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.

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Validation of a Thirty-Year-Old Process for the Manufacture of L-Asparaginase from *Erwinia chrysanthemi*

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Abstract

A 30-year-old manufacturing process for the biologic product L-asparaginase from the plant pathogen *Erwinia chrysanthemi* was rigorously qualified and validated, with a high level of agreement between validation data and the 6-year process database. L-asparaginase exists in its native state as a tetrameric protein and is used as a chemotherapeutic agent in the treatment regimen for Acute Lymphoblastic Leukaemia (ALL). The manufacturing process involves fermentation of the production organism, extraction and purification of the L-asparaginase to make drug substance (DS), and finally formulation and lyophilisation to generate drug product (DP). The extensive manufacturing experience with the product was used to establish ranges for all process parameters and product quality attributes. The product and in-process intermediates were rigorously characterised, and new assays, such as size-exclusion and reversed-phase UPLC, were developed, validated, and used to analyse several pre-validation batches. Finally, three prospective process validation batches were manufactured and product quality data generated using both the existing and the new analytical methods. These data demonstrated the process to be robust, highly reproducible and consistent, and the validation was successful, contributing to the granting of an FDA product license in November, 2011.

Keywords: L-asparaginase, *Erwinia*, process validation

1. Introduction

L-asparaginase is routinely used in chemotherapeutic regimens for the treatment of ALL [1], to enzymatically convert the amino acid asparagine to aspartic acid. As asparagine is essential for growth of leukaemic cells, the L-asparaginase lowers circulating levels of asparagine, thereby depriving the cancerous cells of this amino acid [2]. Clinically, L-asparaginase preparations from two species are available (*Escherichia coli* and *Erwinia chrysanthemi*). Although most ALL patients receive the *E. coli* L-asparaginase, a significant proportion of those patients develop a hypersensitivity to it [3, 4]. In these cases, treatment with the *E. chrysanthemi* L-asparaginase may be pursued.

Erwinase® or Erwinaze® are the proprietary names for L-Asparaginase derived from *E. chrysanthemi*. The *E. chrysanthemi* L-asparaginase exists as a 140,000 Da tetramer in its active form which consists of four identical subunits, and has an isoelectric point of pH 8.6[5]. The product was first developed in the late 1960s and early 1970s [6,7] and has been marketed in many countries around the world. Erwinase® was first approved in the UK by the Medicines Control Agency (now the MHRA) in 1986, and is manufactured by the Health Protection Agency (formerly Centre for Applied Microbiology & Research) at Porton Down, UK. The product is marketed and sold by EUSA Pharma, and until recently, was not available commercially in the US; it was only available there under a treatment IND. In 2010, a Biologics License Application (BLA) was submitted to the US FDA for the product by EUSA Pharma, and in November 2011 the product was approved by the US FDA.

The *E. chrysanthemi* L-asparaginase manufacturing process (Fig. 1) has not been altered significantly since UK licensure in 1986. The process consists of four main stages:

fermentation, extraction, purification and formulation. After each of these major process stages, intermediates may be held frozen prior to further processing.

Process validation is a regulatory requisite for registering new biopharmaceutical products for human use, to show that manufacturing processes are under control and produce materials that are consistent, safe and efficacious. In 1987 and also later in 2011, the US FDA published [8, 9] guidance documents that detail the procedures and practices necessary to validate biological manufacturing processes. Often, these principles are applied to the scale-up of new biopharmaceutical entities with little manufacturing or clinical experience. However, process validation may also be applied retrospectively to existing processes, developed prior to the issue of these regulatory guidances. In addition to the regulatory requirements, process validation may have additional benefits to manufacturers of licensed biopharmaceutical products, including greater process understanding and, potentially, better process yields.

Recently, in line with on-going continuous improvement efforts, a programme of process validation was undertaken for the production of *E. chrysanthemi* L-asparaginase. In contrast to new biopharmaceutical products, there was an extensive history of manufacturing and clinical experience available for this product. This historical database was translated into an effective process validation strategy. The validation of the *E. chrysanthemi* L-asparaginase process, described in detail below, confirmed that the material produced by this process is consistent and of high product quality.

