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ORIGINAL ARTICLE

Mechanism of salt-induced activity enhancement of a marine-derived laccase, Lac15

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Abstract Laccase (benzenediol: oxygen oxidoreductases, EC1.10.3.2) is a multi-copper oxidase capable of oxidizing a variety of phenolic and other aromatic organic compounds. The catalytic power of laccase makes it an attractive candidate for potential applications in many areas of industry including biodegradation of organic pollutants and synthesis of novel drugs. Most laccases are vulnerable to high salt and have limited applications. However, some laccases are not only tolerant to but also activated by certain concentrations of salt and thus have great application potential. The mechanisms of salt-induced activity enhancement of laccases are unclear as yet. In this study, we used dynamic light scattering, size exclusion chromatography, analytical ultracentrifugation, intrinsic fluorescence emission, circular dichroism, ultraviolet–visible light absorption, and an enzymatic assay to investigate the potential correlation between the structure and activity of the marine-derived laccase, Lac15, whose activity is promoted by low concentrations of NaCl. The results showed that low concentrations of NaCl exert little influence on the protein structure, which was partially folded in the absence of the salt; moreover, the partially folded rather than the fully folded state seemed to be favorable for enzyme activity, and this partially folded state was distinctive from the so-called 'molten globule' occasionally observed in active enzymes. More data indicated that salt might promote laccase activity through mechanisms involving perturbation of specific local sites rather than a change in global structure. Potential binding sites for chloride ions and their roles in enzyme activity promotion are proposed.

Keywords Intrinsically disordered protein · Laccase · Marine · Molten globule · Salt · Structure

Abbreviations

2,6-DMP	2,6-Dimethoxyphenol
ABTS	2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
CD	Circular dichroism
DLS	Dynamic light scattering
SEC	Size exclusion chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGZ	Syringaldazine
TFE	Trifluoroethanol
TMAO	Trimethylamine *N*-oxide

Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC1.10.3.2) are multi-copper oxidases capable of oxidizing a wide range of substrates. They have great application

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potential in diverse industries including environmental protection, medicine, organic synthesis and biodegradation (Abadulla et al. 2000; Gupta et al. 2015; Moreno et al. 2012; Riva 2006; Rodgers et al. 2010; Spadaro et al. 1994; Wellington and Kolesnikova 2012; Wellington et al. 2013). The structure of laccase is composed of two or three beta-barrel domains consisting largely of beta-strands (Giardina et al. 2010; Hakulinen and Rouvinen 2015). The active center of laccase consists of one type 1 copper (T1), one type 2 copper (T2) and two type 3 copper (T3). The T1 extracts electrons from the substrate and transfers them to T2/T3 where reduction of oxygen takes place (Giardina et al. 2010; Hakulinen and Rouvinen 2015). Most of the laccases that have been well studied are from plants and fungi. Generally speaking, most plant and fungal laccases are sensitive to halide ions and inhibited by salts (Kittl et al. 2012; Mueangtoom et al. 2010). This restricts their application, especially in cases where high salt concentration is encountered as for dye degradation and wastewater processing. Interestingly, some bacterial laccases, e.g., the CotAs from *Bacillus clausii* and *Bacillus subtilis*, were found to be tolerant to and even activated by salts (Brander et al. 2014). Other salt-tolerant laccases include: laccase PPO1 from *Marinomonas mediterranea*, which can tolerate Cl^- up to 1000 mM (Jimenez-Juarez et al. 2005), and laccase Lbh1 from *Bacillus halodurans* C-125, which was stimulated by NaCl up to 450 mM (Ruijssenaars and Hartmans 2004). Recently, our laboratory obtained a salt-tolerant laccase, Lac15 (GenBank no. ADM87301.1), from a marine microbial metagenome library of the South China Sea. Lac15 exhibited prominent salt (NaCl) tolerance with an IC_{50} of 1500 mM. Furthermore, the activity of Lac15 can be enhanced up to two times below 700 mM NaCl (Fang et al. 2011). Therefore, these laccases could be good candidates for applications in industries involving high concentrations of salt (Singh et al. 2009). Elucidation of the mechanisms through which they tolerate salt and how salts promote their activity may help to improve their application potential.

The mechanisms of proteins adapting to high salt environments have been extensively studied. Compared with mesophilic proteins, halophilic or salt-tolerant proteins contain relatively more negatively charged residues, particularly Asp, and fewer positively charged ones, especially Lys, favoring proper folding of the proteins under extreme conditions as surface acidic residues may interact with hydrated ions to stabilize a folded conformation (Ebel et al. 2002; Fukuchi et al. 2003; Paul et al. 2008). For halophilic enzymes, increasing salt may alter them from a monomeric unfolded state with an opened conformation to an oligomeric folded state with a closed conformation that is catalytically active (Dym et al. 1995; Ortega et al. 2011; Rao et al. 2009). In the case of laccases, it has been proposed that some of them lack specific sites where halide ions could bind to and hamper enzyme activity, which results in salt tolerance (Brander et al. 2014; Enaud et al. 2011; Farnet et al. 2008). However, the mechanism of salt activation of laccases has not been fully understood. Therefore, whether the above general salt adaptation mechanisms for halophilic proteins are applicable to salt activated laccases is unclear. In this study, we used Lac15 as an example to investigate the mechanisms by which salt promotes laccase activity specifically in terms of structural changes. Using dynamic light scattering (DLS), size exclusion chromatography (SEC), intrinsic fluorescence, circular dichroism (CD) and ultraviolet (UV)–visible light absorption, we demonstrated that NaCl does not promote Lac15 activity by stabilizing the protein's global structure. Moreover, we found that a partially folded state rather than completely folded state is favorable to the enzyme activity, which is unusual in enzymes, and that the halide ion may enhance the enzyme activity by binding to specific sites. Our results will contribute to a better understanding of the properties of marine-derived proteins and help with their rational design and application.

