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THE EFFECT OF DEGRADATION ON THE EFFICACY AND UTILITY OF THE
BIOPHARMACEUTICAL ENZYME ERWINIA CHRYSANTHEMI L-
ASPARAGINASE

DAVID PAUL GERVAIS

A thesis in partial fulfilment of the requirements of Oxford Brookes University for the
degree of Doctor of Philosophy on the basis of published work

Submitted May 2015

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I wish to dedicate this thesis to the memory of my late wife, Lisa (Ferris) Gervais, who lost her battle with leukaemia, and inspired me to try to help in some small way towards fighting this disease.

ABSTRACT

A common problem in the development of biopharmaceutical proteins is the lack of understanding around the implications of aggregation, or of their gradual, often partial degradation. Often aggregated or degraded protein variants comprise a small percentage of a biopharmaceutical protein sample, so it is difficult to assess their true impact on product quality and clinical efficacy. The assumption that biopharmaceutical producers adopt is that partially degraded or aggregated protein variants are necessarily undesirable, which may not always be the case. This problem is relevant to the enzyme L-asparaginase from the plant pathogen *Erwinia chrysanthemi* (ErA, or Erwinase), which is used as a treatment for acute lymphoblastic leukaemia (ALL). This research programme has addressed this issue by examining the effect of potentially adverse changes in ErA relative to its *in vitro* kinetic properties, and ultimately, to its *in vivo* pharmaceutical efficacy. Two factors potentially impacting ErA, deamidation and aggregation, were studied in detail. With respect to deamidation, an improvement on published methods for its detection in ErA was developed using a combination of capillary isoelectric focussing (cIEF) and strong chemical denaturants, and this novel method should be widely applicable to other proteins. Deamidation of ErA at selected, theoretically labile residues (N41 and N281, classed as labile using the sequence motif), through expression, purification and characterisation of engineered deamidated mutants, was found to improve, not hinder, the catalytic performance of the enzyme. The structural and kinetic changes imparted by deamidation at these sites were also found to closely mimic an enzyme with high identity and homology (L-asparaginase from *Erwinia carotovora*). Deamidation of ErA was shown to be at least partially induced during the cell lysis stage of manufacturing, and strategies for minimising creation of deamidated degradants were proposed. ErA aggregation was also studied by examining the aggregate profile in the insoluble, sub-visible 2 - 10 μ m range.

Protein particulates in this size range had been previously postulated as playing a role in immune-mediated allergic reactions during clinical administration of protein biopharmaceuticals such as L-asparaginases. Statistical analyses of allergic response during clinical use of ErA were compared to the lot-to-lot quantification of sub-visible protein particles. The results indicated that ErA allergic response was essentially independent of the level of sub-visible particulates. In summary, the outputs of this research have shown that ErA deamidation and aggregation products are not necessarily deleterious to enzymatic function and clinical efficacy. The work has also demonstrated a set of empirical strategies that may be employed more widely in the development of biopharmaceutical products.

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Background 2,295

Summary of Research 2,123

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Conclusions 449

LIST OF ABBREVIATIONS

ALL	Acute Lymphoblastic Leukaemia
Asn	L-Asparagine
CD	Circular Dichroism
cIEF	Capillary Isoelectric Focussing
CZE	Capillary Zone Electrophoresis
DLS	Dynamic Light Scattering
DP	Drug Product
EcA	<i>Escherichia coli</i> L-asparaginase
ErA	<i>Erwinia chrysanthemi</i> L-asparaginase
EwA	<i>Erwinia carotovora</i> L-asparaginase
FIM	Flow-Imaging Microscopy
Gln	L-Glutamine
HPA	Health Protection Agency
HPLC	High Performance Liquid Chromatography
IEF	Isoelectric Focussing
IEX	Ion-Exchange HPLC
k_{cat}	Catalytic Constant (Michaelis-Menten Kinetics)
K_m	Michaelis Constant
LC-MS	Liquid Chromatography coupled Mass Spectrometry
LO	Light Obscuration
MHRA	Medicines and Healthcare Products Regulatory Agency
MS	Mass Spectrometry
PHE	Public Health England
pK _a	Logarithmic Acid Dissociation Constant
pI	Isoelectric Point

PIMT	Protein L-Isoaspartyl Methyltransferase
PTM	Post-Translational Modification
RMM	Resonant Mass Measurement
RNA	Ribonucleic Acid
SbVP	Sub-Visible Particulates
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEC	Size-Exclusion Chromatography
USD	United States Dollar
UV	Ultra Violet
WT	Wild Type

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1.1 BACKGROUND

1.2 Introduction

Proteins are complex molecules which are ubiquitous in nature and perform functions in almost all biological processes. They are constructed from one or more linear chains of covalently-linked amino acids. The polypeptide chains typically assume a three-dimensional shape after translation, in a process known as folding (Cooper, 2000). Under most circumstances, a correctly-folded protein should adopt a native conformation to perform its function, as is the case for proteinaceous enzymes, proteins which facilitate a chemical reaction. It is important to note that not only proteins can act as enzymes; for instance, ribozymes are ribonucleic acid (RNA) molecules that can catalyse certain biochemical reactions in a manner analogous to proteinaceous enzymes (Doudna and Cech, 2002).

There are many proteins and enzymes that are manufactured at a large scale and used in a variety of ways. A protein from a given organism or cell line can be produced by culturing at a suitable scale using fermenters, isolation from the host cell, and purification using techniques such as affinity chromatography. Modern biotechnology techniques, such as recombinant DNA technology, have made the production of proteins and enzymes a routine endeavour in both industry and research environments. Enzymes may be employed as industrial biocatalysts to perform complex organic reactions with stereoselectivity, and in many cases they are specifically designed for enhanced activity using techniques such as directed evolution (Dalby, 2011; Hibbert and Dalby 2005). Another significant use for proteins and enzymes produced at industrial scale is as biopharmaceutical products for the medical treatment of disease in humans. Although important from a medical and ethical point of view, the production of biopharmaceutical

products is increasingly significant in economic terms. The world-wide biopharmaceutical market was estimated at USD 140 billion in 2012, with annual growth projected year-on-year as approximately 15 – 18% (Langer, 2012).

Proteins that are used as biopharmaceutical products must undergo rigorous analysis before use in humans, and this activity is strictly controlled by governmental oversight. New biopharmaceutical products are intensely studied for their *in vivo* efficacy and safety during pre-clinical (animal models) and clinical (human) studies. After successful passage through these stages, the potential product is approved by governmental regulatory bodies (in the UK, the Medicines and Healthcare Products Regulatory Agency or MHRA) and subsequently may be produced for release to the market. Each batch of the protein or enzyme that is produced must be analysed using a wide variety of techniques, to assure that the quality of the product is consistent and fit for medicinal use. Some of the factors which should be analysed include quantification of the proportion of protein material which is degraded or altered, as these factors may impact the efficacy of the protein as a drug (e.g., increased immune reactivity or decreased enzymatic activity).

Pharmaceutical manufacture and storage may result in chemical and physical changes to the protein such as deamidation, aggregation and oxidation. In particular, deamidation and oxidation may be classed as post-translational modifications (PTM), which occur not only during *in vitro* handling (as for biopharmaceuticals) but also during many biological processes *in vivo* and after long-term storage. Protein aggregation is another process that can occur during pharmaceutical manufacture and may result in reduced biological activity. For biopharmaceuticals, the results of these changes (aggregation and deamidation) are classed as degraded protein variants, the presence of which may affect the stability, efficacy and/or clinical impact of the product. Often, proteins used as biopharmaceuticals may contain a small proportion of such degraded variants at the time

of clinical administration. Due to the influence of regulatory bodies during biopharmaceutical development, it is usually assumed that these degraded variants are undesirable or deleterious to clinical use, which may not always be the case. Thus, a common problem in the development of biopharmaceutical proteins is a lack of understanding around the implications of product degradation.

