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Cross-linking and immobilisation of different proteins with recombinant *Verrucomicrobium spinosum* tyrosinase

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ABSTRACT

This paper reports on the cross-linking and immobilisation of various proteins by the recombinant tyrosinase from *Verrucomicrobium spinosum* (*Vs*-tyrosinase). In general it is found that *Vs*-tyrosinase can readily cross-link proteins with a low degree of complexity, such as casein, but that the enzyme cannot readily cross-link well folded protein substrates such as lysozyme, myoglobin, cytochrome *c* or *Candida antarctica* lipase B (CALB). However, the inclusion of phenolic compounds (phenol or caffeic acid) to reaction mixtures of these proteins can greatly enhance the levels of cross-linking. For example it is possible to prepare cross-linked aggregates of industrially applicable enzymes such as CALB by simply incubating it with *Vs*-tyrosinase and phenol. The resulting aggregates can be collected by centrifugation and retain high levels of activity and may find applications in biocatalysis.

1. Introduction

The cross-linking of proteins has a diverse range of applications such as the changing of textures by modification of food proteins (Gerrard, 2002), the formation of enzymes into aggregates for biocatalyst recovery (Sheldon, 2007) to surface immobilisation of proteins for microarray applications (Endrizzi et al., 2006). There are several reported ways to cross-link proteins. Chemical reagents such as glutaraldehyde have been used extensively (Migneault et al., 2004). Alternatively, cross-linking has also been achieved using enzymes. For example, transglutaminase is well known to be able to directly cross-link polypeptide chains (Yokoyama et al., 2004) while oxidoreductases such as tyrosinase (Mattinen et al., 2008), peroxidase (Matheis and Whitaker, 1984) and laccase (Steffensen et al., 2008) can indirectly lead to cross-linking of proteins by activation of tyrosine residues on proteins. We here describe studies of the ability of a novel tyrosinase enzyme we have recently cloned and expressed from the Gram negative bacterium *Verrucomicrobium spinosum* (Fairhead and Thöny-Meyer, 2010) to perform such protein cross-linking reactions.

The tyrosinases are a family of binuclear copper enzymes found in many species of animals, fungi and bacteria which use phenol-like starting materials to produce a variety of pigments including melanin (Claus and Decker, 2006; Halaouli et al., 2006; Marusek et al., 2006). These type III copper proteins are capable of two activities, monophenolase or cresolase activity (E.C. 1.14.18.1) and diphenolase or catecholase activity (EC 1.10.3.1). Both reactions result in the formation of reactive quinones, and these species are important intermediates in the biosynthesis of melanin and related polyphenol compounds.

Tyrosinase has been proposed for use in a variety of biotechnological and biocatalysis applications (Halaouli et al., 2006). Given the ability of the enzyme to react with phenols and its di-copper redox centres it has been used as an electrochemical biosensor for a range of phenolic compounds (Gu et al., 2009). As mentioned above the enzyme can also react with tyrosine residues found on polypeptides, and the reactive quinones formed allow for protein cross-linking to chitosan films as well as protein-protein cross-linking (Lewandowski et al., 2006; Thalmann and Lotzbeyer, 2002). The applications of tyrosinase mediated cross-linking are wide-ranging. For example, tyrosinase mediated immobilisation of protein A has been used to greatly enhance the sensitivity of affinity membranes (Ahmed et al., 2006). Tyrosinase has also been used to modify the gel forming properties of chicken breast myofibrils (Lantto et al., 2007). In addition, the enzyme has been used to immobilise various proteins to chitosan films (Chen et al., 2001) as well as to create a capture and purification system based around chitosan beads and a penta-tyrosine tag (Lewandowski et al., 2006).

Although most studies use the commercially available tyrosinase from mushroom (Ahmed et al., 2006; Chen et al., 2001; Thalmann and Lotzbeyer, 2002), it has been reported that tyrosinases from different sources, e.g. *Pyncoporus* sp. (Halaouli et al., 2005) and *Trichoderma reesei* (Lantto et al., 2007), have the ability to more efficiently cross-link proteins than mushroom tyrosinase

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Table 1
Some molecular properties of the proteins used in this work.

Protein	Abbreviation	Molecular weight (KDa)	Number of tyrosine and lysine residues	
Bovine casein sodium salt	BNaC	12–15% αS1-casein 3–4% αS2-casein 9–11% β-casein 2–4% κ-casein	N/A	N/A
Bovine α-casein (S1/S2)	BαC	23/24.3	10/12	14/24
Bovine β-casein	BβC	23.6	4	11
Bovine κ-casein	BκC	19	9	9
Horse myoglobin	HMb	17	2	19
Horse heart cytochrome *c*	HHC	11.7	4	19
Hen egg white lysozyme	HEWL	14.3	3	6
Candida antarctica lipase B	CALB	33.4	9	9
V. spinosum tyrosinase	*Vs*-TYR	37.9	10	9

Information in the table is taken from the product information from Sigma–Aldrich and from public databases such as NCBI (http://www.ncbi.nlm.nih.gov/protein/).