Materials and methods

Materials

Trifluoroethanol (TFE) and the substrates of laccase except for 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and syringaldazine (SGZ) were purchased from Aladdin Co., Ltd. (Shanghai); ABTS was from Sangon Biotech, Co., Ltd., China; SGZ and trimethylamine *N*-oxide (TMAO) were from Sigma-Aldrich.

Sequence analysis

The amino acid compositions of Lac15 and other halophilic proteins were calculated using the online program ProtParam in EXPASY (http://web.expasy.org/protparam/).

Protein expression and purification

Escherichia coli BL21(DE3) containing pET22b (+)-*lac15* was cultivated at 37 °C in 400 ml LB medium to OD_{600} of 0.6–0.8. IPTG was added to a final concentration of 0.2 mM to induce the expression of *lac15*. After an additional incubation of 20 h at 16 °C, the culture was centrifuged at 8000*g* for 20 min to collect cells. The cells were disrupted by sonication, and the supernatants were applied to HiTrap™Capto™ Q affinity chromatography (GE Healthcare) to purify target protein. The column was eluted with a linear gradient of NaCl (0.1–1 M) in 20 mM Tris–HCl buffer (pH 8.0) at a flow rate of 1 ml/min. The purified protein

was dialyzed against a 50 mM Na_2HPO_4-KH_2PO_4 buffer (pH 7.5).

The homogeneity of the protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 15% polyacrylamide gel. The protein concentration was assayed at A_{595} based on the Bradford method, with bovine serum albumin as the standard (Sangon Biotech, Co., Ltd., China).

Dynamic light scattering

The samples for dynamic light scattering contained 0.5 mg/ml laccase protein in 50 mM Na_2HPO_4-KH_2PO_4 buffer (pH 7.5), with 100 μM $CuSO_4$ and different concentrations of NaCl. The samples were filtered with a 0.22- or 0.02-μm microfilter just before measurement to remove large particles. The measurements were carried out on a Zetasizer Nano-ZS90 (Malvern), with a 10-mm-thick cuvette and 1 ml volume.

Size exclusion chromatography

The state of Lac15 in different concentrations of NaCl was further assayed by size exclusion chromatography on a Hiload 16/60 Superdex200 column (GE Healthcare), which was equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing the same concentration of NaCl as the protein sample and eluted with the same buffer at a flow rate of 1 ml/min. The protein contents were monitored by ultraviolet absorbance at 260/280 nm.

Analytical ultracentrifugation

Sedimentation velocity experiments were performed in a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter, Brea, CA, USA), equipped with AN-60Ti rotor (4-holes) and conventional double-sector aluminum centerpieces of 12-mm optical path length, loaded with 380 μl of samples (the conditions were the same as those for DLS except for the protein concentration of 0.7 mg/ml) and 400 μl of buffer (50 mM Na_2HPO_4-KH_2PO_4, pH 7.5, with 100 μM $CuSO_4$ and different concentrations of NaCl). Before the run, the rotor was equilibrated for approximately 1 h at 20 °C in the centrifuge before experiments were carried out at 20 °C and 41,000 rpm, using the continuous scan mode and a radial spacing of 0.003 cm. Scans were collected in 3-min intervals at 280 nm. The fitting of absorbance versus cell radius data was performed using SEDFIT software (https://sedfitsedphat.nibib.nih.gov/software) and the continuous sedimentation coefficient distribution $c(s)$ model, covering a range of 0–25 S. Biophysical parameters for the buffer and protein partial specific volume (V-bar) were calculated using the program Sednterp. The molecular weight of the protein was estimated by the Svedberg equation, with the protein assumed to be a spherical particle based on the frictional ratio.

Intrinsic fluorescence spectroscopy

The compositions of the samples for intrinsic fluorescence spectroscopy were the same as those for dynamic light-scattering measurement. The experiments were carried out on an F-4500 FL spectrophotometer (Hitachi Limited, Japan), with excitation wavelength of 293 nm and emission wavelength range of 300–400 nm. All measurements were performed at 20 °C. The spectra of the buffers were subtracted from those of the samples to obtain the spectra of the protein.

Circular dichroism spectroscopy

The samples for CD spectroscopy studies contained 0.1–0.2 mg/ml (far UV) or 1–2 mg/ml (near UV) laccase in 50 mM Na_2HPO_4-KH_2PO_4 buffer (pH 7.5), with 10 μM $CuSO_4$ and different concentrations of NaCl. The measurements were carried out on a Biologic MOS-500 spectrometer (Biologic, France), with a 1-mm-thick cuvette of 400 ml volume (far UV) or a 10-mm-thick cuvette of 4 ml volume (near UV). The scanning ranges were 200–250 nm (far UV) and 250–300 nm (near UV). Every sample was scanned three times to obtain average data, and the data for the sample containing only buffer were subtracted. The secondary structure contents were calculated using a built-in function of the specific software for the spectrometer.

Ultraviolet–visible light absorption spectroscopy

The samples for UV–visible light absorption measurement contained 6 mg/ml laccase protein in 50 mM Na_2HPO_4-KH_2PO_4 buffer (pH 7.5), with 100 μM $CuSO_4$ and different concentrations of NaCl. The measurements were carried out on Nucleic Acid/Protein Analyzer DU®730 (Beckman Coulter), with wavelength range of 300–800 nm and step of 1 nm. All measurements were performed at 20 °C. The spectra of the buffers were subtracted from those of the samples to obtain the spectra of the protein.

