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The fusions of elastin-like polypeptides and xylanase self-assembled into insoluble active xylanase particles

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ABSTRACT

We fused the genes of elastin-like polypeptides (ELPs) and xylanase and then expressed them in *Escherichia coli*. Unexpectedly, the fusion proteins self-assembled into insoluble active particles as the ELPs underwent a hardly reversible phase transition. The specific activity of the particles was 92% of the native counterparts, which means it can act as a pull-down handler for converting soluble proteins into active aggregates. We evaluated the characterizations of the insoluble active xylanase particles in detail and the results were encouraging. The pH optimum (6.0) of the particles was the same as the free one, but the optimum pH range was 5–7, while the free xylanase was 6–7. The free xylanase had an optimum temperature of 50 °C, whereas the insoluble active xylanase particles shifted to 70 °C. The pH stability, thermostability and storage stability of the xylanase particles increased significantly when compared with the free xylanase. We also observed an increase of the K_m values of the free xylanase from 0.374 g L^{-1} to 0.980 g L^{-1} at the insoluble state. The considerable higher activity and stability of the xylanase particles were much like immobilized xylanases and could be valuable for its industrial application.

1. Introduction

Elastin-like polypeptides (ELPs) are polymers composed of repeats of the pentapeptide (Val-Pro-Gly-Xaa-Gly)*n*, where Xaa is the amino acid except Pro; *n* describes the number of repeats (Meyer and Chilkoti, 2004). ELPs have been synthesized and used for applications such as protein purification, tissue engineering and drug delivery (Meyer and Chilkoti, 1999). To be purification tag is one of the most important uses of ELPs. Till now, the green fluorescence protein, blue fluorescence protein, thioredoxin, chloramphenicol acetyl transferase, and calmodulin have been purified successfully (Trabbic-Carlson et al., 2004; Chow et al., 2006; Lim et al., 2007). Similar to other affinity tags, a ELPs tag can be genetically engineered into recombinant proteins. The proteins or peptides fused to ELPs show similar stimulus responsive behavior. The ELPs fusion proteins are soluble at temperatures lower than their transition temperature (T_t) and become insoluble aggregates above the T_t. Thus, the ELPs fusion proteins can be purified without chromatography using a technique termed inverse thermal cycling (ITC) (Luan et al., 1990; Meyer and Chilkoti, 2002). Usually, the transition is reversible and the aggregates dissolve when the temperature is below T_t. However, in our case, we found most of the fusions of ELPs and xylanase became insoluble particles at the temperatures below T_t when they experienced an ITC. At the same time, the insoluble particles showed high xylanase activity and stability. They would be potentially useful biocatalysts.

In recent years, there was an increasing trend towards using enzymes for biocatalysis (Faber and Kroutil, 2005; Kaur and Sharma, 2006). More and more researchers paid attention to the active insoluble protein aggregates, which is commonly known as active inclusion bodies (IBs). (García-Fruitós et al., 2005; Arié et al., 2006). For example, an ionic self-assembling peptide ELK16 attached to the carboxyl termini of four model proteins could effectively induce to form active IBs in *E. coli*. The proteins included lipase A, amadoriase II (AMA), β-xylosidase (Xyn B), and green fluorescent protein (GFP), and three of the aggregates retained comparable specific activities with the native counterparts (Wu et al., 2011). Other tags could also induce the formation of active IBs under conditions of heterologous expression in *E. coli*. These tags included the cellulose binding domain from *Clostridiu mcellulovorans* (Shoseyov and DoI, 1990; Goldstein et al., 1993; Nahalka and Nidetzky, 2007), the human β-amyloid peptide Ab42 (F19D) (García-Fruitós et al., 2005) and a modified apolipoprotein A-I mimetic amphipathic peptide 18A (Wu et al., 2011). The peptide-mediated protein aggregations have potential applications in immobilized biocatalysis (Roessl et al., 2010), bioassays (Nahálka et al., 2009), and biomaterials (García-Fruitós et al., 2009).

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In this paper, we will report the stable aggregates of the fused xylanase with ELPs as the tag. To purify the xylanase by the ITC, the fusions self-assembled into aggregates and became insoluble when the temperature was lower than T_t. What's more, the aggregates retained comparable specific xylanase activities and higher stability. We regarded the insoluble active particles as immobilized xylanase, and compared its catalytic properties with the free one. This is the first report of active xylanase particles on ELPs.

2. Materials and methods

2.1. Materials

E. coli strains BL21 (DE3), BLR (DE3), plasmid pUC-19-ELPs, and ELPs [KV8F-20] were preserved in our lab. Restriction endonucleases including *PflM I*, *Bgl* I, *Nde* I, *EcoR* I, *Sfi I*, were purchased from Shanghai Generay. The protein and DNA Marker were obtained from Takara, and the birchwood xylan was from Sigma. The xylanase, namely the Sox-M2, was from *Streptomyces*, with five mutations N12H, N13D, F15Y, 16 F in the N-terminal sequence (Zhang et al., 2010). The amino acid sequence of the coil S was (AGAGAGPEG)10. The coil S between ELPs and xylanase was designed to decrease the mutual interference of ELPs and xylanase (Wheeldon et al., 2009). For facilitating the subsequent non-chromatographic purification, we modified the PET-22b with the sequence of CATATGAGCAAAGGGCCGGGCTGGCCGTGATAAGAATTC to replace the histidine tag. The gene of Sox-M2 and S were synthesized and sequenced by Shanghai Biological Technology Co.

