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Control of process-induced asparaginyl deamidation during manufacture of *Erwinia chrysanthemi* L-asparaginase

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ABSTRACT

During the manufacture of the chemotherapeutic enzyme *Erwinia chrysanthemi* L-asparaginase, a small proportion (approximately 5–15%) of acidic variants, including deamidated species, are observed. Although the deamidated forms appear to have similar specific activity and quaternary structure to the unmodified enzyme, monitoring and control of these forms is important from a regulatory perspective. The extent of Asn to Asp deamidation directly correlates with the time taken to thaw the *Erwinia* cells. *Erwinia* L-asparaginase is a tetrameric enzyme containing one site, Asn_{281}, theoretically very labile to deamidation due to the sequence Asn-Gly. Structurally, this part of the protein sequence is completely buried inside the tetramer, but solvent-exposed upon tetramer dissociation. During the cell thawing and alkaline lysis sequence of the process, lengthening the cell thaw times by up to 24 h allowed tetramer to reassociate, protected Asn_{281} from deamidation and reduced the acidic species content of the L-asparaginase from approximately 17% to 9% as measured by weak cation-exchange (WCX) HPLC. The correlation of cell thaw time with acidic species content was also confirmed using capillary zone electrophoresis (CZE) and peptide mapping. These studies demonstrate that cell thaw time is an important, if unexpected, control variable for L-asparaginase deamidation.

1. Introduction

L-Asparaginase is an important chemotherapeutic enzyme used for the treatment of acute lymphoblastic leukaemia (ALL) [1]. It functions by depriving leukaemic cells of Asn, an amino acid which is essential for their growth and proliferation by conversion to Asp [2]. Commercially, the enzymes available for clinical use are *Escherichia coli* L-asparaginase (EcA) and *Erwinia chrysanthemi* L-asparaginase (ErA). Presently EcA is used in a majority of patients, but some patients develop a hypersensitivity to EcA, in which case ErA is utilised [3,4].

The proprietary name of ErA for clinical use is Erwinase® or Erwinaze®. ErA exists as a 140,000 Da homotetramer in its active form, and has an isoelectric point of pH 8.6 [5]. Erwinase® was first developed in the late 1960s and 1970s [6,7] and is marketed in many countries around the world.

As a part of an on-going process understanding programme for ErA manufacture, a number of sensitive analytical characterisation techniques have been developed. These techniques have been validated and subsequently utilised to confirm the process robustness and the product quality of ErA. Monitoring of product variants, such as deamidated and oxidised forms of the protein, is an important part of process and product characterisation. Although deamidated variants of ErA appear to retain their activity and function, characterisation of them is nonetheless important from a regulatory perspective.

Deamidation is one of the most frequent non-enzymatic degradation reactions to occur in proteins and peptides [8] and is most common in Asn residues but is also possible in Gln and others. In a deamidation reaction involving an Asn residue, the α-nitrogen C-terminal to the Asn attacks the Asn side-chain carboxylate group, resulting in a cyclic succinimide and evolution of ammonia. The cyclic succinimide intermediate is then hydrolysed to form a mixture of Asp and isoAsp [9]. Completion of the reaction leads to a lowering of the protein pI due to the change in residue charge from neutral to negative. This causes charge heterogeneity in protein products and creation of acidic protein species. Deamidation in protein biopharmaceuticals is undesirable, as deamidation at certain sites may lead to loss of secondary structure, tertiary structure, protein function, and/or enzyme activity [10,11].

Deamidation in L-asparaginase was first observed for EcA using isoelectric focusing in the 1970s [12,13], where it was noted that the change in isoelectric point (pI) had no apparent effect on enzyme activity. Deamidation of EcA was also studied by other researchers [14] who investigated deamidated forms using electrophoresis and

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amino acid analysis. More recently, researchers have used two-dimensional gel electrophoresis to separate post-translationally modified forms (including deamidated forms) of EcA and ErA [15].

The purpose of the work described in this paper was to further understand the design space of the ErA manufacturing process, by linking process conditions to the extent of protein deamidation in the final product. We show how the tertiary and quaternary structure of the ErA homotetramer protects an apparently labile Asn residue from deamidation under normal conditions, and how dissociation of the tetramer can lead to alkali-induced conversion of Asn residues to Asp.

2. Materials and methods

2.1. Cell thaw experiments

Reagents used were obtained from Sigma (Gillingham, UK) unless otherwise indicated. Samples of *E. chrysanthemi* cell paste and associated process streams were obtained from the HPA Development & Production, Porton Down, UK. Frozen cell pastes, stored at −20 °C, were thawed at room temperature and once thawed, the 24 h held portion was held at 2–8 °C until further processing. The pH and conductivity during cell thaw experiments was adjusted using NaOH and acetic acid and pH and conductivity were monitored using a Jenway (Staffordshire, UK) Model 3540 metre and associated pH and conductivity probes and calibration standards. Resin capture was accomplished using Whatman (Maidstone, UK) CM32 microgranular cation exchange cellulose, and eluate concentration was performed using Amicon (Millipore, Watford, UK) Ultra-15 centrifugal filter units with a 10 kDa MWCO regenerated cellulose membrane.