Enzyme assay

Laccase activity was quantified using SGZ as substrate. The assay mixture consisted of 10 μl appropriately diluted laccase protein and 970 μl of 50 mM Na_2HPO_4-KH_2PO_4 buffer (pH 7.5) containing 10 μl 10 mM SGZ ($\varepsilon_{525} = 65{,}000\ M^{-1}\ cm^{-1}$) and 10 μl 10 mM $CuSO_4$. After incubation at 45 °C for 5 min, the mixture was transferred into an ice-water bath for 30 s to stop the reaction. The absorbance was measured at 525 nm. One activity unit (U) was defined as the amount of

laccase required to oxidize 1 µmol of substrate per minute. Reactions with heat-treated laccase were used as controls.

The effect of NaCl on Lac15 activity was investigated for different substrates. The substrates and absorption wavelengths of the corresponding products were as follows: ABTS, 420 nm; 2,6-dimethoxyphenol (2,6-DMP), 468 nm; SGZ, 525 nm; guaiacol, 465 nm; dopamine hydrochloride, 475 nm; potassium ferrocyanide trihyrate, 405 nm. Optimum pH for catalytic activity was determined for the substrates by measurement of the product formation rate in 50 mM Na_2HPO_4-KH_2PO_4 buffer (pH 6.0–7.5), 50 mM Tris–HCl (pH 8.0–9.0) and CH_3COONa-CH_3COOH (pH 4.0–5.5). The concentration of NaCl was varied between 0 and 3 M. All reaction solutions included, besides of the substrates in the buffer at their optimum pH, 100 µM $CuSO_4$ and Lac15 at appropriate concentrations. All reactions were carried out at 45 °C, and the reaction time varied for different substrates: ABTS, 3 min; 2,6-DMP, 5 min; SGZ, 5 min; guaiacol, 15 min; dopamine hydrochloride, 5 min; potassium ferrocyanide trihyrate, 5 min. The reaction was terminated by placing the reaction mixture in an ice-water bath for 30 s. The enzyme activity was calculated from the absorbance of the products. Reactions with heat-treated laccase and particular concentrations of NaCl were used as controls.

Effect of pH on the salt dependence of Lac15 activity was measured at pHs ranging from 6.0 to 8.5. The buffers used for different pHs were 50 mM Na_2HPO_4-KH_2PO_4 (pH 6.0–7.5) and 50 mM Tris–HCl (pH 8.0).

The kinetic parameters of the enzyme were tested under optimum conditions, and K_m and V_{max} were calculated by nonlinear regression of the Michaelis-Menten equation (Eq. 1) with OriginLab 8.5 (OriginLab Corp., USA). All measurements were performed in triplicate and repeated at least twice:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]}. \quad (1)$$

Results and discussion

Lac15 has some characteristics of halophilic proteins

Lac15 is a protein of 439 amino acids, of which negatively charged residues (particularly Asp) account for 12.7% (Asp accounts for 6.8%) and positively charged residues (Lys) account for 7.7% (0.9%). As a comparison, the laccase from the halophilic archaeon *Haloferax volcanii*, LccA, whose activity is promoted by and tolerant to NaCl (up to 1.4 M) (Uthandi et al. 2010), contains 16.9% (8.3%) negatively charged residues (Asp) and 5.9% (0.7%) positively charged ones (Lys). Another two proteins from *H. volcanii*, the ubiquitin-like proteins SAMP1 and SAMP2, which show significant halophilicity (Liao et al. 2013; Ye et al. 2013), contain 22.7% (12.1%) and 19.5% (10.6%) negatively charged residues (Asp) and 9.1% (3.0%) and 5.7% (1.1%) positively charged ones (Lys), respectively. Its relatively much lower ratio of acidic residues and comparable proportion of basic residues indicate that Lac15 is not a classic halophilic protein. This is consistent with the fact that Lac15 displays substantial activity in the absence NaCl, and the activity is initially enhanced and subsequently suppressed by increasing salt, with the maximum activity occurring in 200 mM NaCl (Fang et al. 2011).

NaCl has a minor effect on the predominance of monomeric active Lac15 in solution

For some salt-activated proteins, increasing salt promotes their transformation from monomer to oligomer, with the former being inactive and the latter active (Dym et al. 1995; Rao et al. 2009). To test whether this is the case for Lac15, we used DLS and SEC to measure the states of the enzyme in different concentrations of NaCl. When measured with DLS, Lac15, filtered with a 0.22-µm filter, showed an increasing diameter along with rising NaCl concentration (Fig. S1 in online resource 1), denoting salt-enhanced protein aggregation. After filtering the samples with a 0.02-µm microfilter, DLS identified a main component with a diameter of about 7 nm (Fig. 1a), indicating the predominance of the monomeric state among the low-molecular-weight components. To evaluate the proportions of the aggregated and monomeric components, protein concentration and enzyme activity were measured for Lac15 in different concentrations of NaCl before and after filtration with a 0.02-µm filter. The results showed that the protein concentration and enzyme activity were almost completely maintained after the filtration (data not shown), indicating the aggregates accounted for only a minor proportion of the Lac15 in solution and that the monomeric state is the active form of the enzyme. The low-molecular-weight components of Lac15 in solution were further assayed by SEC, and the result showed that in all tested concentrations of NaCl most Lac15 was eluted at around 79–82 ml, with the elution volume increasing with NaCl concentration (Fig. 1b). After calibration with a protein reference, the apparent molecular sizes of the main component of Lac15 in various concentrations of NaCl were calculated to be 43–52 kDa, with the size increasing with inverse correlation to NaCl concentration (Table S1 in online resource 1). This supports the view that the monomeric state is the predominant species in solution, and this was confirmed by AUC measurements. The AUC results showed that there was a roughly linear anti-correlation between apparent sedimentation coefficient (s) of the main component of Lac15 and salt concentration. Given that the protein was a spherical particle in various concentrations of NaCl, judged