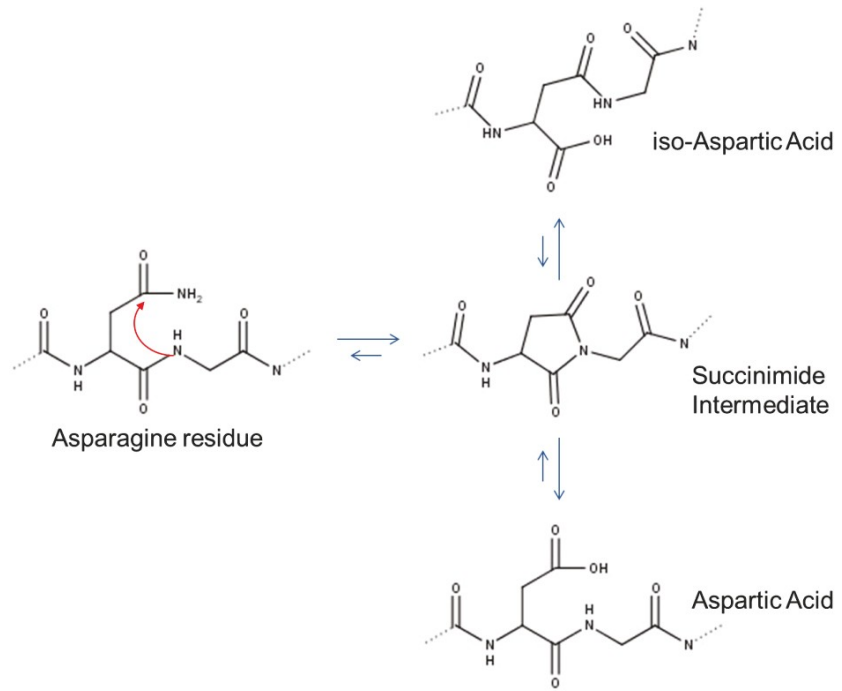
This problem is particularly relevant to the enzyme L-asparaginase from the plant pathogen *Erwinia chrysanthemi* (ErA, or Erwinase®), which is used as a treatment for acute lymphoblastic leukaemia (ALL). Therefore, the aim of the research programme described here is to relate the effect of degradative changes in ErA to its *in vitro* kinetic properties, and ultimately, to its *in vivo* pharmaceutical efficacy. Two of the most common undesirable changes (deamidation and aggregation) to ErA have been studied during the course of this research. The relevant literature for L-asparaginase deamidation and aggregation is summarised in the sections below.

1.3 Protein Degradation by Deamidation

The term deamidation refers to the removal of an amide group from an organic compound *via* a chemical reaction. In the context of proteins and peptides, deamidation refers to a loss of an amide group from the side-chain residue of either asparagine (N or Asn) or glutamine (Q or Gln). Deamidation reactions may be mediated by enzymatic means or may occur due to non-enzymatic reactions (Aswad, 1995). Deamidation reactions that occur naturally *in vivo* have been hypothesised to serve as intra-cellular regulators, or ‘molecular clocks’, for the overall control of the timing of biological processes (Robinson and Robinson, 2004).

Non-enzymatic deamidation is one of the most frequent degradation reactions to occur in proteins and peptides (Yang and Zubarev, 2010) and is much more common in Asn residues compared with Gln residues. In a deamidation reaction involving an Asn residue (Figure 1), the α -nitrogen C-terminal to the Asn attacks the side-chain carboxylate group, resulting in a succinimide intermediate and the release of ammonia. The cyclic succinimide intermediate is then spontaneously hydrolysed to form a mixture of Asp and isoAsp (Aswad *et al.*, 2000). Completion of the reaction leads to a lowering of the protein pI due to the change in residue charge from neutral to negative. This degradation reaction causes charge heterogeneity in protein products and the creation of acidic protein variants.

Deamidation is a common degradation reaction in proteins and peptides, and may occur even at physiological pH. However, an alkaline environment or an increase in temperature may increase the propensity of Asn residues to undergo deamidation. The neighbouring residues in the protein primary structure also play a role in determining whether a given Asn residue will undergo deamidation. The amino acid residue C-terminal to Asn has been shown to be critical in determining the rate of deamidation, with glycine (sequence of Xxx-Asn-Gly-Xxx) resulting in the highest rates of deamidation, up to 70-fold more than other residues (Tyler-Cross and Schirch, 1991; Patel and Borchardt, 1990).



LJXUH 1. 0HFKDQLVP IRU QRQ-HQ}\PDWLF GHDPLGDWLRQ

In the context of biopharmaceutical proteins, deamidation is undesirable, but perhaps to a certain extent unavoidable. Deamidation may lead to loss of secondary, tertiary and/or quaternary structure thus compromising protein function and/or enzyme activity (Gupta and Srivastava, 2004; Dutta *et al.*, 2012). However, the impact of a particular deamidation event is difficult to characterise fully. Understanding the impact of deamidation (and charge-variants in general) usually involves isolation by preparative HPLC and may be extremely laborious (Zhang *et al.*, 2011; Khawli *et al.*, 2010). The isolated species may have multiple post-translational modifications, making it difficult to address the impact of an individual modification on protein or enzyme function. Finally, the HPLC-isolated charge variants may have a different stability profile, further complicating their characterisation.

An alternative strategy to study and characterise the impact of protein deamidation is by production of recombinant deamidated variants. Production of a recombinant deamidated form of a protein by heterologous expression and subsequent purification offers an alternative route to study the variant in isolation and understand its true biochemical, biophysical and functional properties. Recombinant deamidated variants have not been previously described in the literature in the context of understanding biopharmaceutical degradation and the impact on efficacy. However, multiple researchers have studied recombinant deamidated forms of lens crystallin proteins α A (Gupta and Srivastava 2004a; Chaves *et al.*, 2008), α B (Gupta and Srivastava 2004b; Mafia *et al.*, 2008), and β B1 and others (Lampi *et al.*, 2001; Takata *et al.*, 2008) in the context of understanding the impact of deamidation on human cataract formation. Also, outside of a biopharmaceutical context, deamidation at certain amino acid residue positions was shown to have an impact on enzyme activity using recombinant variants of a serine hydroxymethyltransferase (di Salvo *et al.*, 1999).

As an established biopharmaceutical product, L-asparaginase is a good model for studying the effect of deamidation, as the activity of the enzyme depends on maintaining its quaternary structure. Thus, in addition to deamidation near the active site residues, deamidation at a site that changes the propensity of the 35kDa monomeric protein to assemble as a tetramer is expected to affect its enzyme activity. Deamidation of L-asparaginase was first observed for the *Escherichia coli* enzyme (EcA) using isoelectric focusing (IEF) in the 1970s (Laboureur *et al.*, 1971a; Laboureur *et al.*, 1971b), 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 (Wagner *et al.*, 1969) who investigated enzyme isoforms using electrophoresis and 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 (Bae *et al.*, 2011). However, in all of these studies, the effect of L-asparaginase deamidation was only examined in a crude, qualitative way. Prior to the programme of research detailed in this thesis, there has been no systematic study of the effects of deamidation on L-asparaginase for biopharmaceutical use.

1.4 Protein Degradation by Aggregation

Protein aggregation is another common phenomenon affecting protein structure and function both *in vivo* (e.g., in amyloid plaque formation in Alzheimer's disease and as a result of macromolecular crowding) and *in vitro* during biopharmaceutical production. Protein aggregation may be reversible or irreversible, and aggregates may be ordered (e.g. amyloids) or amorphous (such as inclusion bodies) (Fink, 1998). Aggregates may range in size from dimers (two proteins or protein assemblies) to large, insoluble aggregates of

thousands of subunits. They may be held together by covalent means (disulphide bonds), hydrophobic interactions, or electrostatic interactions. Although aggregates may retain functional or enzymatic properties, more commonly protein aggregation alters protein biological function (Invernizzi *et al.*, 2012; Zhou *et al.*, 2008).

Protein aggregates may be classified and categorised according to size. Two of the categories of interest are small aggregates (with mean diameters of nm or tens of nm) and large aggregates including sub-visible particulates (SbVP) (Rathore and Rajan, 2008) with mean diameters in the low-micron range. SbVP are usually insoluble aggregates that cannot be detected by the human eye. In the context of biopharmaceutical protein products, the presence and number of sub-visible particulates (SbVP) is an important factor to quantify and understand. This is particularly true with regard to parenteral (injectable) biopharmaceutical products such as ErA, where after clinical administration, SbVP may readily interact with human tissues and the immune system.

In order to avoid the possibility of such deleterious effects, formation of protein aggregates in biopharmaceutical products such as ErA must be understood and controlled. Repeated freezing and thawing of ErA in water is known to generate a degree of small, soluble aggregates detectable by size-exclusion chromatography (SEC) (Jameel *et al.*, 1995; Jameel *et al.*, 1997). Lyophilisation, a process that is used in ErA formulation to remove water from the protein matrix and enhance stability, must be regarded as a potential aggregation-inducing step (Lueckel *et al.*, 1998) requiring further analysis with respect to SbVP. Multi-subunit proteins, such as the tetrameric 140kDa (35kDa per subunit) L-asparaginase, may also face issues during lyophilisation such as loss of quaternary structure and possible loss of activity (Hellman *et al.*, 1983; Ward *et al.*, 1999). The degree to which aggregation may occur in reconstituted lyophilized drug product (DP) formulations varies from protein to protein, and the degree of aggregation may be reduced

or lowered by additions of excipients such as sucrose or trehalose (Adams 1991; Arakawa *et al.*, 1993).