2.2. Protein and activity assays

The protein content of all samples described in this work was determined using the technique of Lowry [16]. The asparaginase activity assay used is based on the Berthelot reaction and methods described in the literature [17,18].

2.3. Dissociation and reassociation experiments

Native PAGE analysis was conducted using the Blue Native (BN) technique developed by Schägger and von Jagow [19] with Invitrogen™ (Paisley, UK) Novex™ 4–16% Native PAGE Bis-Tris gels, Novex™ Native PAGE running buffers and sample buffers, fixing in methanol/acetic acid, and destaining in acetic acid solution. Size-exclusion chromatography (SEC) was performed using TSK gel G3000SWXL column (Tosoh, King of Prussia, PA, USA) and a Waters HPLC workstation (Elstree, UK) in an isocratic mobile phase consisting of 100 mM sodium chloride in pH 7.2 sodium phosphate buffer.

2.4. WCX assay

The acidic species content in process samples was monitored using a Weak Cation-Exchange (WCX) HPLC assay, using a Dionex ProPac WCX-10 column (Dionex, Leeds, UK) and a Waters HPLC workstation (Elstree, UK). The column was run using a gradient from 10 to 300 mM sodium chloride in pH 6.2 sodium phosphate buffer and monitored at a wavelength of 220 nm.

2.5. Capillary electrophoresis analysis

The Capillary Zone Electrophoresis (CZE) analyses of ErA samples were performed using a PEREGRINE high-performance capillary electrophoresis (CE) system from deltaDOT (London, UK) equipped with a 512 diode array detector with detection at 214 nm. Bare fused silica capillaries were obtained from CM Scientific (Silsden, UK).

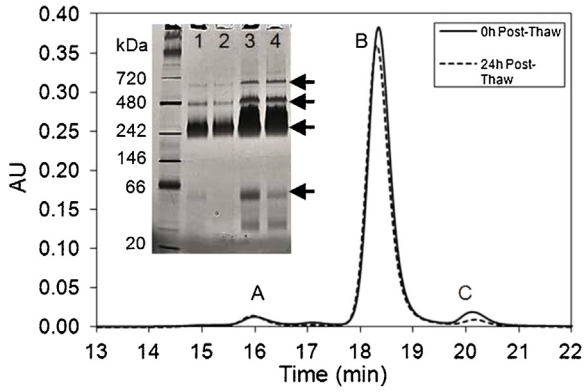

Fig. 1. Effect of time on freeze–thaw induced ErA tetramer dissociation. The main frame shows an SEC trace of purified ErA at 0 h and 24 h post-thaw time, with the aggregate (A), tetramer (B) and monomer subunit (C) marked on the trace. Inset figure shows a Native PAGE gel image of the same experiment, with the location of monomeric (bottom arrow), tetrameric (middle arrow) and higher-order aggregate (top two arrows) species indicated. Lane 1 – 1 μg/well at 0 h post-thaw, Lane 2 – 1 μg/well at 24 h post-thaw, Lane 3 – 5 μg/well at 0 h post-thaw, and Lane 4 – 5 μg/well at 24 h post-thaw.

2.6. Peptide mapping assay

Peptide mapping of purified ErA samples was conducted using tryptic digest followed by reversed-phase UPLC. Samples were denatured for 10 min at 65 °C in the presence of Rapigest SF (Waters, Elstree, UK) in pH 7.4 phosphate buffer, cooled, then digested in pH 7.4 phosphate buffer for 2 h at 37 °C using sequencing-grade trypsin (Promega, Southampton, UK). The digests were terminated by addition of guanidinium HCl and injected into an ACQUITY UPLC BEH C18 (Waters, Elstree, UK) reversed-phase 2.1 mm × 150 mm column with 1.7 μm particle size and 130 Å pore size using a Waters UPLC workstation. The column was run in the presence of 0.1% TFA using a gradient between 1% acetonitrile in water and 100% acetonitrile.

3. Results and discussion

The manufacturing process for ErA has been described previously [6,20] and comprises the fermentation of *E. chrysanthemi*, alkaline cell lysis and extraction of ErA [21], purification of the ErA using chromatography, and formulation of the final drug product using lyophilisation. Between each of these major process stages, the ErA intermediates are held frozen, and thawed before further processing occurs.

Freezing and thawing of ErA has been shown [22,23] to cause partial dissociation (approximately 40% as measured using the activity assay) of the active ErA tetramer into the inactive monomeric 35 kDa subunits. The dissociation and subsequent loss of activity was reported [23] to be more severe when ErA is frozen at low temperatures (−40 °C and lower). When ErA is frozen at −20 °C, we have observed this dissociation to be less extensive as well as reversible, such that dissociated 35 kDa monomers reassemble into the 140 kDa tetramer over a time scale of approximately 24 h. This effect, which has been observed over the course of many years of ErA process and assay development, is demonstrated in Fig. 1. Two samples of purified ErA were frozen, and one thawed and left at 2–8 °C for 24 h, the other thawed immediately before simultaneous analyses using both SEC and native PAGE. Disappearance of the monomeric ErA subunit (peak C by SEC) over the 24 h time scale is