2. Materials and Methods

2.1 *E. chrysanthemi* L-asparaginase Manufacturing Process Description

E. chrysanthemi L-asparaginase is manufactured using a four-stage process (fermentation, extraction, purification and formulation) as shown in Figure 1. The fermentation stage begins with expansion of a vial of *E. chrysanthemi* from the working cell bank (WCB) to produce a working seed inoculum. The inoculum is further expanded in a 50 L intermediate fermentation before the production fermentation at 750 L scale. Cells are harvested from the production fermentation by continuous flow centrifugation to form a cell paste, which is stored frozen. The extraction stage comprises processing of approximately three lots of cell paste by cell lysis, an L- asparaginase capture using cation-exchange resin and ultrafiltration to yield a crude enzyme preparation. During the purification stage (Fig. 2), approximately ten extracts (equivalent to thirty fermentations) are pooled and processed through a series of column chromatography and other protein purification steps to yield a single batch of purified asparaginase DS. The DS may be stored frozen until required for formulation, during which the DS is lyophilised to form the final DP presentation.

2.2 Description of Analytical Methods

Unless otherwise indicated, samples for analysis were derived from the *E. chrysanthemi* L-asparaginase manufacturing process described above. The protein content of these samples was determined using a validated method based on the technique of Lowry [10]. The asparaginase activity assay is based on the Berthelot reaction and methods described

in the literature [11,12]. SDS-PAGE analysis was conducted using standard techniques with Invitrogen™ (Paisley, UK) Novex™ 4 – 12% Bis-Tris gels, Novex™ MES running buffer, reducing agent and LDS sample buffer and Invitrogen™ SimplyBlue™ safestain staining reagent. Size-exclusion HPLC assays were performed using a TSK gel G3000SWXL column (Tosoh, King of Prussia, PA, USA) and a Waters HPLC workstation (Elstree, UK). The reversed-phase HPLC assays were conducted using a Waters Acquity™ UPLC 1.7 µm, 300 Å, 2.1 x 100 mm column and a Waters UPLC workstation.

2.3 Process Validation Strategy

The process validation exercise for *E. chrysanthemi* L-asparaginase required an approach different to that applied to new processes. This was due to the unusual situation of having a large body of manufacturing and clinical data extending over many years prior to performing this process validation study. The historical manufacturing database utilised consisted of eight purification batches (sequentially numbered A-H), corresponding to approximately six years of manufacturing experience. As multiple fermentation cell pastes are used to make one L-asparaginase purification batch, this database included approximately 300 fermentation batches. These historical data were compiled and analysed prior to running three prospective process validation batches. A flow chart depicting the process validation strategy is shown in Figure 3.

During the manufacture of *E. chrysanthemi* L-asparaginase, there are process parameters which are controlled within specified ranges in order to make product that meets pre-defined specifications for quality attributes. These process parameters are the ‘inputs’ to the process which have acceptable ranges defined in the batch manufacturing instructions

and in the product licenses and, in the main, cannot be deviated from. Quality attributes are the 'outputs' of the process and are measurements of the characteristics of the intermediates or final product. Examples of process parameters and quality attributes for three key L-asparaginase manufacturing steps are shown in Table 1. The historical data from the quality attributes for the entire *E. chrysanthemi* L-asparaginase manufacturing process were analysed and 'windows of operation' were generated using standard statistical methods as described later.

For the purposes of increased process understanding during the validation exercise, additional analytical techniques were developed beyond the historical product quality attributes listed in Table 1. The new analytical techniques are listed in Table 2 and were designed to provide information on protein aggregation, oxidation, charge variants, impurities, and the presence of other excipients and reagents present at various stages during manufacture. As there was no existing database for these new analytical techniques, setting specifications prior to process validation was not possible. Therefore, two purification batches (G and H) were fully analysed using the new techniques. The new data were used as guidance for the prospective process validation batches and to help set specifications for these methods for future manufacturing campaigns. Example data generated using these new methods are shown below in Results.

Based on the analyses of the historical database, and the application of the new analytical techniques, a process validation protocol was written. This protocol established how three prospective purification batches (as well as the associated approximately 90 fermentation batches and 30 product extraction batches) would be produced, analysed and the expected ranges for in-process and DS and DP data. In this paper, only the DS stages of the process are discussed.

3.1 Results

3.2 Understanding the Historical Process

The historical database (consisting of eight purification batches incorporating approximately 300 fermentations) was analysed to determine the normal process variance. This was performed for the multitude of various product quality measurements which are routinely captured during *E. chrysanthemi* L-asparaginase manufacture. Selected examples are presented below to illustrate the approach taken for analysis of the historical data.

