with Yang and co-workers' results on [BMIM][BF_4]. It may therefore be suggested that the effect of IL on the water molecules, which is not fully understood, causes a structural change in TyrBm which translates differently towards each substrate. We have previously suggested that different residues take part in each of the two reactions in tyrosinase (Sendovski et al. 2011), and this study further supports this hypothesis. We have not yet succeeded to crystallize the enzyme in the presence of the ILs and subsequently structural data are not available to support this assumption. As in the case of SDS, unsuccessful co-crystallization may be due to slight structural changes in peripheral residues that are critical for lattice formation. The ionic nature of both the ILs and SDS may cause changes in the availability of charged residues that form important crystal contacts. These same effects may also be the source of changes in activity, although this is difficult to ascertain.

This study has further dealt with the effect of SDS on a bacterial tyrosinase. The expected improvement in activity was obtained for the substrates L-tyrosine and L-Dopa, in the presence of SDS concentrations above the CMC and up to 50 mM SDS. Most studies to date, showed a similar effect using 1–2 mM SDS (Cong et al. 2009; Gandia-Herrero et al. 2005; Lopez-Serrano et al. 2002; Saeidian et al. 2007), which is close to the CMC, and these conditions most likely comprise of more free monomers. Cong et al. reported that a non-active hemocyanine could obtain a weak diphenolase activity in the presence of SDS (Cong et al. 2009). This was proposed to occur due to a movement in a blocking domain in the protein facilitated by the presence of SDS. Their study, which determined the CMC of SDS to be ~1 mM, concluded that SDS can modulate the structure of native proteins without destroying them, especially when present above the CMC in the form of micelles. Moreover, a study on the interactions of cellulase with SDS using isothermal titration calorimetry examined the mechanism by which SDS at CMC interacts with proteins. This study found that what may occur is: (1) binding of SDS at monomer level with proteins, (2) interactions between the protein and complete SDS micelles, and (3) SDS induces conformational changes to the protein (Xiang et al. 2006). These possible events may explain the results shown in our study, in which at concentrations well above the CMC, SDS causes an increase in enzymatic activity, probably due to conformational changes caused by interaction of SDS molecules or more likely, micelles. High resistance to SDS was recently shown for a xylanase which retained 100 % of its activity in concentrations up to 100 mM SDS (Zhou et al. 2011). The study suggested that SDS resistance is a property of kinetically stable and proteolytic-resistant proteins. This could also be the case with TyrBm and future work will try to confirm its proteolytic stability. Another possibility which cannot be ruled out is that SDS stabilizes the enzyme in a fashion that causes an increase in the amount of active enzyme molecules in the reaction; this however does not explain the change in substrate specificity, discussed hereinafter.

The 15- to 20-fold improvement in the activity of TyrBm on phenol and catechol, typically poor substrates of this enzyme, obtained by the presence of SDS is unprecedented. The specific activity improved so significantly that TyrBm was more active on phenol than on its native substrate, L-tyrosine. The main difference between the two sets of substrates is the amine and carboxyl side chain on the aromatic ring, which is present in tyrosine and Dopa and causes them to be less hydrophobic than phenol and catechol. The relatively hydrophobic nature of phenol and catechol, or the lack in polar side chain, may be the cause for the low activity of tyrosinase towards them. One of the most important amino acids in the vicinity of the active site of TyrBm, identified in both saturating mutagenesis studies and by X-ray crystallographic studies, was found situated next to a bound SDS molecule in the *TyrBm_SDS* crystal structure. Furthermore, the activity of a variant with a mutation at this residue, R209H, was shown to be affected to an even greater degree by the presence of SDS. It can therefore be suggested that electrostatic changes to this residue—and to other residues yet to be determined—cause the significant change in TyrBm selectivity and stability in the presence of SDS. Alternatively, SDS may cause movement in residue R209 and neighbouring residues such as E158 due to steric hindrance enabling better substrate/product flux.

This study showed for the first time that ILs and SDS can not only improve activity, but influence substrate selectivity. Moreover, the results obtained are especially encouraging from a biotechnological perspective. The application of ILs or SDS as selectivity adjusters for the production of desired catechols of different types, is shown to be possible. Research is currently in progress to evaluate the possibility of combing the two additives in order to promote both enhanced selectivity and activity.