Concerns over the potential for undesired immunogenic reactions from SbVP in parenteral products (Rosenberg 2006; Carpenter *et al.*, 2009) has led to further study in this area in the past few years. Early research in this area established a relationship between IFN- α aggregates and levels of anti-IFN- α antibodies in murine models, suggesting that aggregates were undesirable from an immunogenicity perspective (Braun *et al.*, 1997). Of particular interest is the size range between 2 and 10 μ m; however the measurement of SbVP in this range is a technically challenging and evolving area for both regulators and industry (Carpenter *et al.*, 2009; Singh *et al.*, 2010). Normally for routine quality-control testing of biologic products, the light obscuration (LO) technique is applied, but this is not ideal for the 2 - 10 μ m range for biologic products with translucent protein SbVPs (Narhi *et al.*, 2009; Cao *et al.*, 2009). One difficulty in the measurement of protein aggregates is that no one sizing technique is suitable across the entire spectrum from the angstrom/nanometer level up to microns (Narhi *et al.*, 2009), with the 2 - 10 μ m range particularly troublesome. Researchers have also measured protein aggregates using both Dynamic Light Scattering (DLS) and Resonant Mass Measurement (RMM), but these techniques are more applicable to particles in the smaller 20 – 1000nm size range (Panchal *et al.*, 2014). A newer technique for measurement of SbVP is flow-imaging microscopy (FIM) which has the capability to classify particulate matter as well as provide size distributions and particulate counts (Sharma *et al.*, 2007; Sharma *et al.*, 2010). FIM algorithms can be produced in order to classify particles based on image characteristics such as shape or translucency (Weinbuch *et al.*, 2013; Strehl *et al.*, 2012). Prior to the research described in this thesis, no FIM studies of ErA had been described in the literature. Furthermore, to the best of my knowledge and that of the peer reviewers for my

manuscript, the clinical impact of SbVP had not been described in the literature for any biopharmaceutical protein product.

2.1 SUMMARY OF THE RESEARCH

2.2 Aims of the Research Programme

Due to their complex nature, proteins may degrade into related variants *via* a number of non-enzymatic means, such as deamidation, aggregation and oxidation. This is particularly true when manufacturing proteins at large scale for biopharmaceutical use. The degraded protein variants may or may not have the same stability, efficacy and/or clinical impact as the parent compound. Often, proteins used as biopharmaceuticals may contain a small proportion of degraded variants at the time of administration. In biopharmaceutical development it is usually assumed that these degraded variants are undesirable or deleterious to clinical use, which is a common conclusion (D'Souza *et al.*, 2012; Torosantucci *et al.*, 2013) but may not always be the case (Haberger *et al.*, 2014). Thus, a common problem in the development of biopharmaceutical proteins is the lack of understanding around the implications of degradation.

This problem is relevant to the enzyme L-asparaginase from the plant pathogen *Erwinia chrysanthemi* (ErA, or Erwinase), which is used as a treatment for acute lymphoblastic leukaemia (ALL). The overall programme of research described here aims to deepen the understanding of the robustness of ErA production, the degradation processes that the enzyme may undergo, and how those degradation processes impact the safety and efficacy of the product in its intended therapeutic use. In particular, the study aimed to:

1. Develop an understanding of the effect of deamidation on ErA, including novel methods for measuring deamidation in ErA, and the potential causes of deamidation during ErA manufacture.
2. Understand how ErA deamidation at the most labile sites in the molecule affects the structure and function of the enzyme.

3. Perform a systematic study of the effect of subvisible ErA aggregates in the 2-10 μ m size range on the onset of allergic reactions observed after ErA clinical use.
4. Provide a rational framework to understand the variability of enzyme produced by the normal ErA manufacturing process, as well as demonstrating the potential for the application of this framework to other biopharmaceutical products.

2.3 Summary of the Published Work

The research programme described above was carried out at Public Health England (PHE, formerly the Health Protection Agency) at Porton Down, UK between 2010 and 2014. The output from this programme includes a total of five peer-reviewed primary research publications, which are listed in Table 1. A brief summary of the scope of each publication is provided in the sub-sections below.

Table 1. List of publications.

Reference Number	Reference
1	Gervais D, King D. Capillary isoelectric focussing of a difficult-to-denature tetrameric enzyme using alkylurea-urea mixtures. <i>Analytical Biochemistry</i> 2014; 465:90-95.
2	Gervais D, Foote N. Recombinant deamidated mutants of <i>Erwinia chrysanthemi</i> L-asparaginase have similar or increased activity compared to wild-type enzyme. <i>Molecular Biotechnology</i> 2014; 56(10):865-877.
3	Gervais D, Corn T, Downer A, Smith S, Jennings A. Measurement of subvisible particulates in lyophilised <i>Erwinia chrysanthemi</i> L-asparaginase and relationship with clinical experience. <i>AAPS J</i> 2014; 16(4):784-790.
4**	Gervais D, O'Donnell J, Sung M, Smith S. Control of process-induced asparaginylation during manufacture of <i>Erwinia chrysanthemi</i> L-asparaginase. <i>Process Biochem</i> 2013; 48(9):1311-1316.
5*	Gervais D, Allison N, Jennings A, Jones S, Marks T. Validation of a 30-year-old process for the manufacture of L-asparaginase from <i>Erwinia chrysanthemi</i> . <i>Bioproc Biosyst Eng</i> 2013; 36(4):453-460.

* Later referred to as Gervais *et al.*, 2013a.

** Later referred to as Gervais *et al.*, 2013b.

2.2.1 Quantitative Measurement of Deamidation in ErA (Paper 1)

Paper 1 (Gervais and King, 2014) describes a novel method developed to assess the extent of ErA deamidation using capillary isoelectric focussing (cIEF). As a quantitative method, cIEF is a good tool for the analysis of deamidation, as this PTM results in a shift in the protein isoelectric point (pI). However, it is important in these applications that the protein is fully denatured to ensure that all the amino acid residues, including those that are buried in the native state, contribute to the measured pI. Typically in cIEF, 8M urea is used to denature the protein, but in the case of ErA, a stronger denaturant was found to be required. Denaturants used in both gel IEF and cIEF must be non-ionic in nature, since the presence of salts in IEF cause the electrical current to increase. High concentrations of salts (or ionic chaotropic denaturants such as guanidinium hydrochloride) cause such an increase in current that the electrophoretic medium (capillary or gel) may be destroyed. Substituted alkylureas were identified from the literature (N-methylurea, N-ethylurea, etc) which had been shown to be more powerful denaturants than urea alone (Kumar *et al.*, 2014; Poklar *et al.*, 1999). Alkylureas had not previously been utilised in cIEF applications, and our paper demonstrated that they can be used in a robust and reproducible assay for ErA deamidation. The method we reported can be widely applicable to the study of proteins that do not easily denature in urea.

2.2.2 Effect of Deamidation on ErA Structure and Activity (Paper 2)

Understanding the impact of deamidation on ErA was critical to the success of this research programme. Detailed study of the effects of deamidation was not possible using routine ErA samples from production, as they may contain a maximum of only 5 – 10% deamidated material. Therefore, a programme of work was undertaken to express, purify

and fully characterise three ErA mutants which contained genetically engineered deamidated residues, and this research is documented in Paper 2 (Gervais and Foote, 2014). Multiple techniques were used to characterise the thermal and chemical stability of variants, including denaturation, isoelectric focusing, HPLC, SDS-PAGE, circular dichroism, and *in silico* structural analyses. As part of this work, an enzyme purification strategy was developed, using modifications of reported substrate-affinity chromatography techniques (Kotzia and Labrou, 2005; Lee *et al.*, 1989). This strategy proved to be successful, and allowed a better understanding of the effect of deamidation at multiple sites, the relationship of deamidation to ErA enzyme kinetics, and the relationship between deamidation and enzyme structure and stability. Certain mutations rendered an ErA protein that closely resembles a homologue of high amino acid sequence conservation (*Erwinia carotovora* L-asparaginase, or EwA). The similarity extends to the stability, the hydrogen bonding network near the mutations and the enzyme kinetic properties of the proteins (Papageorgiou *et al.*, 2008; Gervais and Foote, 2014).

2.2.3 Effect of Aggregation on ErA Pharmaceutical Efficacy (Paper 3)

Protein aggregation is another common phenomenon that can alter the efficacy of biopharmaceuticals. Aggregates of enzyme products, such as ErA, may have reduced activity and efficacy due to masking of the enzyme's active site. Sub-visible aggregates, in the size range between 2 and 10 μ m, have been further hypothesised as being implicated in undesired immunogenicity (and allergic response) to biopharmaceuticals (Carpenter *et al.*, 2009, Cao *et al.*, 2009). Therefore, in the research described in Paper 3 (Gervais *et al.*, 2014), ErA aggregation was studied as another mechanism with the potential for adverse effects towards efficacy and potency. Using flow-imaging microscopy, the ErA

aggregation profile in the sub-visible size range was characterised. The research went on to relate the aggregation data to the effectiveness in the clinical use of the enzyme. Using statistical analyses it was demonstrated that the incidence of allergic events, such as hypersensitivity, was essentially independent of the level of sub-visible ErA aggregates in the materials used in the clinic. Studies such as this, aiming to define the relationship between protein aggregates and allergic response, had not previously been documented in the literature for any biopharmaceutical product.

