Bioresource Technology 101 (2010) 331–336

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Improved cross-linked enzyme aggregates for the production of desacetyl β-lactam antibiotics intermediates

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ARTICLE INFO

Article history:
Received 10 June 2009
Received in revised form 5 August 2009
Accepted 5 August 2009
Available online 3 September 2009

Keywords:
CLEA
Biocatalysis
Acetyl xylan esterase
Cephalosporin
Particle size

ABSTRACT

Cross-linked enzyme aggregates (CLEAs) are reported for the first time for a recombinant acetyl xylan esterase (AXE) from *Bacillus pumilus*. With this enzyme, CLEAs production was most effective using 3.2 M (80%-saturation) ammonium sulfate, followed by cross-linking for 3 h with 1% (v/v) glutaraldehyde. Particle size was a key determinant of the CLEAs activity. The usual method for generating particles, by short-time vortexing was highly inefficient in terms of enzyme activity yields. In contrast, the use of long-time vortexing increased activity recovery, and a novel approach consisting in the utilization of a commercial mechanical cell disruptor which is based on a reciprocating movement recovered all the enzyme activity in few seconds. In the CLEAs thus produced, the enzyme was much more resistant to shear, heat and extreme pH values than the soluble enzyme. The CLEAs were highly effective in transforming fully 7-amino cephalosporanic acid and cephalosporin C into their corresponding desacetyl derivatives, which are important advanced intermediates in the production of semisynthetic β-lactam antibiotics. The operational stability of such CLEAs was remarkable, with a half life of 45 cycles. Therefore, the new methodology used here should decrease the industrial cost of the CLEAs, both in terms of biocatalyst production and reusability.

1. Introduction

Enzymes are useful tools in industry because of their possible applications in stereo- and regio-specific catalysis. Numerous efforts are devoted to immobilize various enzymes by binding them to solid carriers, with the goal of facilitating their use in industrial processes (Hartmeier, 1988). An unavoidable consequence of this approach to immobilization is the dilution of the enzyme by the presence of the inert carrier, decreasing space–time yields and lowering productivity (Tischer and Kasche, 1999). In principle, an alternative approach for production of enzymes in particulate form without requiring a solid carrier is the aggregation of enzymes molecules by adding salts such as ammonium sulfate or organic solvents such as acetone, acetonitrile or isopropanol (Brown and Glatz, 1986). Apparently, the native conformation of the protein is stabilized in the aggregate. Treatment of the aggregated protein using a chemical cross-linking agent (generally a suitable dialdehyde) can render the enzyme permanently particulate and generally provides additional stabilization (Clair and Navia, 1992; Fernandes et al., 2005). Hence, cross-linked enzyme aggregates (CLEAs), in addition to exhibiting good mechanical stability, can be highly active, since they do not include large amounts of foreign particulate non-enzymatic material, and they can have increased stability under the frequently non-optimal or even harsh conditions used in industrial processed (Roberge et al., 2009) such as in synthetic organic chemistry, in biomedical applications and in environmental catalysis (Kim et al., 2008; Wong and Whitesites, 1994), favourably affecting the recycling of the catalyser for repeated use. However, an improvement in the production of CLEAs is needed since with the cross-linking reaction, washing periods, centrifugations and filtrations, the size of CLEAs aggregates increases. As a result, internal mass-transfer limitations are unavoidable, which reduces global activity. Mixing with magnetic stirrers or long vortexing periods resulted in a great loss of activity (Aytar and Bakir, 2008).

In recent years, there has been growing interest on esterases due to their broad array of substrate specificities and versatility for catalysis (Jaeger and Reetz, 1998; Jaeger and Eggert, 2002; Bornscheuer, 2002). Therefore, these enzymes are good candidates for CLEAs design. In this search, acetyl xylan esterases (AXEs) are of particular interest since they can remove the acetyl group from the

Abbreviations: CLEAs, cross-linked enzyme aggregates; AXE, acetyl xylan esterase; *p*-NPA, *p* -nitrophenyl acetate; 7-aminocephalosporanic acid, 7-ACA; cephalosporin C, CPC.

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doi:10.1016/j.biortech.2009.08.016

3-position of cephalosporin C (CPC) and of 7-amino cephalosporanic acid (7-ACA), yielding desacetylated advanced intermediates which are highly valuable for the production of semisynthetic β-lactam antibiotics.

This paper reports the production of improved cross-linked aggregates of the purified recombinant AXE from *Bacillus pumilus* CECT5072 by using a fast mechanical mixing device (FastPrep). The temperature stability, pH stability and specificity towards the above indicated advanced β-lactams support the value of this novel CLEAs for use in industrial processes.

2. Methods

2.1. Materials

Recombinant AXE with a 36-amino-acid N-terminal extension containing a six-histidine tag was produced as previously reported (Martínez-Martínez et al., 2007) from *Escherichia coli* Rosetta (DE3)pLys cells (from Novagen, USA) transformed with plasmid pET28a6His–AXE, a pET28a (Novagen, USA) derivative which contains the cloned AXE gene from *B. pumilus* CECT5072. To maximize enzyme purity, the reported purification procedure (Martínez-Martínez et al., 2007) was modified by adding, prior to the Ni^{2+} chelating affinity chromatography step, an ion exchange chromatography step using an 5-mL HiTrapCaptoQ column (GE Healthcare) with elution of the enzyme using a 0–500 mM NaCl gradient in 50 mM Tris–HCl, pH 8.0. CPC, 7-ACA and their corresponding desacetyl derivatives were obtained from Bioferma Murcia S.A. (Spain). High purity glutaraldehyde (*BioChemika*) and ammonium sulfate (*BioUltra*) were obtained from Fluka. Other reagents, including Bradford, were from Sigma (Madrid, Spain).