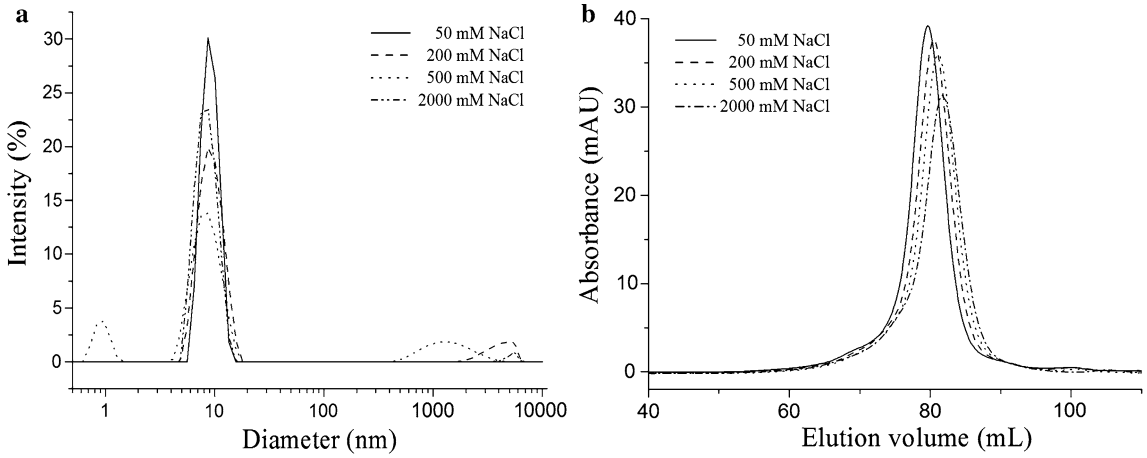

Fig. 1 Hydrodynamic properties of Lac15 in different concentration of NaCl after filtration with a 0.02-µm microfilter, measured by **a** dynamic light scattering and **b** size exclusion chromatography [on Superdex200 (16/60) column]

by similar frictional ratios, the reduction in *s* at higher salt concentration might be due to the increased solvent density and viscosity suppressing diffusion of the protein (Table S2 in online resource 1). The molecular weights of the main components of Lac15 in different concentrations of NaCl, estimated using the sedimentation coefficients, were consistent with a monomeric protein as expected (Fig. S2 and Table S3 in online resource 1). As the ratio of monomeric Lac15 to the oligomeric states did not change in line with the enzyme activity in low concentrations of NaCl (Table S3 in online resource 1), we assume there is no correlation between the protein oligomerization and the activity enhancement of Lac15.

Notably, in the SEC result there was variation between the apparent molecular sizes of monomeric Lac15 in different concentrations of NaCl (Table S1 in online resource 1). Speculatively, this might be due to a variation in protein conformation, i.e., Lac15 may be more compact in higher concentrations of NaCl. This was supported by the AUC data, which also showed a decreased sedimentation coefficient for monomeric Lac15 when NaCl increased (Fig. S2 in online resource 1) although we cannot distinguish the contributions of the protein conformation and buffer density used in these data.

NaCl hardly perturbs the tertiary structure of Lac15

In folded, soluble, globular proteins, hydrophobic cores bury aromatic side chains. As these aromatic groups are sensitive to the polarity of their surroundings, they can be used as a probe for the tertiary structure of the proteins. Lac15 has 20 Phe, 4 Tyr and 7 Trp, so its tertiary structure was readily measured using intrinsic fluorescence especially that of Trp. As shown in Fig. 2a, in the absence of NaCl, Lac15 exhibited a maximum emission wavelength of about 345 nm, slightly blue shifted from 360 nm under denaturing conditions (6 M

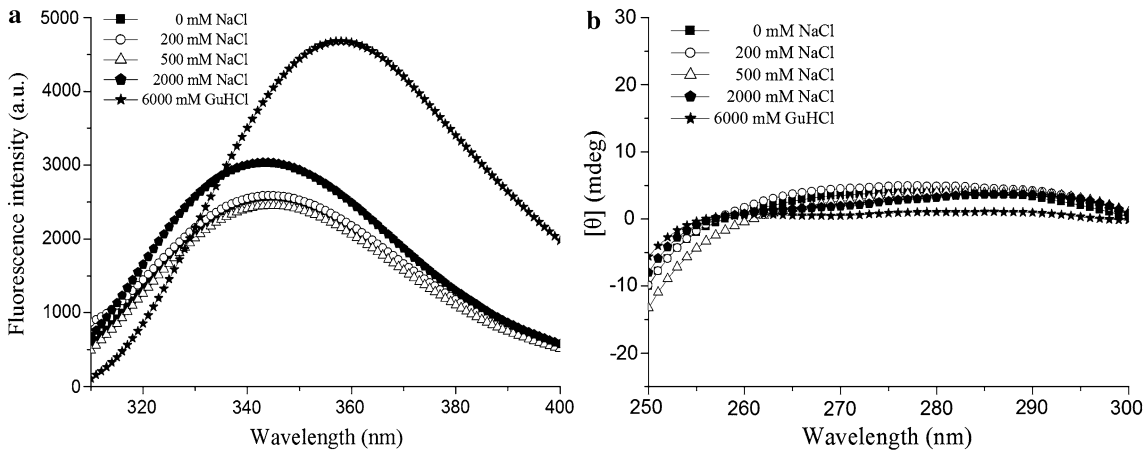

Fig. 2 **a** Intrinsic fluorescence emission and **b** near UV circular dichroism spectra of Lac15 in different concentrations of NaCl

GuHCl). This indicated that without salt, the protein adopted a partially folded state, in which some aromatic residues were packaged into a somewhat hydrophobic environment. As the salt concentration increased, the emission maximum stayed at 345 nm, denoting the hydrophobic environment had been maintained. This implied that the tertiary structure of Lac15 was hardly perturbed by NaCl. The above data were supported by near UV CD, where the slight signal deviation from near zero under unfolding conditions (6 M GuHCl) denoted the non-polar environment of aromatic side chains and the nearly overlapping spectra indicated the environment was only slightly perturbed by the salt (Fig. 2b).