Historical data are presented in Figure 4 for two product quality attributes (culture age and specific activity) from the fermentation stage of the process. Each point in the graphs represents a data point from one fermentation batch. The culture age represents the total process time spent in the production fermenter before the fermentation process end points (600 nm absorbance plateau and step change in CO_2 evolution) are achieved. The culture age specification range for this process is 8 – 18 hours, but it is clear from the presentation of the historical data that actual operation occurs in a much narrower window. In Figure 5, historical data are presented for two product quality attributes (total protein and specific activity) from the cation-exchange chromatography step. Although in Figure 4B it may appear that there is a slight upwards trend in the specific activity over time, this may simply be due to slight variations in complex raw materials such as yeast extract, and in subsequent batches (data not shown) this trend did not continue.

The data in these Figures show the normal variation in these attributes over the course of manufacture of the historical batches and illustrate the batch-to-batch reproducibility of the *Erwinia* L-asparaginase production process. The mean and standard deviation (SD) of the data were calculated for each of these quality attributes. The mean, mean ± two SD

and mean ± three SD are plotted along with the data points. For these data, the mean ± three SD values are taken to be the normal 'operating window' for each parameter, since the data in most cases are well described by a normal distribution around the mean. For example, the 98.3% of the data in Figure 4A are within ± two SD and 99% are within ± three SD.

3.3 Process Validation

Once the historical 'operating windows' were established for the various process parameters and quality attributes, process validation batches were conducted. Three prospective process validation batches were conducted at the purification stage. However, approximately thirty fermentation batches are required to make one lot of drug substance, so in order to make these three process validation batches in the purification and drug product stages of the process, ninety fermentation batches had to be made. Due to the large number of batches, process parameters and quality attributes studied during validation, only a small subset of the data are provided here.

The fermentation stage of the process showed a high degree of consistency during the process validation batches with the data in reasonable agreement (Figure 6) with the historical operating windows. The specific activity data at fermentation harvest (Figure 6B), tare observed to largely comply with the historical operating windows. Interestingly, a step change was observed during process validation in the culture age (or process time) of the fermentation step (Figure 6A). Although the culture ages for all fermentation batches shown in the figure were within the specification limits of 8 – 18 hours, the step change resulted in process performance outside the narrow historical operating windows. These step changes were also observed in the culture age data for the inoculum fermenter

(data not shown) and were shown due to introduction of a new batch of yeast extract at the time. This raw material is common to both inoculum and production culture steps. Some variability in fermentation processes due to raw materials is to be expected, and has been specifically discussed for fermentation of *Erwinia* using complex media, where small changes in media components may have a dramatic effect on enzyme production and growth [13]. The slight change in culture age had no impact on the product quality at DS (data not shown). As the step change in the data could be easily explained, it was not considered to negatively influence the process validation exercise.

Similarly, for the purification stage of the process, the prospective validation data showed good agreement with the historical operating windows and demonstrated a high degree of reproducibility and consistency. Two example data sets are presented here (Figure 7) for total protein and specific activity throughout the purification process. In all cases apart from the cation-exchange II step, the data agree with the historical operating windows as shown in the figure. The process intermediate after the cation-exchange II step had a slightly higher specific activity when compared to the data in the historical database. As specific activity is a measure of product purity, one batch being slightly more pure than expected at this intermediate stage should not be taken as a major process issue and was ascribed to normal process or assay variation. The final drug substance for this batch met all quality attributes and specifications (data not shown).

The process validation data generated using the new analytical techniques also showed a high degree of batch-to-batch consistency and reproducibility. Many analytical techniques were developed to fully characterise the product, and a selection of data are presented here. The HPLC-based techniques (Table 3) were used to look at product purity, oxidation and aggregation states. SDS-PAGE was also used to show product purity, and selected data are shown in Figure 8. Other analytical techniques were used, such as

peptide mapping, host-cell protein content, residual DNA content, bioburden content, endotoxin content and isoelectric charge variant content (data not shown). Taken together as a whole, these data confirm that the *Erwinia* L-asparaginase manufacturing process is consistent and reproducible and results in a product with consistent quality and purity.