2.2.4 Control of Deamidation During ErA Manufacture (Paper 4)

Control of in-process deamidation is a critical part of manufacturing any biopharmaceutical product. Deamidation of asparagine residues is mediated by a non-enzymatic reaction at pH values greater than 7, and the product of the reaction is either an aspartic acid or isoaspartate residue. In addition to the change in the net charge of the protein, the substitution of asparagine for the acidic aspartic acid residue may have an impact on protein structure and function. In the research described in Paper 4 (Gervais *et al.*, 2013b), the causes of ErA deamidation during manufacture were investigated. ErA contains one asparagine residue that is theoretically prone to deamidation due to the short sequence, NG motif (Tyler-Cross and Schirch, 1991). However, the NG motif is only surface-exposed when the tetramer is disassembled; it is normally buried within the tetrameric structure and thus protected from deamidation when the quaternary structure is intact. During the course of this research, the freezing and thawing of *Erwinia chrysanthemi* cells was found to cause partial and temporary disassembly of the tetrameric ErA, exposing the labile asparagine residue and making it susceptible to deamidation during cell lysis. It was found that allowing time for the tetramer to re-form after cells

thaw protected the labile asparagine residue of the NG motif, thus preventing protein deamidation.

2.2.5 Consistency of ErA Manufacturing (Paper 5)

The overall research programme was reliant on the consistent and reproducible production of ErA enzyme. Therefore, in the research described in Paper 5, Gervais *et al.*, 2013a (which was the first paper chronologically), it was demonstrated that ErA is made using a robust and reproducible process. Because the quality of the enzyme is dependent on its quaternary structure, as ErA is a tetrameric enzyme (four identical 35kDa subunits) that must be fully assembled in order to be active, it was important to prove that the subsequent studies on ErA degradation were not dependent on how the protein samples were purified. The ErA manufacturing process is an established, long-standing process, so the approach to validating the process had to be different compared with new biopharmaceutical entities. Communication of this non-standard, alternative approach to process validation for older products was the main aim of this paper.

2.3 Contributions of the Candidate

A summary of the candidate's contributions to each of the five papers which comprise the published work is provided in Table 2. Statements for each of the papers, signed by all co-authors and indicating agreement with the candidate's contributions, are also available in Appendix 1.

Table 2. Contributions of the Candidate

Contribution of Candidate	Co-authors in agreement
<p>Paper 1. <i>Capillary isoelectric focussing of a difficult-to-denature tetrameric enzyme using alkylurea-urea mixtures.</i></p> <p>David Gervais had the lead role in this research. He identified the need for better analytical tools for ErA deamidation measurements, identified and procured the instrumentation, and proposed alkylureas as cIEF-compatible denaturants. He developed the methods, researched the literature, performed the experiments, analysed the data, wrote the manuscript and corresponded with reviewers. The co-author contributed some of the analyses with the instrument and methods to integrate the electropherograms.</p>	D King
<p>Paper 2. <i>Recombinant deamidated mutants of Erwinia chrysanthemi L-asparaginase have similar or increased activity compared to wild-type enzyme.</i></p> <p>David Gervais had the lead role in this research. He proposed the generation of recombinant deamidated variants of ErA using heterologous protein expression in <i>E. coli</i>. David Gervais designed the variant constructs, and performed the protein expression, purification and characterisation including <i>in vitro</i> techniques and <i>in silico</i> structural analyses. He also analysed the data and wrote the manuscript. David Gervais served as the lead author and addressed the reviewers' comments. N Foote contributed the enzyme activity and K_m measurements.</p>	N Foote
<p>Paper 3. <i>Measurement of subvisible particulates in lyophilised Erwinia chrysanthemi L-asparaginase and relationship with clinical experience.</i></p> <p>David Gervais had the lead role in this research. He determined the instrumentation necessary to make the aggregation size measurements and played a lead role designing the methods and in implementation of the technique. He directed the research and analysed all the data, wrote the manuscript, and served as corresponding author with the journal reviewers. Co-authors provided clinical data, provided some of the analytical runs on the instrument, and helped with manuscript revision.</p>	T Corn A Downer S Smith A Jennings

Contribution of Candidate	Co-authors in agreement
<p data-bbox="316 320 1279 392">Paper 4. <i>Control of process-induced asparaginyl deamidation during manufacture of Erwinia chrysanthemi L-asparaginase.</i></p> <p data-bbox="316 427 1279 685">The paper contains a summary of the contributions to this work within the text of the published manuscript. David Gervais had the lead role in this work, with the initial proposal that thaw time and deamidation may be related. He designed the research, performed the research and the research experiments, analysed the data, and wrote the manuscript. He also responded to reviewers' comments as corresponding author. Co-authors provided analytical methods and supported data evaluation.</p>	<p data-bbox="1305 320 1460 353">J O'Donnell</p> <p data-bbox="1305 398 1406 432">M Sung</p> <p data-bbox="1305 477 1406 510">S Smith</p>
<p data-bbox="316 723 1279 795">Paper 5. <i>Validation of a 30-year-old process for the manufacture of L-asparaginase from Erwinia chrysanthemi.</i></p> <p data-bbox="316 831 1279 1048">David Gervais had the lead role in this manuscript. Although the data were generated by the Erwinase manufacturing team, David Gervais influenced the validation strategy, analysed the data, and wrote the manuscript. He also responded to reviewers' comments as corresponding author. Co-authors provided analyses of historical data, supported data evaluation, and supported manuscript revision.</p>	<p data-bbox="1305 723 1428 757">N Allison</p> <p data-bbox="1305 801 1439 835">A Jennings</p> <p data-bbox="1305 880 1401 913">S Jones</p> <p data-bbox="1305 958 1412 992">T Marks</p>

3.1 CRITICAL APPRAISAL OF THE PUBLISHED WORK

The critical appraisal of the published work includes an assessment of the contribution to knowledge offered by each work (Section 3.1), as well as a detailed analysis of each publication grouped by sub-topic (Section 3.2).

3.2 Assessment of Candidate's Contribution to Knowledge

The published work contributes to the present state of knowledge in the field in several different ways:

1. It provides a description of a set of mutants of *Erwinia chrysanthemi* L-asparaginase in terms of structural, kinetic and physicochemical properties. Observed differences in the kinetic properties are related to the structural characteristics at the mutation points in the molecule. These types of findings are of great interest in the structural biology, protein chemistry and biotechnology areas, and may help efforts to engineer L-asparaginase variants that are more stable, more active and which persist longer during clinical use.
2. It provides an understanding of the consequences of degradation of *Erwinia chrysanthemi* L-asparaginase by two of the most common means: deamidation and aggregation. Typically in development of protein biopharmaceuticals, the products of these degradation processes are assumed to have a negative impact on enzymatic function, stability or clinical use. However, in this work it is shown that these degradants are not necessarily detrimental, and that these product-related variants should be studied using orthogonal techniques to

establish their true properties. The methodology used in this work should be widely applicable to the study of proteins being used as biopharmaceuticals.

3. To the best of our knowledge, it provides the first data set known in the literature relating protein aggregate levels to immunogenicity. The topic of undesired immune response in protein biopharmaceuticals is a topic of great interest. However, no other paper published to date seems to have attempted to understand these potential immune-mediated reactions in a causal way.
4. It contributes a novel, improved method for routine measurement and quantitation of deamidated protein species using alkylureas and capillary isoelectric focussing. The use of alkylureas as powerful denaturants was reported in the literature previously, but application of these compounds to quantitative capillary electrophoretic techniques was not known. The agreement of the data with isoelectric point predictions using pK_a values provides good validation of the technique presented in the published work. Capillary electrophoresis is an important technique for detection and quantitation of protein species. The published work provides the community with a new and useful tool for analysis of deamidated variants in protein preparations.
5. It contributes a framework for proving the robustness of older protein manufacturing processes through validation. The principles of validation are usually applied to new products in development, and their application to older products presents a unique set of challenges not covered in the literature prior to this paper.

3.3 Critical Analysis of the Published Work

3.3.1 Effect of Deamidation on ErA

Prior to the start of the research programme, there was little information available on the measurement or effects of post-translational modifications (such as deamidation) on *Erwinia chrysanthemi* L-asparaginase. However, with respect to ErA, there was already a rich body of literature in the public domain. Some of the relatively recent references on ErA include publication of the crystal structure of the ErA enzyme (Lubkowski *et al.*, 2003; Aghaiypour *et al.*, 2001; Miller *et al.*, 1993) and a closely related variant (Papageorgiou *et al.*, 2008). Further published studies on ErA include cloning and expression of recombinant forms of the enzyme (Kotzia and Labrou, 2007), and use of mutations to engineer thermal stability (Kotzia and Labrou 2009) and resistance to proteolytic cleavage (Kotzia *et al.*, 2007). However, none of these studies included research into the deamidation (or aggregation) of the enzyme.