NaCl varies the secondary structure of Lac15

Since the tertiary structure of Lac15 was hardly perturbed by NaCl, the difference in activity might result from a change in secondary structure rather than variation in the aggregation state. To demonstrate this, a CD spectrum was recorded for Lac15 with different concentrations of NaCl. As expected, in the absence of NaCl, Lac15 displayed a spectrum signifying a partially unfolded state in which the characteristics of beta structures (trough near 216 or 220 nm for parallel or antiparallel beta sheets, respectively; the main secondary structure component of laccases) were faint, while the signal for the random coil (trough near 200 nm) was strong (Fig. 3). As the salt concentration increased, the signals became more profound for the beta structure and weaker for the random coil, denoting that a more folded structure had formed. This was consistent with the SEC and possibly AUC results, which indicated that Lac15 was a little more compact in high concentrations of NaCl. Interestingly, in 200 mM NaCl where Lac15 exhibited maximum activity (Fang et al. 2011), the protein displayed a spectrum of a partially folded/unfolded rather than a fully folded state, close to that in the absence of NaCl (Fig. 3). Moreover, at a higher concentration (2000 mM) of NaCl, although Lac15 contained a more folded structure, with the signal of the beta sheet around 220 nm more profound (Fig. 3), its activity was suppressed rather than enhanced. One explanation for these seemingly odd results may be that NaCl on the one hand drives Lac15 to fold to enhance the enzyme activity, while on the other hand salt binds to the protein at specific sites to inhibit the activity; in low concentrations of salt, the former effect prevails, while at high concentrations the situation reverses.

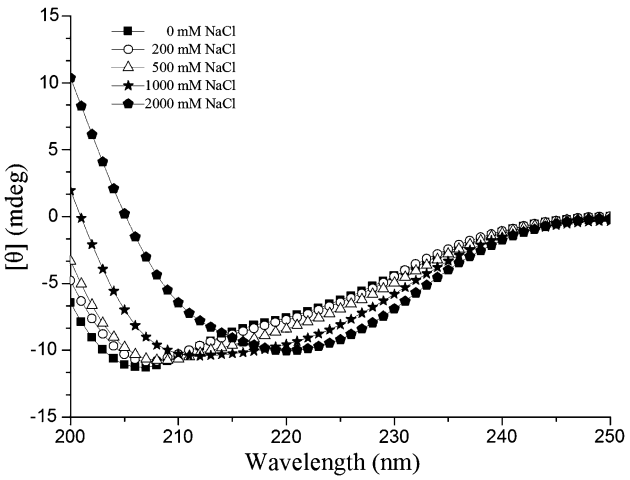

Fig. 3 CD spectra of Lac15 in different concentrations of NaCl

TFE and TMAO affect Lac15 in patterns somewhat similar to that of NaCl

To examine the above assumption, we measured the effects on the structure and activity of Lac15 of a reagent that has a generic effect on the secondary structure of proteins but does not bind to specific sites. As Lac15 is perturbed by NaCl mainly in its secondary structure, we used TFE, which facilitates the intramolecular hydrogen bonding that stabilizes secondary structures (Dammers et al. 2015; Roccatano et al. 2002; Shao 2014), to promote protein folding. The CD spectra showed that, like NaCl, TFE increased the beta structure content of the protein, e.g., with 39% beta sheet in 20% TFE (Fig. 4a). Also similar to NaCl, TFE promoted enzyme activity at low concentrations, with maximum activity occurring at 2%, while suppressing the activity at higher concentrations (Fig. 4b). Moreover, at TFE concentrations conveying maximum and minimum activities, the protein was folded to extents comparable to those seen with NaCl (Fig. S3 in online resource 1). All these data suggest that the conformation of Lac15 with a fully folded structure is not beneficial for its enzyme activity. However, it should be noted that the structure induced by TFE, especially at high concentrations, is not necessarily the same as what the protein presents under physiological conditions. In addition, we could not exclude that TFE may also have some unknown specific effects on the protein.

As TFE may induce a non-native structure and therefore lead to an erroneous conclusion, we measured the effects of TMAO, a natively occurring osmolyte that stabilizes proteins by influencing the hydrophobic force (Liao et al. 2017; Mondal et al. 2015), on the structure and activity of Lac15. TMAO is produced by some marine organisms to maintain a folded active state of proteins under stress conditions and thus frequently is used to induce unfolded proteins to fold (Baskakov and Bolen 1998; Liao et al. 2017; Wang and Bolen 1997; Yancey and Siebenaller 2015). As expected, TMAO induced Lac15 to form more secondary structure as its concentration increased (Fig. 5a). However, the content of the secondary structure induced by TMAO, e.g., a 35% beta sheet at 1200 mM, was relatively low compared to that induced by NaCl and TFE. Given the fact that the structure-promoting