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References

Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr Sect D Biol Crystallogr 66: 213–221.

Brünger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallogr Sect D Biol Crystallogr 54: 905–921.

Burton SG (2003) Oxidizing enzymes as biocatalysts. Trends Biotechnol 21: 543–549.

Claus H, Decker H (2006) Bacterial tyrosinases. Syst Appl Microbiol 29: 3–14.

Cong Y, Zhang Q, Woolford D, Schweikardt T, Khant H, Dougherty M, Ludtke SJ, Chiu W, Decker H (2009) Structural mechanism of SDS-induced enzyme activity of scorpion hemocyanin revealed by electron cryomicroscopy. Structure 17: 749–758.

Decker H, Tuczek F (2000) Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. Trends Biochem Sci 25: 392–397.

Decker H, Schweikardt T, Nillius D, Salzbrunn U, Jaenicke E, Tuczek F (2007) Similar enzyme activation and catalysis in hemocyanins and tyrosinases. Gene 398: 183–191.

Emsley P, Cowtan K (2004) *Coot*: model-building tools for molecular graphics. Acta Crystallogr Sect D Biol Crystallogr 60: 2126–2132.

Gandia-Herrero F, Jimenez-Atienzar M, Cabanes J, Garcia-Carmona F, Escribano J (2005) Differential activation of a latent polyphenol oxidase mediated by sodium dodecyl sulfate. J Agric Food Chem 53: 6825–6830.

Halaouli S, Asther M, Sigoillot JC, Hamdi M, Lomascolo. A (2006) Fungal tyrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. J Appl Microbiol 100: 219–232.

Hernandez-Romero D, Sanchez-Amat A, Solano F (2006) A tyrosinase with an abnormally high tyrosine hydroxylase/dopa oxidase ratio. FEBS J 273: 257–270.

Itoh S, Fukuzumi S (2007) Monooxygenase activity of type 3 copper proteins. Acc Chem Res 40: 592–600.

Karbassi F, Haghbeen K, Saboury AA, Rezaei-Tavirani M, Ranjbar B (2004) Calorimetric, spectrophotometric and circular dichroism studies on the impact of sodium dodecyl sulfate on the mushroom tyrosinase structure. Biologia 59: 319–326.

Kawamura-Konishi Y, Tsuji M, Hatana S, Asanuma M, Kakuta D, Kawano T, Mukouyama EB, Goto H, Suzuki H (2007) Purification, characterization, and molecular cloning of tyrosinase from *Pholiota nameko*. Biosci, Biotechnol, Biochem 71: 1752–1760.

Klabunde T, Eicken C, Sacchettini JC, Krebs B (1998) Crystal structure of plant catechol oxidase containing a dicopper center. Nat Struct Biol 5: 1084–1090.

Leslie AGW (1992) Joint CCP4+ESF-EAMCB Newsletter on protein crystallography: No. 26.

Lopez-Serrano D, Sanchez-Amat A, Solano F (2002) Cloning and molecular characterization of a SDS-activated tyrosinase from *Marinomonas mediterranea*. Pigment Cell Res 15: 104–111.

Martinez MV, Whitaker JR (1995) The biochemistry and control of enzymatic browning. Trends Food Sci Technol 6: 195–200.

McCoy AJ (2007) Solving structures of protein complexes by molecular replacement with Phaser. Acta Crystallogr Sect D Biol Crystallogr 63: 32–41.

Moore BM, Flurkey WH (1990) Sodium dodecyl sulfate activation of a plant polyphenoloxidase. Effect of sodium dodecyl sulfate on enzymatic and physical characteristics of purified broad bean polyphenoloxidase. J Biol Chem 265: 4982–4988.

Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr Sect D Biol Crystallogr 53: 240–255.

Neeley E, Fritch G, Fuller A, Wolfe J, Wright J, Flurkey W (2009) Variations in IC_{50} values with purity of mushroom tyrosinase. Int J Mol Sci 10: 3811–3823.

Nillius D, Jaenicke E, Decker H (2008) Switch between tyrosinase and catecholoxidase activity of scorpion hemocyanin by allosteric effectors. FEBS Lett 582: 749–754.