(Mattinen et al., 2008). Given the aforementioned reports and the wide range of applications, we investigated in the work presented here the cross-linking activities of a new example of these enzymes.

2. Materials and methods

2.1. Materials

In general chemicals and proteins were purchased from Sigma–Aldrich (Buchs Switzerland). The tyrosinase used in this work from the Gram negative bacterium *V. spinosum* is an enzyme, which unlike the well studied *Streptomyces* species tyrosinases, does not require a caddy protein for activation. Rather, the enzyme activity is stimulated by proteolytic removal of a C-terminal peptide. The recombinant trypsinised pro-tyrosinase from *V. spinosum* was prepared as previously described (Fairhead and Thöny-Meyer, 2010). The various model proteins used in this work (all purchased from Sigma–Aldrich) and some of their relevant molecular properties are described in Table 1.

2.2. Protein cross-linking

Protein cross-linking experiments were performed by incubating 1 μL of 10 mg/mL *Vs*-tyrosinase with 100 μL of 2 mg/mL substrate protein (see Table 1) in 0.1 M sodium phosphate buffer pH 7.0 plus either a further 100 μL of buffer or 100 μL of 0.2 mg/mL caffeic acid or 100 μL of 0.2 mg/mL phenol. Samples were then incubated for 1 h at room temperature in a rotary mixer (18 rpm) and an aliquot was removed for analysis by SDS PAGE.

2.3. Preparation of phenol enzyme aggregates

Candida antarctica lipase B (CALB) was immobilised into tyrosinase generated phenol aggregates by incubating 50 μL of 0–10 mg/mL CALB plus 200 μL of 0.1 M sodium phosphate pH 7.0 containing 0–50 mg/mL phenol plus 1 μL of 0–10 mg/mL *Vs-tyrosinase*. Samples were then incubated at room temperature in a rotary mixer (18 rpm). After 1 h 749 μL of buffer were added and a 20 μL sample of the mixture was removed (for activity analysis) and the sample centrifuged at 20,000 *g* for 10 min. A 20 μL sample of the supernatant was removed (for activity analysis) and the pellet resuspended in 980 μL of buffer. A 20 μL sample of the resuspended pellet was then taken, also for activity analysis. The pellet was washed a further 4 times and finally resuspended in 960 μL of buffer, and a 20 μL sample was taken for activity analysis.

2.4. CALB enzyme assay

The activity of CALB was measured using *p*-nitrophenylbutyrate (pNPB) (Martinelle et al., 1995). An aliquot of the enzyme preparation (typically 20 μL) was added to 280 μL of assay buffer in a 96 well micro titre plate. The assay buffer contained 0.1 M sodium phosphate buffer pH 7.0 and 2 mM pNPB. The absorbance increase at 405 nm over 30 min was then recorded on a SynergyMX plate reader (Biotek Instruments GmbH). The linear rate of absorbance increase per minute at 405 nm was then calculated from the first 10–20 min of data. All reactions were performed at a constant temperature of 30 °C and at least 3 separate experiments were performed.

2.5. SDS PAGE

SDS PAGE analysis was performed using standard methods (Laemmli, 1970) and gels were subsequently stained with Coomassie brilliant blue. The PageRuler plus prestained protein ladder was used as a marker in all gels (Fermentas, GmbH).

2.6. Heme staining

SDS Gels were stained for heme (using peroxidase activity as an indicator of its presence) using *o*-dianisidine as a substrate (Braun and Thöny-Meyer, 2004). Gels were run as normal (except samples were not heated prior to loading as was the case for Coomassie stained gels, i.e. 95 °C for 10 min), rinsed for 10 min in distilled water and then fixed for 10 min in a 10% solution of trichloroacetic acid. The gel was then rinsed for 10 min in distilled water twice more and placed in the assay solution for around 5 min. Colour development was then stopped by rinsing the gel thoroughly in distilled water. The assay solution contained 50 mM sodium citrate buffer pH 4.4 plus 0.7% hydrogen peroxide and 1 mg/mL *o*-dianisidine.

3. Results and discussion

3.1. Vs-tyrosinase mediated protein cross-linking

It has been reported by several authors that tyrosinase enzymes may be useful in cross-linking proteins and that this may have several applications, such as in the food industry (Anghileri et al., 2007; Burzio et al., 2000; Dabbous, 1966; Mattinen et al., 2008). This activity is hypothesised to arise from exposed tyrosine residues on the surface of the target protein being converted to a quinone like species and that this unstable compound further reacts with either free amino or sulfhydryl groups or other activated tyrosines (Mattinen et al., 2008) to give a cross-linked product (supplementary information Fig. S1). To investigate this potential application we performed cross-linking experiments using the *Vs*-tyrosinase and various proteins as model substrates (Table 1). The results of these experiments are presented in Fig. 1.