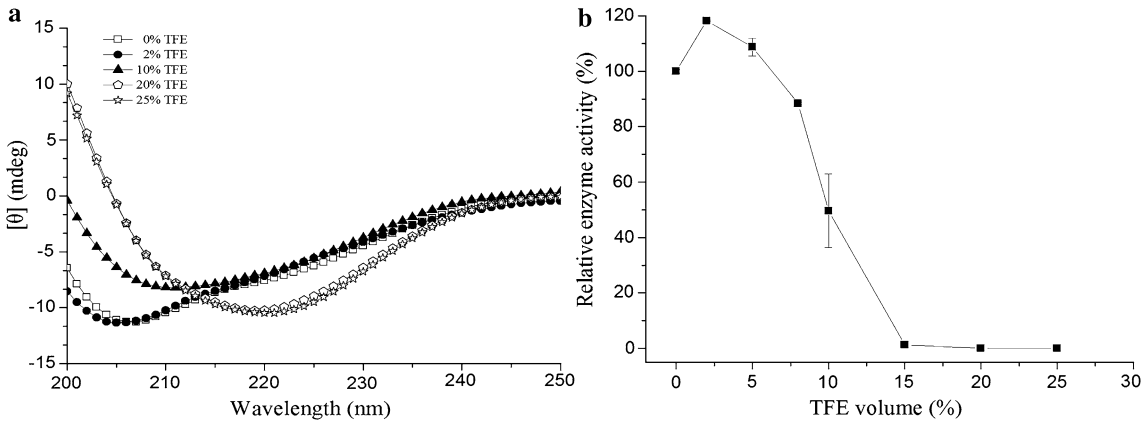

Fig. 4 Effect of TFE on **a** the CD spectrum and **b** enzyme activity of Lac15

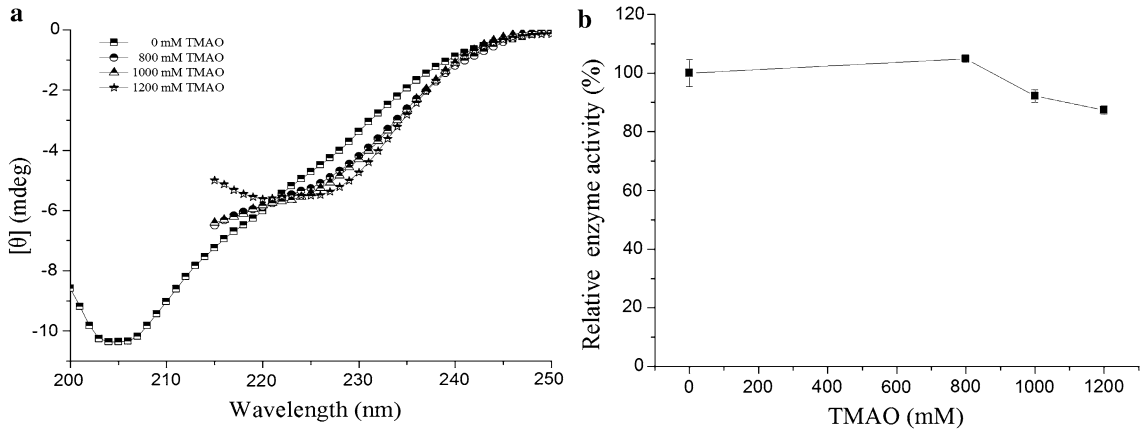

Fig. 5 Effect of TMAO on **a** the CD spectrum and **b** enzyme activity of Lac15

force of TMAO is derived mainly from its chaotropic effects, which enhance hydrophobic interactions (Wang and Bolen 1997), its weak strength in driving hydrogen bond formation and inducing secondary structure is understandable. The mild structural variation caused by TMAO might explain why corresponding changes in enzyme activity were relatively faint (Fig. 5b). In summary, the patterns of NaCl, TFE and TMAO affecting Lac15 were similar to some extent: they promoted the enzyme activity when the protein was partially folded to a certain degree and suppressed the activity when the protein was further folded beyond that.

NaCl influences both the substrate affinity and catalytic rate of Lac15

All the above results suggested that NaCl does not promote the activity of Lac15 by inducing a folded state of the protein. Therefore, it was proposed that the impact of NaCl might be attributed to some sites in the protein where the salt could bind specifically. To show this, we measured the enzyme kinetic parameters of Lac15 in different concentrations of NaCl. The results showed that both K_m and k_{cat} varied with the concentration of NaCl (Fig. S4 in online resource 1; Table 1), indicating both substrate binding and catalysis were perturbed by the salt in both enhancement and inhibition effects. This implied the halide ion interferes with not only the sites the substrate interacts with, but also those involved in electron/proton transfer. Notably, neither of NaCl's effects on the substrate affinity and catalytic rate was simple promotion or inhibition. The salt enhanced or reduced K_m and k_{cat}, depending on the concentration, implying the halide ion acted through more than a simple competitive, uncompetitive or any other single mechanism. In addition, K_m and k_{cat} changed in the same directions, upwards then downwards, with increased NaCl, indicating potential correlation between the effects of the salt on substrate affinity and catalytic rate. However, when NaCl increased, the k_{cat}/K_m did not stay constant; it changed in the opposite direction to K_m and k_{cat}. This implied the correlation between the effects of NaCl on the substrate affinity and catalytic rate is a complex instead of simple one.

Table 1 Enzyme kinetic parameters of Lac15 in different concentrations of NaCl, measured with SGZ as substrate

Enzyme	NaCl (mmol L^{-1})	V_{max} (μmol L^{-1} min^{-1})	K_m (μmol L^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μmol^{-1} L s^{-1})
Lac15	0	9.44 ± 0.89	36.09 ± 2.26	9.39	0.26
	200	9.80 ± 0.70	41.13 ± 4.35	9.75	0.24
	500	7.50 ± 0.50	27.80 ± 2.58	7.46	0.27
	2000	3.64 ± 0.20	12.52 ± 1.42	3.62	0.29
	2500	2.42 ± 0.09	7.75 ± 0.79	2.41	0.31

Salt dependence of Lac15 activity at different pHs is similar

Specific interaction of halides with laccases has been extensively studied. Evidence indicates an impact of high pH on the salt dependence of laccases, which suggests competition between OH^- and halide ions (Xu 1997; Xu et al. 1998). The salt dependence of Lac15 at different pHs showed similar profiles, with the inhibition effect of NaCl decreased at higher pH (Fig. 6). This is consistent with the idea that OH^- competes with halide ions for the same sites, with higher affinity and a less inhibitory effect (Xu 1997; Xu et al. 1998). Like the inhibitory effect, the enhancement effect of NaCl was decreased when pH increased (Fig. 6). In addition, at pH 8, the optimum NaCl concentration of Lac15 was higher than at lower pHs. This indicates that the halide ion promotes the enzyme activity through a similar mechanism to that of inhibition, i.e., binding to specific sites by negative charge.