4. Conclusions

Using the process validation strategies detailed in this paper, the manufacturing process for *Erwinia* L-asparaginase was successfully validated. The historical database was consulted and for each process parameter or quality attribute, operating windows were defined, each consisting of the mean value ± three SD around the mean. New analytical techniques were employed to fully characterise the quality of the product and applied to a subset of historical batches. Three prospective process validation batches were then conducted and characterised using both routine and new analytical methods. The results from all these measurements were compared against the historical operating windows (for routine measurements) or for new measurements, the existing historical data. All analytical measurements performed on the process validation campaign samples confirmed the robustness and reproducibility of the *Erwinia* L-asparaginase manufacturing process. Furthermore, the product quality of both the process intermediates and the final drug substance was shown to be reproducible and consistent. In some cases, studying process performance using historical operating windows can lead to process understanding, such as step changes in performance when new raw materials are introduced.

The results of the process validation of *Erwinia* L-asparaginase demonstrate that combining historical data mining with rigorous application of new analytical techniques,

older biologic processes can be successfully characterised and validated to comply with modern regulations. The framework described in this paper could easily be applied to other legacy biopharmaceutical products. As a result of the successful completion of the validation programme described above the *Erwinia* L-asparaginase manufacturing process was considered validated, and an US FDA product license was granted for Erwinaze® in November, 2011.

Acknowledgements

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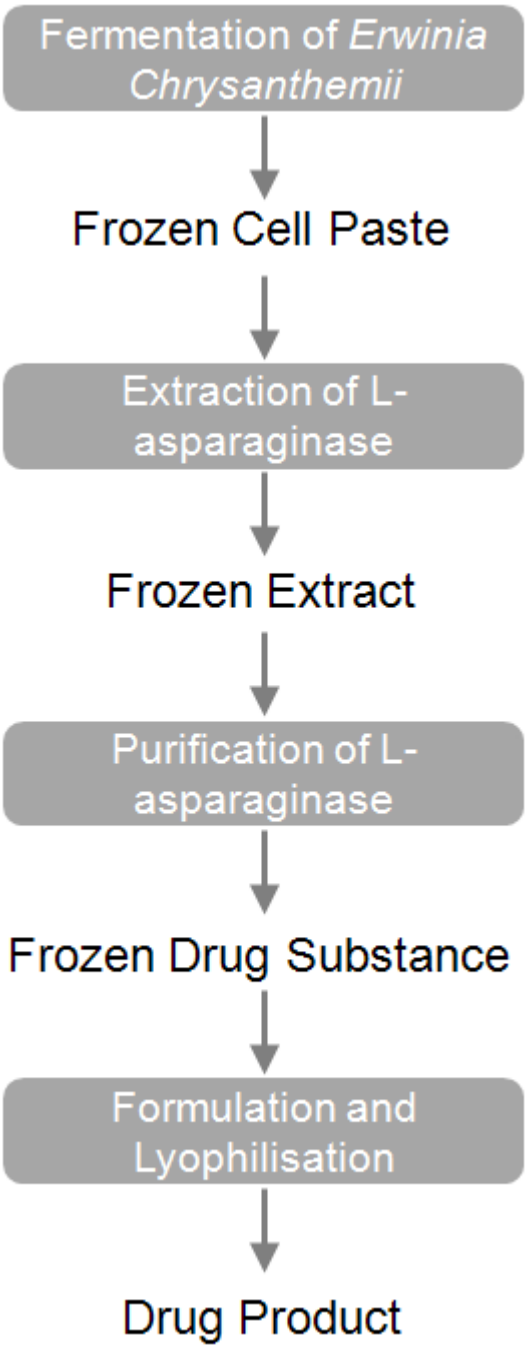


Figure 1. *E. chrysanthemi* L-Asparaginase Manufacturing Process Overview.