Early research on the closely-related *Escherichia coli* L-asparaginase (EcA) (Laboureur *et al.*, 1971a; Laboureur *et al.*, 1971b; Wagner *et al.*, 1969) may have identified the existence of deamidated forms of the enzyme, but they were only detected, and not characterised. A more recent reference (Bae *et al.*, 2011) included a consideration of EcA and ErA deamidation in the context of all post-translational modifications (PTM) using two-dimensional gel electrophoresis and mass spectrometry analyses of commercial preparations. However the work presented was qualitative, not quantitative for deamidation (or any other PTM). The impact of any specific deamidation site (or PTM) on the properties of the enzyme was also not considered in the paper by Bae *et al.* (2011).

Three of the papers presented in this thesis are relevant to the topic of ErA deamidation and significantly extend this area of knowledge. The published work includes an analysis

of the causes of ErA deamidation during manufacture (Gervais *et al.*, 2013b), a full characterisation of the deamidated forms of the enzyme (Gervais and Foote, 2014), and novel methods for measurement of ErA deamidation (Gervais and King, 2014).

Deamidation is a primary structure change (a change at the amino acid sequence level) and is perhaps the most common PTM observed. However, measurement of protein deamidation is difficult. Most often, mass spectrometry (MS) is utilised (Robinson *et al.*, 2006) with most modern MS instruments able to resolve the +1Da difference that results from a deamidation event (Rivers *et al.*, 2008). Although MS can detect deamidation, specialised and expensive equipment is required for such analyses and the technique is not always suitable for routine protein measurements, particularly in a biopharmaceutical production setting. Use of MS to measure deamidation may be further complicated during sample workup by creation of deamidated artifacts not originally present in the analyte sample. The protein tertiary structure has been shown to play a role in the rate of deamidation, with buried residues protected from nonenzymatic deamidation (Rivers *et al.*, 2008). Therefore, depending on the proteolytic digestion, denaturation, and sample workup conditions used prior to mass spectrometry analyses, significant artefacts may be introduced as protected Asn residues become exposed to solvent.

Another alternative approach to deamidation measurement is to use assays based on the enzyme protein L-isoaspartyl methyltransferase (PIMT). PIMT preferentially methylates isoaspartate residues in the presence of S-adenosyl-L-methionine, facilitating conversion of the isoaspartate back to aspartate (Johnson *et al.*, 1987). A commercial assay using this principle and HPLC has been developed (Johnson and Aswad, 1991) for the detection of isoaspartate in samples of biopharmaceutical proteins. Isoaspartate residues do not occur naturally in proteins on translation, but may occur as the product of a racemisation reaction involving Asp residues. Since the product of an asparaginyl deamidation event is

a mixture of isoaspartate and aspartate residues in a distribution of approximately 2:1 isoAsp to Asp (Aswad *et al.*, 2000), the assay is only an indirect measure of protein deamidation. However, in the case of ErA, this commercial assay (Promega, Southampton, UK) was attempted on a number of occasions without success (data not shown), possibly due to the buried nature of the labile Asn residues in the enzyme.

Consequently, alternative means of assessing ErA deamidation were required. The assay described in the first paper of the published work is based on a change in net charge, a further property of deamidated proteins. In this paper, it is shown that ErA deamidation can be quantified using capillary isoelectric focussing (cIEF) (Paper 1, Gervais and King, 2014). cIEF methods are more suitable for quantitative analysis than gel-based techniques, as the detection by UV absorbance is more sensitive than gel densitometry. The cIEF outputs, or electropherograms, can be integrated to provide quantitative information in the same way as HPLC traces. The cIEF approach was an improvement on the previously-published two-dimensional gel electrophoresis method for ErA (Bae *et al.*, 2011) which used a gel-based IEF separation and was not quantitative. The novel cIEF technique utilises mixtures of urea and N-ethylurea to provide a level of protein unfolding appropriate for measurement of the impact of charge over the entire 327 residue length of the ErA monomer. This strategy, coupled with the quantitative sensitivity of cIEF, allowed an assessment of the levels of PTMs present in ErA samples from the manufacturing plant. In Paper 1, it is demonstrated through forced-degradation experiments that the assay is appropriate for the detection of deamidated variants if they are present. The method should be broadly applicable to other enzymes and proteins beyond ErA.

The cIEF approach, however, is not without drawbacks. One particular point is that ErA acidic species (peaks with a pI less than the main peak pI of 7.35) cannot be definitely

classed as deamidation, using this assay. Rather, it is appropriate to say that this method is capable of detecting deamidated residues if they are present in ErA samples, as demonstrated in Paper 1 with forced degradation and recombinant mutants. These approaches provided assurance that deamidation could be detected, and that the low values of ErA acidic species observed in routine manufacturing samples show that deamidation levels may be very low (1.0 – 3.5% of total UV-absorbing species). With this important issue of data interpretation clarified, it can be argued that the MS approach is superior, as the mass signals obtained enable the analyst to definitively classify the nature of any PTMs present, including deamidation. The cIEF approach should therefore be regarded as a convenient method for high-throughput situations where there are numerous samples, or where expensive MS equipment is not available.

While measurement of deamidation is an important factor to consider, so too is the effect of deamidation on the characteristics and performance of the enzyme. The second paper from the published work (Paper 2; Gervais and Foote, 2014) describes the production and characterisation of recombinant ErA mutants with engineered deamidation sites. This paper has been cited (Pokrovskaya *et al.*, 2014) by investigators characterising mutant versions of *Rhodospirillum rubrum* L-asparaginase. In general, the production of recombinant deamidated variants had been described in the literature (e.g., Takata *et al.*, 2008) but not in the context of studying the impact on biopharmaceutical properties or efficacy. Furthermore, the ErA mutants generated and studied in this research programme had not been previously described. Production of recombinant deamidated variants is a good strategy to help understand the impact of this PTM on the structure and function of the enzyme. However, one particular drawback of this approach is the inability to genetically engineer variants incorporating isoAsp residues. As discussed above, isoAsp residues are a normal product of non-enzymatic deamidation reactions. If an isomerase

were available, such as an aspartate racemase, that had activity on protein-bound amino acid residues (instead of free, solvated amino acids), it may have been possible to further study the influence of isoAsp versus Asp deamidation products, but this is beyond the scope of the work presented here.

Prior to generation of the ErA recombinant deamidated mutants, it was expected that the mutants would display inferior properties compared with the wild type (WT) enzyme. The published work (Gervais and Foote, 2014) includes a structural and functional description of how the deamidation events change ErA. Interestingly, it was discovered that the mutations studied (N41D, N281D and a double mutant containing both substitutions) increased, rather than decreased, the catalytic performance of the enzyme (expressed as k_{cat}). Furthermore, the N281D mutation was observed to impart an increase in the Michaelis constant K_m compared with WT (denoting a decrease in substrate affinity). The properties of ErA mutants carrying the N281D mutation were similar to those of the wild-type L-asparaginase (EwA) from the related specie *Erwinia carotovora* as described by Papageorgiou *et al.* (2008), in terms of thermal stability and function (i.e., specificity, catalytic constants, and activity). A structural analysis *in silico* was carried out using the CHIMERA software package (Pettersen *et al.*, 2004) (Figure 2) by comparing published structures for ErA (PDB: 1HG1, 1O7J) with the published structure for EwA (PDB: 2JK0). This analysis revealed that hydrogen bond formation in the regions of interest around the ErA N281D site had also changed to be more akin to that of the published EwA structure. The agreement of both the empirical and *in silico* results with published data from a closely related enzyme with high sequence identity to ErA, provides a compelling validation of the data discussed in Paper 2.