NaCl has different influences on the activities of Lac15 toward different substrates

To prove NaCl interferes with the substrate binding of Lac15, the activities of Lac15 on different substrates at different concentrations of NaCl were measured. As shown in Fig. 7, on one hand, the profiles of NaCl dependence of Lac15 activity to different substrates differed significantly from each other; on the other hand, the extent of the enzyme activity promoted, if any, by NaCl and the optimum concentration of the salt varied greatly between the substrates. These data support the suggestion that NaCl does not affect Lac15 activity via global protein structural changes, or else all the profiles for different substrates should be similar. In addition, these data confirmed that NaCl may influence Lac15 activity by interfering with substrate binding; therefore, around the T1 where the substrates bind there might be some specific binding sites for NaCl.

NaCl interferes with Cu^{2+} binding

One of the ways NaCl could interfere with the catalysis of Lac15 is by impacting the Cu^{2+} constitution, which is critical for electron/proton transfer by laccases. To test this, a UV–visible light absorption spectrum was recorded for Lac15 in different concentrations of NaCl. The results showed that in 200 mM NaCl the protein displayed a similar spectral profile to that in 0 mM NaCl (Fig. 8), denoting the Cu^{2+} constitution was not perturbed by low concentrations of NaCl. However, when NaCl increased further, the spectra displayed varied profiles particularly at around 370 nm, denoting the Cu^{2+} constitution specifically of T2/T3 was

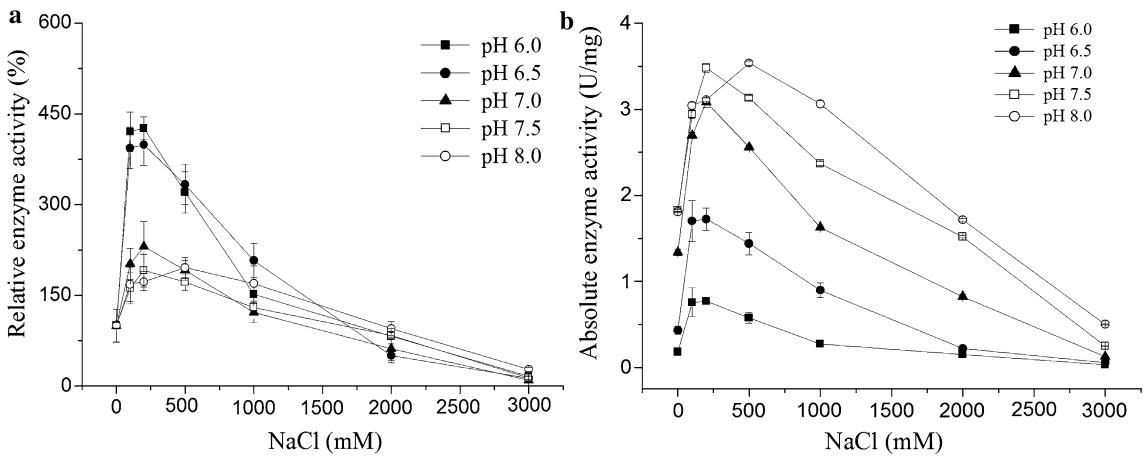

Fig. 6 NaCl dependence of **a** the relative and **b** absolute enzyme activities of Lac15 at different pHs

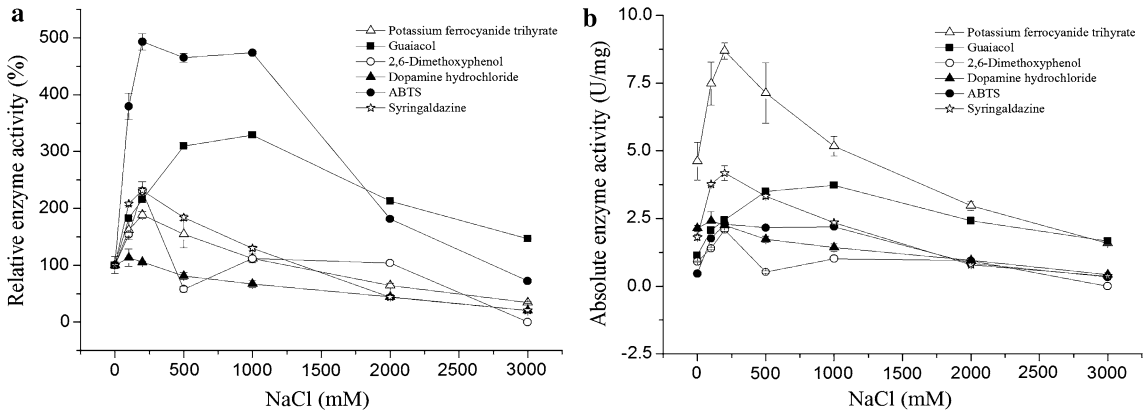

Fig. 7 Effects of NaCl on **a** the relative and **b** absolute activities of Lac15 toward different substrates