2.2. Construction and expression of the SoxB-M2-S-ELPs fusion gene

The molecular biology techniques, such as preparation, transformation of cells, plasmid extraction and enzyme digestion, are from reference (Sambrook et al., 1989). SoxB-M2-S-ELPs gene was synthesized, prepared by *Nde*, and *Hind*III digestion and then connected to pUC19 and got the cloning vector pUC19-SoxB-M2-S-ELPs. Then the cloning vector was digested by *Nde I*, and *Hind*III and the obtained SoxB-M2-S-ELPs gene was inserted into the modified pET-22b to yield the expressing vector pET-22b-SoxB-M2-S-ELPs. After confirming the sequence, the expression plasmids were introduced into *E. coli* BL21 and *E. coli* BLR (DE3). The condition of the strain incubation and recombinant gene inducing were described in detail in an earlier paper (Fu et al., 2012).

2.3. Preparation and quantification of the insoluble active xylanases

The fusions were aggregated by increasing the temperature of the cell lysate to 40 °C with 0.8 mol L^{-1} Na_2CO_3. The aggregates were separated from solution by centrifugation at 35–45 °C at 10,000–15,000 × *g* for 15 min. The supernatant was decanted and discarded, and the pellet containing the fusion protein was resolubilized in PBS by agitation in cold. The insoluble protein aggregates were separated from the clarified soluble fractions at 4 °C by centrifugation (15,000 × *g* for 15 min), then the soluble protein was decanted and retained. The insoluble fractions were the active xylanase aggregates, we washed the aggregates once with PBS and resuspended in the same volume of PBS. The amounts of xylanase in both fractions were determined densitometrically by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using bovine serum albumin (BSA) as standard (Peternel et al., 2008). Protein concentration was determined using BSA as the protein standard.

2.4. Dynamic Light Scattering (DLS) measurements

The average size and size distribution of the insoluble active xylanase particles was determined by DLS using Zetasizer Nano (Malvern Instruments, Worcestershire, UK), configured with a 173° scattering angle and equipped with a HeNe laser (633 nm) with an output power of 10 mW. Solutions were vigorously stirred before analysis and each sample measured in triplicate.

2.5. Xylanase activity assays

The activities of the xylanase in both purification and immobilization were measured by the dinitrosalicylic acid (DNS) method (Bailey et al., 1992). Briefly, 200 μL of diluted xylanase were mixed with 200 μL of a 10 mg/mL suspension of birchwood xylan (Sigma–Aldrich) and incubated for 30 min at the optimum temperature. Then 400 μL of DNS reagent were mixed and incubated for 5 min at 100 °C. One international unit (IU) is defined as the amount of xylanase that releases 1 μmol of reducing sugar per minute.

2.6. pH and temperature properties of the free and insoluble active xylanases

The effects of pH on the free and insoluble active xylanases were studied by assaying both preparations at different pH. The pH values range from 5 to 10 (100 mmol L^{-1} Na_2HPO_4 citric acid buffer for pH 4.0–5.0; 100 mmol L^{-1} phosphate buffer for pH 6.0–8; 100 mmol L^{-1} Na_2CO_3–$NaHCO_3$ buffer for pH 9.0–10.0.). To determine the pH stability of them, xylanases were incubated 1 h in buffers of various pHs (as mentioned above) and the residual activity was measured by the standard assay procedure.

The optimum temperature for the free and insoluble xylanases was determined by incubating them with substrates at temperature ranging from 40 to 80 °C. To determine the thermal stability of xylanase, the free and insoluble xylanases were incubated at 40, 50, 60 and 70, 80 °C for 60 min (pH 6.0). Aliquots of the free and insoluble xylanase were withdrawn at different time intervals, and the remaining activities were measured under the standard conditions.

2.7. Kinetic parameters of the free and insoluble active xylanase

Kinetic parameters for both free and insoluble active xylanase were determined by using a HP8453 UV–vis spectrometer. In brief, Birchwood xylan solutions were prepared at different concentrations (0, 1.1, 1.25, 1.43, 1.67, 2, 2.5, 3.33, 5, 10 g L^{-1}) in PBS buffer (0.05 mol L^{-1}, pH7.0). A fixed volume of the free and insoluble active xylanase was added to each birchwood xylan solution and incubated for 10 min separately. Then each reaction mixture was centrifuged immediately, and the absorbance of the supernatant was monitored at 540 nm. The substrate saturation curves of various xylanase samples with birchwood xylan were fitted into Michaelis–Menten kinetics and the corresponding K_m and V_{max} were obtained from Lineweaver–Burk plot.

2.8. Storage stability of the free and insoluble active xylanase

Storage stability of the free and insoluble active xylanase was investigated by calculating the remaining activity periodically during 44 days of incubation at 4 °C and 30 °C, respectively. The residual activities were calculated as percentage of the initial activity.