Nolan LC, O'Connor KE (2007) Use of *Pseudomonas mendocina*, or recombinant *Escherichia coli* cells expressing toluene-4-monooxygenase, and a cell-free tyrosinase for the synthesis of 4-fluorocatechol from fluorobenzene. Biotechnol Lett 29: 1045–1050.

Olivares C, Solano F (2009) New insights into the active site structure and catalytic mechanism of tyrosinase and its related proteins. Pigm Cell Melanoma R 22: 750–760.

Olivares C, Garcia-Borron JC, Solano F (2002) Identification of active site residues involved in metal cofactor binding and stereospecific substrate recognition in mammalian tyrosinase. Implications to the catalytic cycle. Biochemistry 41: 679–686.

Rodriguez-Lopez JN, Escribano J, Garciacanovas F (1994) A continuous spectrophotometric method for the determination of monophenolase activity of tyrosinase using 3-methyl-2-benzothiazolinone hydrazone. Anal Biochem 216: 205–212.

Saeidian S, Keyhani E, Keyhani J (2007) Effect of ionic detergents, nonionic detergents, and chaotropic agents on polyphenol oxidase activity from dormant saffron (*Crocus stivus* L.) corms. J Agric Food Chem 55: 3713–3719.

Selinheimo E, NiEidhin D, Steffensen C, Nielsen J, Lomascolo A, Halaouli S, Record E, O'Beirne D, Buchert J, Kruus K (2007) Comparison of the characteristics of fungal and plant tyrosinases. J Biotechnol 130: 471–480.

Sendovski M, Kanteev M, Shuster V, Adir N, Fishman A (2010) Primary x-ray crystallographic analysis of a bacterial tyrosinase from *Bacillus megaterium*. Acta Crystallogr Sect F Struct Biol Crystallogr 66: 1101–1103.

Sendovski M, Kanteev M, Shuster Ben-Yosef V, Adir N, Fishman A (2011) First structures of an active bacterial tyrosinase reveal copper plasticity. J Mol Biol 405: 227–237.

Shuster Ben-Yosef V, Sendovski M, Fishman A (2010) Directed evolution of tyrosinase for enhanced monophenolase/diphenolase activity ratio. Enzyme Microb Technol 47: 372–376.

Shuster V, Fishman A (2009) Isolation, cloning and characterization of a tyrosinase with improved activity in organic solvents from *Bacillus megaterium*. J Mol Microbiol Biotechnol 17: 188–200.

Skubak P, Murshudov GN, Pannu NS (2004) Direct incorporation of experimental phase information in model refinement. Acta Crystallogr Sect F Struct Biol Crystallogr 60: 2196–2201.

van Holde KE, Miller KI, Decker H (2001) Hemocyanins and invertebrate evolution. J Biol Chem 276: 15563–15566.

Wang G, Aazaz A, Peng Z, Shen P (2000) Cloning and overexpression of a tyrosinase gene *mel* from *Pseudomonas maltophila*. FEMS Microbiol Lett 185: 23–27.

Xiang J, Fan J-B, Chen N, Chen J, Liang Y (2006) Interaction of cellulase with sodium dodecyl sulfate at critical micelle concentration level. Colloids Surf B Biointerfaces 49: 175–180.

Yang Z (2009) Hofmeister effects: an explanation for the impact of ionic liquids on biocatalysis. J Biotechnol 144: 12–22.

Yang Z, Pan W (2005) Ionic liquids: green solvents for nonaqueous biocatalysis. Enzyme Microb Technol 37: 19–28.

Yang Z, Yue YJ, Xing M (2008) Tyrosinase activity in ionic liquids. Biotechnol Lett 30: 153–158.

Yang Z, Yue YJ, Huang WC, Zhuang XM, Chen ZT, Xing M (2009) Importance of the ionic nature of ionic liquids in affecting enzyme performance. J Biochem (Tokyo) 145: 355–364.

Zhou J, Shi P, Zhang R, Huang H, Meng K, Yang P, Yao B (2011) Symbiotic *Streptomyces* sp. TN119 GH 11 xylanase: a new pH-stable, protease- and SDS-resistant xylanase. J Ind Microbiol Biotechnol 38: 523–530.