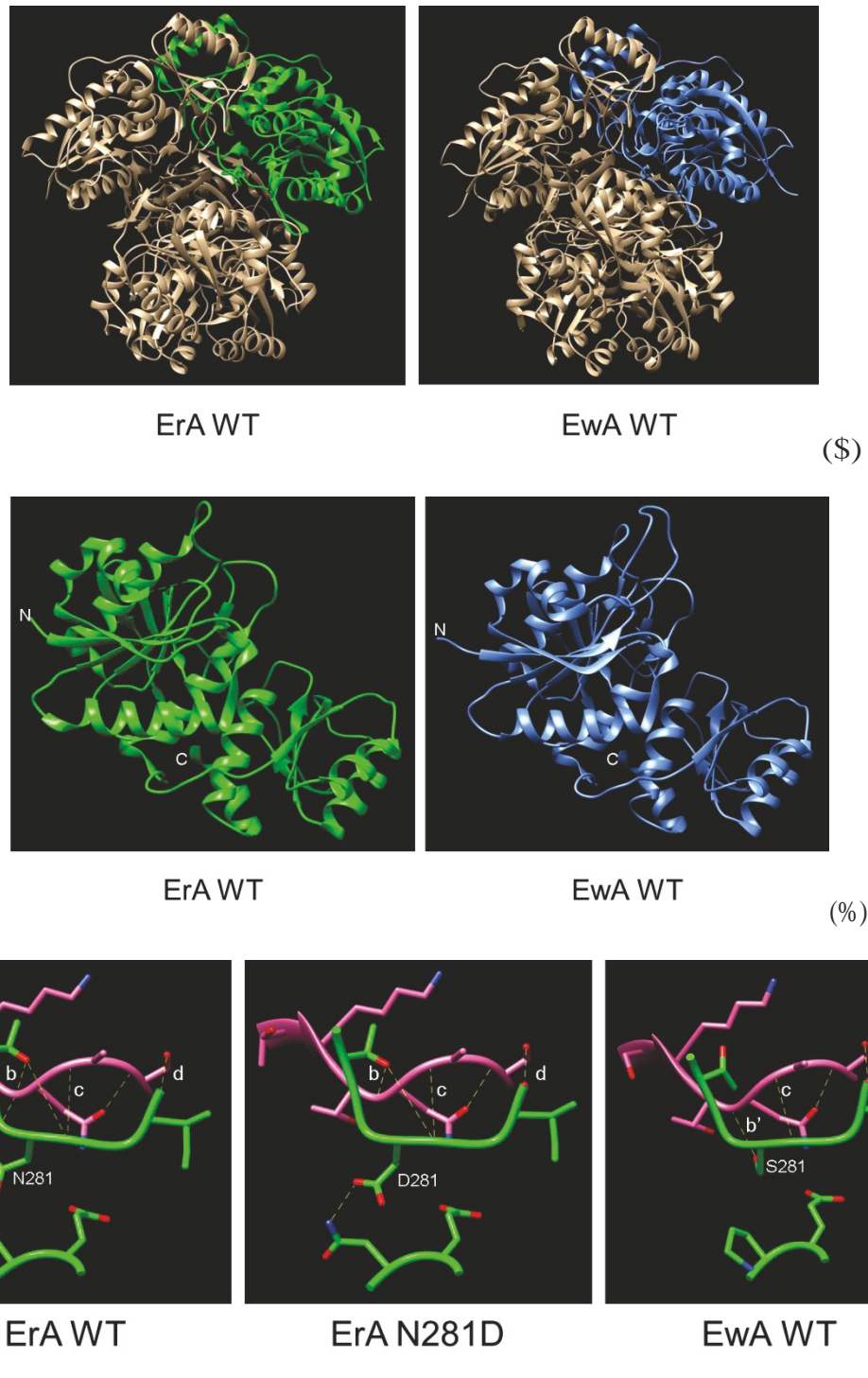


Figure 2. Structural comparison of *L*-asparaginase from *Erwinia chrysanthemi* (ErA) with *Erwinia carotovora* (EwA). Images were generated using CHIMERA software and PDB entries 1HG1 and 2JK0. (A) Tetramer structure with one subunit shown in green (ErA) or blue (EwA), (B) Comparison of 35kDa subunit structure with the N and C termini indicated, and (C) Comparison of hydrogen bonding opportunities around N281/D281 for WT and mutant ErA and S281 (EwA) with the A chain depicted in green, the C chain depicted in pink, and the location of inter-chain bonds labelled a, b, c, or d. The N281D mutation in ErA causes the loss of a hydrogen bonding opportunity (labelled 'a') between the N281 amide and the peptide bond carbonyl group in A171 on the opposing chain.

One interesting observation regarding the research described in Paper 2 was that one of the mutations (N281D) had an effect on both the thermal and denaturing stability of the enzyme. For thermal stability, variants not carrying the N281D mutation retained approximately 100% of initial activity at temperatures up to 60°C, while those with the N281D deamidation showed a decrease in activity at temperatures above approximately 45°C. Similarly, ErA without the N281D mutation retained activity after exposure to all concentrations of urea up to 7.2M, where those enzymes with the N281D mutation displayed reduced activity after exposure to approximately 1.8M urea and higher. Protein stability is a major consideration with regard to the production of biopharmaceuticals, and a lowering of protein stability may be a concern to manufacturers, developers and regulators. However, it should be noted that ErA is an unusually stable enzyme and that the stability change effected by the N281D deamidation also only occurs during higher-than-normal temperatures (>45°C) and conditions (>1.8M urea). Restriction of handling, storage and clinical administration conditions may be the only steps required to limit stability problems due to ErA deamidation, and this understanding should be considered as one of the useful outputs of this research programme.

Although production of the recombinant deamidated ErA mutants was an acceptable strategy that provided convincing and useful results, it should be noted that alternative strategies exist and could have also been explored. The inability to genetically engineer isoAsp residues, as discussed above, is one drawback of the chosen methodology. Another approach would have involved crystallisation of purified enzyme from the recombinant mutants and comparison of mutant x-ray structures with that of the wild-type, in order to gain further structural insight. This strategy should have been more informative than the circular dichroism (CD) and *in silico* analyses used in Paper 2, but the strategy was not pursued due to lack of access to the facilities needed to complete such

studies. However, due to the good agreement between the published work and previously published studies (Papageorgiou *et al.*, 2008) on structure and function of the recombinant deamidated ErA variants and wild-type EwA, it could also be argued that the crystallisation approach was superfluous in this instance. A further alternative approach would have been to make mutants of ErA deamidated at different sites to N41 and N281; however, these latter sites were chosen based on their theoretical propensity to deamidate based upon literature data (Tyler-Cross and Schirch, 1991) and were therefore thought to represent the most likely scenarios in ErA degradation. Finally, the research described in Paper 2 could have been approached from the point of view of intentionally introducing deamidation into the WT protein (e.g., in forced-degradation studies); however, under this strategy, it would have been impossible to direct the deamidation to selected sites of interest. With these alternatives in mind, it seems reasonable to conclude that the approach used in this research programme (characterisation of selected recombinant deamidated mutants) was appropriate given the research questions under investigation.

In addition to measurement of deamidation and the effects of deamidation, the research programme also focussed on the causes of deamidation during ErA manufacture. This is the subject of the fourth paper (Paper 4; Gervais *et al.*, 2013b), in which deamidation-labile ErA residues were identified, along with the point in the manufacturing process where they were most susceptible. Chronologically, this part of the research programme was conducted prior to the research detailed in Papers 1 and 2, so that the cIEF method was not available, nor was the understanding of the impact of deamidation. The deamidation methods that were available included ion-exchange HPLC (IEX), capillary zone electrophoresis (CZE) and a limited subset of peptide-map LC-MS. Using size-exclusion chromatography (SEC) it was determined that ErA tetramers fall apart after freeze-thaw and take some time to reassociate. Allowing a longer freeze-thaw time is

beneficial, as the monomers can then reassemble into tetramers thereby protecting one of the theoretically most deamidation-prone residues (N281).

In terms of a critical evaluation of Paper 4, there are obvious parts of the strategy that could have been approached differently. Use of the superior cIEF technique would have been preferable compared with IEX and CZE, but at the time cIEF was not available. Furthermore, availability of recombinant deamidated mutants would have been very advantageous, as the mutants could have been used in spiking and proof-of-concept experiments directed at particular residues. In hindsight, deamidated versions of peptides from the mapping assay used in Paper 4 (e.g., a synthetic peptide TGDGIVPP...) would have also provided a good control mechanism for the data described in the paper. However, it should be noted that the deamidated mutants described in Paper 2 have been subjected to the same tryptic digest and peptide mapping assay (data of P. Kanda, PHE, not shown or published) and found to behave as expected, i.e., deamidation in the mutant ErA variants N41D and N281D is easily detected using MS.

In summary, the published work extends the state of knowledge on the effects, measurement and causes of ErA deamidation. Little was described in the literature on ErA deamidation before the research programme was undertaken. Although alternative means could have been used to describe the effects, causes and quantitation of ErA deamidation, the published data obtained compare well with that in the literature from closely-related enzymes with high identity to ErA. Furthermore, the methods used in the published work are broadly applicable to other biopharmaceutical protein products.

3.3.2 Effect of Aggregation on ErA

Protein aggregation is another common problem with respect to the stability of protein biopharmaceuticals such as ErA. Studies of the effect of ErA freezing and thawing cycles on aggregation have previously been described (Jameel *et al.*, 1995; Jameel *et al.*, 1997), but these studies focussed on small, soluble aggregates detectable by SEC, such as ErA dimers (two 140kDa tetramers held together) or trimers. Aggregates may also take the form of much larger particles consisting of many subunits, with sizes up to the micron size range or larger. Prior to the start of this research programme, no studies of larger, sub-visible ErA aggregates in the 2 – 10 μ m size range (sub-visible particulates or SbVP) had been described in the literature. These larger aggregates were previously postulated to play a role in undesired immune-mediated reactions to some biopharmaceutical treatments. For example, one hypothesis is that allergic reactions may result from immune system cells encountering aggregates containing ordered or partially-ordered arrays of proteins (Carpenter *et al.*, 2009). However, it is important to note that the relationship between SbVP and undesired immunological reactions was only proposed in Carpenter *et al.* (2009), and prior to this research programme no data existed to back up this proposal for ErA or any other biopharmaceutical product.