2.2. CLEAs production

All steps were carried out at 4 °C. Pilot tests of aggregation with ammonium sulfate were carried out by adding to 1 mL of a 1 mg/mL solution of the purified enzyme in 50 mM Tris–HCl pH 8.0, the required volume of saturated ammonium sulfate solution to bring the mixture to the desired degree of saturation with the salt. Pilot tests of aggregation with acetonitrile or with *tert*-butanol (final concentrations up to 90% v/v) were carried out in the same way except for the addition of the cold solvent instead of adding the salt solution. After 1 h, the appropriate amount of a freshly prepared 25% (v/v) aqueous solution of glutaraldehyde was stirred into the suspensions to attain the desired concentration of the dialdehyde (concentrations tested, 0.2%, 0.5%, 1% or 2% glutaraldehyde). At various times from the addition of the glutaraldehyde, 100-μl aliquots were suspended in 900 μl of 150 mM potassium phosphate pH 7.5 and were centrifuged (16000$g \times 2$ min) determining enzyme activity in the supernatant and in precipitates.

The pilot assays yielded optimally active CLEAs when using ammonium sulfate at 80% saturation, and a 3-h cross-linking period with 1% glutaraldehyde. To scale-up CLEAs production, these conditions were used with an initial enzyme solution containing 5.3 mg/mL purified AXE. At the end of the cross-linking period the entire suspension was centrifuged (16000$g \times 2$ min), washing three times the precipitated CLEAs by repeated cycles of suspension in potassium phosphate 150 mM pH 7.5 and centrifugation. The final preparation of the CLEAs, stored at 4 °C, was stable for several weeks.

2.3. CLEAs scanning electron microscopy

Droplets from CLEAs suspensions were placed on moist filter papers and dehydrated serially (10 min each step) by passage through a graded series of acetone solutions (30%, 50%, 70% and 90%) to 100% acetone and dried at the critical point of CO_2 (Anderson, 1951). After gold sputtering, the samples were visualized with a Jeol T6.100 (Japan) scanning electron microscope (SEM) operated at 15 kV.

2.4. CLEAs dispersion and sizing of the resulting particles

Two systems were used for the disruption of the aggregates. One approach is widely used, consisting on vortexing at medium speed with a Heidolph (Germany) Reax Control vortex. A novel approach was used in which the CLEAs were subjected to reciprocating mixing using a FastPrep-24 sample preparation system (M.P. Biomedicals, CA, USA) at a setting of 6.0 m/s. The degree of dispersion attained by both procedures was monitored by bright-field optical microscopy at a final enlargement of 40×, taking digital images that were analyzed planimetrically by image processing (MIP 4.5 image analysis software, Digital Image System, Barcelona, Spain).

2.5. Enzyme assays

Acetyl xylan esterase activity was determined colorimetrically at 25 °C by following the hydrolysis of 2 mM *p*-nitrophenyl acetate (*p*-NPA) to *p*-nitrophenol (ε_{405} = 16980 M^{-1} cm^{-1}) in 50 mM potassium phosphate buffer pH 8.0. For assay of the 7-ACA and CPC desacetylating activities, the enzyme (generally 0.04 mg/mL of CLEAs) was incubated at 20 °C with one or the other of these compounds at the indicated concentration (range, up to 225 mM) in 150 mM potassium phosphate pH 7.5. The reaction was terminated by centrifuging out the CLEAs (16000$g \times 2$ min). Samples from the supernatant were subjected to reversed-phase HPLC using a Kromasil C8 column and a mobile phase consisting of 30% methanol containing 15 mM tetrabutylammonium hydrogen sulfate and 10 mM potassium phosphate buffer pH 6.5, monitoring the optical absorption in the effluent at 254 nm (Martínez-Martínez et al., 2007). Under these chromatographic conditions and at a flow rate of 1 mL/min, the desacetylated products appear as a well resolved peaks from the corresponding substrates ($Rt_{7\text{-ACA}}$ = 3.8 min, $Rt_{\text{desacetyl-7-ACA}}$ = 5.0 min, Rt_{CPC} = 3.3 min, $Rt_{\text{desacetyl-CPC}}$ = 4.6 min). The amount of product was estimated by peak integration, using as standards the desacetylated substrates.

2.6. Enzyme stability assays

Thermal stability was monitored by incubating the enzyme (either in soluble form or in the CLEAs), in a solution of 50 mM Tris–HCl pH 8.0, at the indicated temperatures, taking samples after the periods of time specified for determining enzyme activity in the standard assay at 25 °C using *p*-NPA as the substrate.

For monitoring stability at different pHs soluble or CLEAs enzyme was incubated for 24 h at 30 °C in the appropriate 50 mM buffer at the desired pH (Na acetate, K phosphate, Tris–HCl and boric acid/borate for the respective pH ranges for 3–5, 6–7, 8–9 and 10–11), determining enzyme activity at pH 8.0 in the standards assay by diluting samples of the solutions in the enzyme assay medium.

3. Results

3.1. Optimization of CLEAs production

The AXE enzyme, overexpressed as a soluble protein in *E. coli* Rosetta (DE3)pLys cells and purified by a three step procedure, was highly homogeneous, yielding on SDS–PAGE a single protein