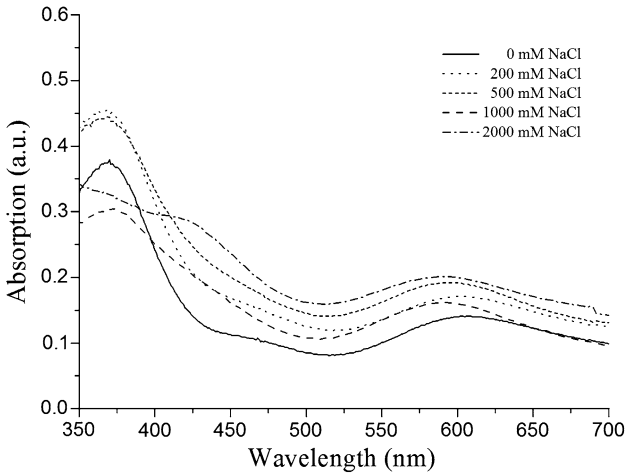

Fig. 8 Ultraviolet–visible absorption spectra of Lac15 in different concentrations of NaCl

changed by high concentrations of NaCl. These results indicated that NaCl did not enhance the enzyme activity of Lac15 by establishing the Cu^{2+} constitution but suppressed the activity by impairing the Cu^{2+} constitution.

Conclusion

As a protein derived from a marine environment, it is not surprising that Lac15 exhibits maximum activity in 200–500 mM NaCl. The effects of halide ions on proteins, particularly laccases, have been studied in molecular detail. The results showed Cl^- may generally stabilize backbone hydrogen bonds by surface interactions (Christensen and Kepp 2013). So, the fact that the secondary structure of Lac15 increased along with NaCl is also understandable. Interestingly, at optimum NaCl concentration, Lac15 is partially folded rather than fully folded, distinguishing it from most halophilic proteins whose maximal activities always correlate with their unique folded states. There are a few enzymes whose activities are not dependent on ordered conformations, with their active forms being intrinsically disordered (Uversky 2013; Vendruscolo 2010). However, all these exceptional enzymes possess a nearly full secondary structure but partial tertiary structure compared with their folded counterparts and are thus referred to as 'molten globular enzymes' (Pervushin et al. 2007; Uversky et al. 1996; Vamvaca et al. 2004, 2008; Woycechowsky et al. 2008; Zambelli et al. 2012). Lac15 appears to be another example of an intrinsically disordered enzyme, with its most active form being a partially folded/unfolded state similar to a molten globule. However, in contrast to molten globular enzymes, the most active form of Lac15 contains an incomplete secondary structure but almost complete tertiary structure. Disruption of the tertiary structure with denaturant GdnHCl caused loss of the secondary structure of Lac15 in 200 mM NaCl as expected (Fig. S5 in online resource 1). These data indicate the dominance of the tertiary structure in Lac15, distinctive from typical molten globule enzymes whose secondary structure is dominant. To our knowledge, Lac15 is the first atypical molten globule example of an intrinsically disordered enzyme. The local flexibility or dynamics in enzymes is believed to be crucial for enzyme catalysis because of their influence on substrate binding, product release and effective barriers for the catalyzed reaction (Hammes-Schiffer and Benkovic 2006; Hammes 2002; Olsson et al. 2006). Particularly, internal motions in the 'molten globular enzymes' may favor the enzyme achieving a rapid turnover (Pervushin et al. 2007). Thus, what the enhanced disorder in the secondary structure of Lac15 means for its function is intriguing.

Since our results here show that NaCl does not promote Lac15 activity by driving the enzyme protein to fold, we

speculate that salt activation of the laccase might be due to a specific interaction between the Cl^- ion and local sites of the protein. Halide binding to the laccase copper cluster has been extensively studied (Brander et al. 2014; Champagne et al. 2013; Enaud et al. 2011; Kepp 2015; Xu 1997). However, most such studies focus on inhibition rather than enhancement of the enzyme activity. Cl^- is assumed to bind to sites around T1 to competitively or non-competitively inhibit the enzyme activity by influencing electron transfer (Champagne et al. 2013; Enaud et al. 2011). Similarly, Cl^- may bind to some sites around T2/T3, e.g., the water exit channel, to affect the enzyme activity by influencing proton transfer (Brander et al. 2014; Kepp 2015). This is supported by the crystal structure of laccase from *Melanocarpus albomyces* (Hakulinen et al. 2002, 2008), in which a Cl^- is coordinated directly to T2. Lac15 has some characteristics similar to the 'halophobic enzymes' reported in these studies, e.g., lining the water exit channel there are residues that could repulse Cl^- (data not shown). However, this may explain only the salt tolerance but not the salt-induced promotion of Lac15 activity.

Our data indicate that Cl^- may bind to the sites around the T1 and T2/T3 copper centers to promote as well as inhibit the enzyme activity of Lac15. Intriguingly, in contrast to some other laccases whose substrate binding as well as catalysis was competitively inhibited by Cl^- bound around T1 (Enaud et al. 2011), Lac15 interacts with the substrate with higher affinity when its activity is inhibited, while, at the optimum NaCl concentration, Lac15 interacts with the substrate with the lowest affinity. This implies that there might be several sites around the T1 of Lac15 where Cl^- could bind and act specifically. Cl^- activation is observed in other enzymes besides laccases. For example, in α-amylases from *Pseudoalteromonas haloplanktis*, Cl^- near the active site is believed to help polarization of the hydrolytic water and protonation of the catalytic residue to activate the enzyme (Aghajari et al. 2002). We therefore speculate that the Cl^- bound around the T2/T3 in laccase may act in a similar way to facilitate oxygen reduction, therefore enhancing the enzyme activity. Further structural and biochemical evidence is needed to demonstrate this hypothesis and provide more details about the mechanism of salt-induced activity enhancement of laccases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no interest conflict.

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