The study of SbVP for other proteins was known in the literature (Singh *et al.*, 2010; Cao *et al.*, 2009) and the severity of allergic reactions to L-asparaginases including ErA had also been studied (e.g., Woo *et al.*, 2000) but no study had been published regarding any potential link between the two phenomena. Therefore, the published work presented here (Paper 3; Gervais *et al.*, 2014) represents not only the first study of its kind for ErA, but also for any protein in clinical use. The levels of sub-visible aggregates were found to have no relationship to the incidence of allergic reactions during clinical use in humans, an important initial step for this field of study.

The data presented in Paper 3 do support the argument that SbVP levels and allergic reaction appear unrelated. However, several factors should also be considered in a critical evaluation of this work, including the methodology in the SbVP measurements as well as the approach to the clinical data. In the paper, SbVP data from two methods (light obscuration or LO, and flow-imaging microscopy, or FIM) are compared. Many lots of ErA lyophilised drug product (DP) were measured using the two techniques. Ideally, these two measurements would have been made together in real-time on the same samples, to separate any temporal or stability-based effects. However, these measurements were made at different times due to logistical and practical issues. The LO measurements were made at the time of release of each DP batch to the clinical market, while the FIM measurements were made shortly prior to writing the manuscript for Paper 3, and in some cases months or years had passed between the two measurements. The reason for this is that LO is used as the regulatory-approved release test for ErA DP and must be conducted at time of release. Further, the LO measurement is carried out off-site by a third-party contractor, and each LO measurement requires approximately 20 vials of a given batch of ErA DP to be reconstituted and pooled to provide sufficient liquid volume for the instrument. At the time of the FIM measurements, it was not possible to go back and re-acquire LO data for each DP batch, as sufficient vials were no longer available for this. The FIM instrument can handle one vial or even partial vials to provide a measurement, but it was not available at the time the LO data were acquired. Another factor is that due to the pooling of vials required for each LO analysis, vial-to-vial variability in the measurements is minimised, while in the FIM analysis inter or even intra-vial differences can be measured. These pooling factors are discussed in Paper 3.

Apart from the logistical issues regarding the LO and FIM measurements, the relationship of these SbVP data with the clinical data must be considered. In Paper 3, a DP lot

containing relatively high levels of 2-10 μ m SbVP was chosen to evaluate for clinical experience versus the rest of the DP lots. Although the lot chosen for evaluation had relatively high levels of SbVP by both LO and FIM, it was not the highest measurement made for either technique. This is due to the fact that there was poor agreement between the LO and FIM data sets, as discussed in Paper 3. Analysis of the clinical data against other DP lots (such as those with the highest FIM or highest LO numbers), to determine any differences in the correlation, was not carried out due to the lack of access to the raw clinical data.

Simultaneous acquisition of the data (LO and FIM), as postulated above, may have improved the agreement of the two SbVP measurements, but the lack of agreement could also be due to basic differences in the particle sizing methodology. LO is a light-obscuration technique which relies on protein particles blocking the passage of light through a flow cell. Although LO is an industry-standard technique for protein parenterals, the translucent nature of protein particulates results in some obvious deficiencies for LO as an analytical test. FIM is a newer technique which uses a microscope mounted to a vertical flow cell, coupled with a computer-based counting system, to image every particle that passes the field of view. In this way, FIM data can be sorted into types of particulates and can also theoretically provide a total count of every particulate present in a given sample.

Even with the logistical (measurement timing) and empirical (lack of agreement between two techniques) drawbacks discussed above, this study indicates that allergic reactions observed during clinical use are not a consequence of ErA SbVP. Most, if not all of the critical points discussed above are a consequence of the fact that this study was conducted retrospectively, instead of in a prospective way. The clinical data and LO data were already complete at the start of the FIM work and data analyses, and simultaneous

assessment of SbVP using multiple methods was not possible. Any future work in this area should consider prospective studies in order to answer some of the questions posed above.

3.3.3 Consistency of ErA Manufacture

Part of the published studies describes the work performed to demonstrate the reproducibility, and consistency of the ErA manufacturing process (Paper 5; Gervais *et al.*, 2013a). The research described in this paper was performed prior to that detailed in the other four papers (Papers 1-4). Paper 5 has been cited in two other publications to date (Keating 2013; Liu *et al.*, 2013).

Paper 5 was written to describe the strategy and approach of performing validation studies on a manufacturing process for a mature biopharmaceutical product, such as ErA. The situation with ErA validation was unusual, as most biopharmaceutical products undergo validation studies at the end of the research and development phase, just as the product is finishing clinical trials in humans and before marketing the drug. In the case of ErA, these studies were performed after the product had already been marketed for many years (apart from in the United States). The strategy detailed in the paper was successfully used to gain acceptance for the product with the US Food and Drug Administration.

Approaching this paper from a critical point of view, it is difficult to find alternative strategies that could have been used. At least in part, the strategy described in the paper was dictated by practical and logistical considerations. For instance, many of the new analytical methods (such as HPLC-based methods) that were developed for the validation exercise were only used to analyse two ‘profiling’ batches (referred to as ‘G’ and ‘H’ in the paper) prior to the prospective validation exercise. In an ideal situation, more of the

historical batches ('E' and 'F', for instance) could have been analysed using these methods, to gain more experience and useful data going forward. However, these new methods took some time to develop and in a practical sense, were not ready for these earlier batches. This, coupled with the frequency with which manufacturing batches are made (at present, only 2 per annum) meant that the profiling batches were limited to two.

Biopharmaceutical validation is defined as the process of ensuring that a protein manufacturing process is robust, reproducible, and produces product of acceptable quality. In the literature, there are numerous articles about biopharmaceutical validation (e.g., Moran *et al.*, 2000; Rathore and Sofer, 2012), as well as industry and regulatory guidances, however most, if not all, of these resources are geared toward new products in development. Validation of existing protein products presents a set of unique issues and challenges, and the published work (Paper 5) represents a strategy for approaching this situation.

4.0 CONCLUSION

Biopharmaceutical proteins, including ErA, may undergo some degree of degradation during processing, storage and in clinical use. The degradation may occur to a smaller or lesser extent depending on handling and storage conditions, and may be controlled to some extent by imposing restrictions on these conditions. However, even small amounts of degraded variants may be present in protein pharmaceutical preparations, and therefore their impact must be assessed. Often, the impact of these species is assumed to be negative, due to difficulties in isolating the degraded species in a sufficient quantity to allow them to be studied. In this research programme, it was shown that in the case of the biopharmaceutical enzyme ErA, the impact of certain types of degradation is not necessarily negative.

One of the main outcomes of this work is the development of alternative tools and strategies that can be used to assess the impact of degraded protein species in biopharmaceutical preparations. For instance, production of recombinant ‘deamidated’ enzyme is a relatively straightforward way of studying the impact of deamidation on protein structure and function. Rather than inferring the effect of deamidation on activity and pharmaceutical efficacy by comparing ErA batches with varying small percentages (5 – 15%) of deamidated species, the effects of deamidation could be studied directly. Similarly, by studying the aggregation state of ErA, it was observed that a low sub-visible protein aggregate content can be tolerated without compromising the efficacy of ErA or increasing the risk of clinical hypersensitivity. Due to the obvious potential benefits these strategies provide, a key output of this research programme is the recommendation that these techniques should be applied more generally in biopharmaceutical development.

These strategies are not without drawbacks. Genetically-engineered deamidated variants of ErA cannot mimic the presence of isoAsp residues, which are a natural result of non-enzymatic deamidation reactions of proteins. Furthermore, the strategy of producing recombinant deamidated protein variants would be self-limiting in cases where there are many labile Asn residues in the protein, as the number of variants that would need to be expressed, purified and characterized may become overwhelming.

Further outputs of the research programme include the detailed characterization of a set of ErA mutants, development of a novel assay to assess protein deamidation, and understanding means of controlling deamidation and process consistency in ErA manufacture. Although this research has demonstrated that certain types of degradation are not necessarily an issue for ErA, it must be stressed that these factors must be considered for each protein on a case-by-case basis. Such endeavours are necessarily largely empirical in nature. It is hoped that the research and strategies presented here may aid others in the characterization and understanding of the effects of protein degradation.

5.0 REFERENCES

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Gervais D, O'Donnell J, Sung M, Smith S. Control of process-induced asparaginyl deamidation during manufacture of *Erwinia chrysanthemi* L-asparaginase. *Process Biochem* 2013; 48(9):1311-1316.

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APPENDIX 1 – The Published Work

Paper 1. Gervais D, King D. Capillary isoelectric focussing of a difficult-to- denature tetrameric enzyme using alkylurea-urea mixtures. *Analytical Biochemistry* 2014; 465:90-95. This paper is available digitally from the publisher (Elsevier) at DOI doi:10.1016/j.ab.2014.08.004.

The author's accepted manuscript version and the publisher's version are both available on RADAR: <https://radar.brookes.ac.uk/radar/items/2bf631a8-5a43-4e1f-b843-9a83008aaf5f/1/>

Paper 2. Gervais D, Foote N. Recombinant deamidated mutants of *Erwinia chrysanthemi* L-asparaginase have similar or increased activity compared to wild- type enzyme. *Molecular Biotechnology* 2014; 56(10):865-877.

This paper is available digitally from the publisher (Springer) at DOI 10.1007/s12033-014-9766-9

The author's accepted manuscript version and the publisher's version are both available on RADAR: <https://radar.brookes.ac.uk/radar/items/47552fb8-d07e-4771-8afa-84de834023ea/1/>

Paper 3. Gervais D, Corn T, Downer A, Smith S, Jennings A. Measurement of subvisible particulates in lyophilised *Erwinia chrysanthemi* L-asparaginase and relationship with clinical experience. *AAPS J* 2014; 16(4):784-790.

This paper is available digitally from the publisher (Springer) at DOI 10.1208/s12248-014-9612-9

The author's accepted manuscript version and the publisher's version are both available on RADAR: <https://radar.brookes.ac.uk/radar/items/d92bbd43-7b14-4a5f-9563-3a293b76accc/1/>

Paper 4. Gervais D, O'Donnell J, Sung M, Smith S. Control of process-induced asparaginyl deamidation during manufacture of *Erwinia chrysanthemi* L- asparaginase. *Process Biochem* 2013; 48(9):1311-1316.

This paper is available digitally from the publisher (Elsevier) at DOI 10.1016/j.procbio.2013.06.024

The author's accepted manuscript version and the publisher's version are both available on RADAR: <https://radar.brookes.ac.uk/radar/items/459fac79-4d86-4216-93b5-00d7339b97da/1/>

Paper 5. Gervais D, Allison N, Jennings A, Jones S, Marks T. Validation of a 30- year-old process for the manufacture of L-asparaginase from *Erwinia chrysanthemi*. *Bioproc Biosyst Eng* 2013; 36(4):453-460.

This paper is available digitally from the publisher (Springer) at DOI 10.1007/s00449-012-0802-5

The author's accepted manuscript version and the publisher's version are both available on RADAR: <https://radar.brookes.ac.uk/radar/items/68035c99-f5a4-43d8-a094-bf89d4fd2c40/1/>

APPENDIX 2 – Co-Authors’ Statements of Candidate Contribution

The co-authors’ statements have also been added to the individual papers.

PhD by Published Work

Statement of contribution by David Paul Gervais

Paper to be considered as part of the PhD by Published Work:

Gervais D, King D. Capillary Isoelectric Focussing of a Difficult-to-Denature Tetrameric Enzyme Using Alkylurea-Urea Mixtures. *Analytical Biochemistry*, 2014. *In press*; DOI 10.1016/j.ab.2014.08.004.

Background: Measurement of the amount of deamidation (asparaginylyl and glutaminylyl) in *Erwinia chrysanthemi* L-asparaginase is an important facet of understanding the degradation profile of the enzyme. Current HPLC techniques measure the enzyme in the native, tetrameric state, which may confound quantitation due to buried residues (the most labile asparagine is internal to the tetramer) or conformational differences leading to charge variants. Therefore, a more robust technique was required.

Contribution of candidate: DP Gervais had the lead role in this research. He determined the instrumentation necessary to make the deamidation measurements, procured the instrument, ran the instrument and generated most of the data. He directed the research in this area including creating and running the forced-deamidation procedure and made the observation that the enzyme was not fully unfolded in the 8M urea denaturant. He analysed all the data, wrote the manuscript, and served as corresponding author with the journal reviewers. D King provided support to running the iCE instrument.

I agree that David Paul Gervais made the aforementioned contribution to this paper.

Name

Signature

Date

Darryl King



14 AUG 2014

PhD by Published Work

Statement of contribution by David Paul Gervais

Paper to be considered as part of the PhD by Published Work:

Gervais D, Foote N. Recombinant deamidated mutants of *Erwinia chrysanthemi* L-asparaginase have similar or increased activity compared to wild-type enzyme. *Molec Biotechnol* 2014; DOI 10.1007/s12033-014-9766-9.

Background: Deamidation is an important post-translational modification of *Erwinia chrysanthemi* L-asparaginase (Erwinase) which may happen during processing or storage of the enzyme. Deamidation is of interest to the regulatory bodies (US FDA) and the impact of deamidation on the enzyme is not well understood. Isolation of deamidated enzyme from the bulk enzyme preparations is tedious and non-trivial.

Contribution of candidate: DP Gervais had the lead role in this research. It was his idea to generate recombinant deamidated variants of Erwinase using heterologous protein expression in *E. coli*. DP Gervais designed the variants, and performed the protein expression, purification and characterisation including *in vitro* and *in silico* analyses. He also analysed the data and wrote the manuscript. DP Gervais served as the lead author and corresponded with the reviewers' comments. N Foote contributed the enzyme activity and K_m measurements.

I agree that David Paul Gervais made the aforementioned contribution to this paper.

Name

Signature

Date

Nicholas Foote

N. Foote

17th July 2014

PhD by Published Work

Statement of contribution by David Paul Gervais

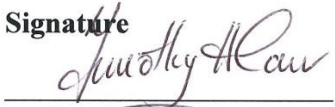
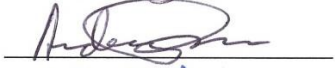

Paper to be considered as part of the PhD by Published Work:

Gervais D, Corn T, Downer A, Smith S, Jennings A. Measurement of subvisible particulates in lyophilised *Erwinia chrysanthemi* L-asparaginase and relationship with clinical experience. *AAPS J* 2014; 16:4, 784 - 790.

Background: Protein aggregation is an important degradation pathway for biopharmaceutical products. Aggregates, including those in the 2 - 10 μ m sub-visible size range, should therefore be quantified, understood and if possible, controlled. However, their impact is also not known *in vivo*. It is thought that they may play a role in the formation of undesired immunological reactions to the product, but there are no available data to support this. A project was undertaken at PHE to measure these species in Erwinase preparations and to link the data to allergic response in the clinical use of the enzyme. The peer reviewers commented that this paper is the first of its kind in the literature.

Contribution of candidate: DP Gervais had the lead role in this research. He determined the instrumentation necessary to make the aggregation size measurements and played a lead role designing the methods and in implementation of the technique. He directed the research and analysed all the data, wrote the manuscript, and served as corresponding author with the journal reviewers.

I agree that David Paul Gervais made the aforementioned contribution to this paper.

Name	Signature	Date
Tim Corn		16 July 2014
Andrew Downer		18 JUL 2014
Stuart Smith		24 JULY 2014
Alan Jennings		04 - AUGUST - 2014

PhD by Published Work

Statement of contribution by David Paul Gervais

Paper to be considered as part of the PhD by Published Work:

Gervais D, O'Donnell J, Sung M, Smith S. Control of process-induced asparaginyl deamidation during manufacture of *Erwinia chrysanthemi* L-asparaginase. *Process Biochem* 2013; 48:9:1311-1316.

Background: In a separate, unpublished study carried out by D P Gervais, it was observed that the thaw time of *Erwinia* cells appeared to have an impact on the extent of post-translational deamidation measured in the final protein drug substance. The experiments described in the paper were conducted in order to further understand, characterise and control the level of asparagine deamidation in this therapeutically-important biopharmaceutical protein.

Contribution of candidate: The paper contains a summary of the contributions to this work. DP Gervais had the lead role in this work. He designed the research, performed the research and the research experiments, analysed the data, and wrote the manuscript. He also responded to reviewers' comments as corresponding author.

I agree that David Paul Gervais made the aforementioned contribution to this paper.

Name	Signature	Date
Jennifer O'Donnell		16th Dec 13
Mei-an Sung		21st July 14
Stuart Smith		24th July 2014

PhD by Published Work

Statement of contribution by David Paul Gervais

Paper to be considered as part of the PhD by Published Work:

Gervais D, Allison N, Jennings A, Jones S, Marks T. Validation of a 30-year-old process for the manufacture of L-asparaginase from *Erwinia chrysanthemi*. *Bioproc Biosyst Eng* 2013; 36:4:453-460.

Background: The article describes the approach, strategy and resulting data used to prove that the process used to manufacture *Erwinia chrysanthemi* L-asparaginase (Erwinase) is robust and reproducible. The data were generated over a long period of time and were used to submit to the US Food and Drug Administration (FDA) to license Erwinase in the US. Before this manuscript was prepared, D Gervais wrote the validation reports that were submitted to FDA, which included a strategy he developed on how best to present the data in a regulatory context. The manuscript was developed subsequently to document this novel approach.

Contribution of candidate: DP Gervais had the lead role in this manuscript. Although the data were generated by the Erwinase manufacturing team, DP Gervais formulated the validation strategy, analysed the data, and wrote the manuscript. He also responded to reviewers' comments as corresponding author.

I agree that David Paul Gervais made the aforementioned contribution to this paper.

Name	Signature	Date
Nigel Allison		22/7/14
Alan Jennings		01-06-2014
Shane Jones		23/07/14
Trevor Marks		18/7/14