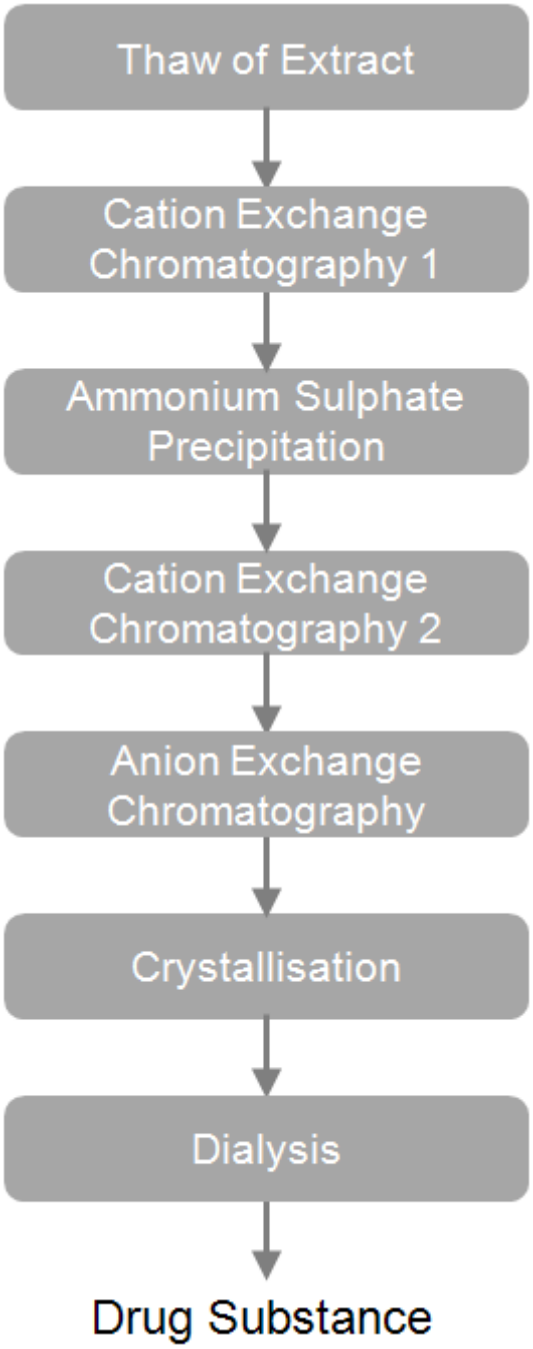


Figure 2. Overview of Purification Process for *E. chrysanthemi* L-asparaginase.

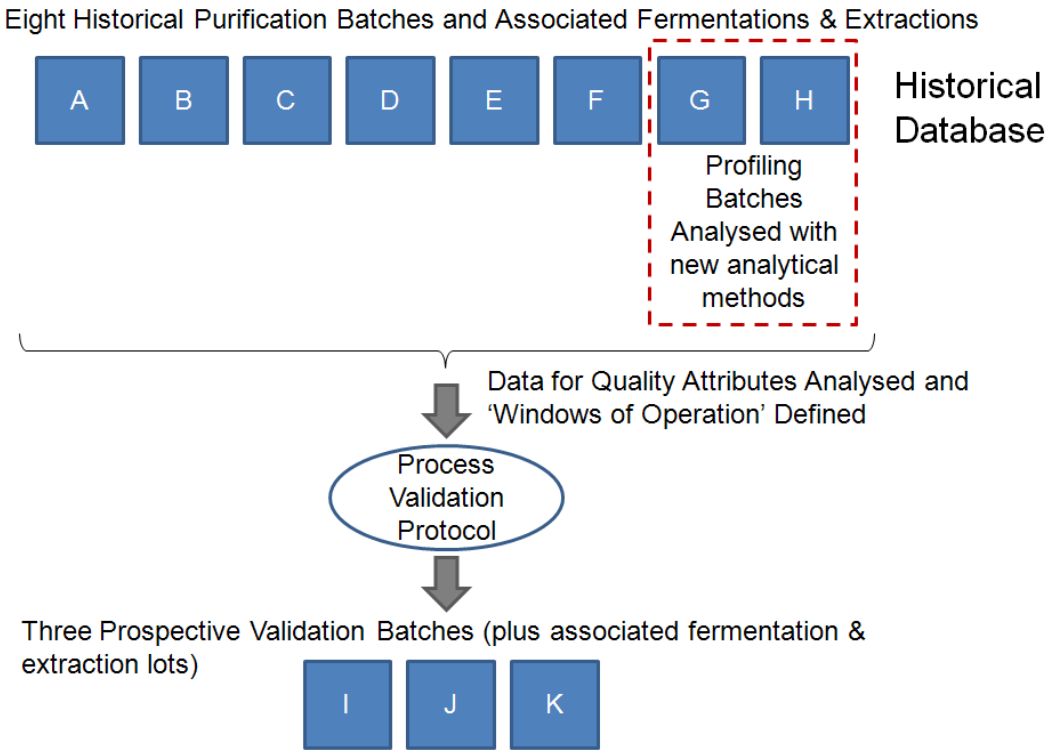
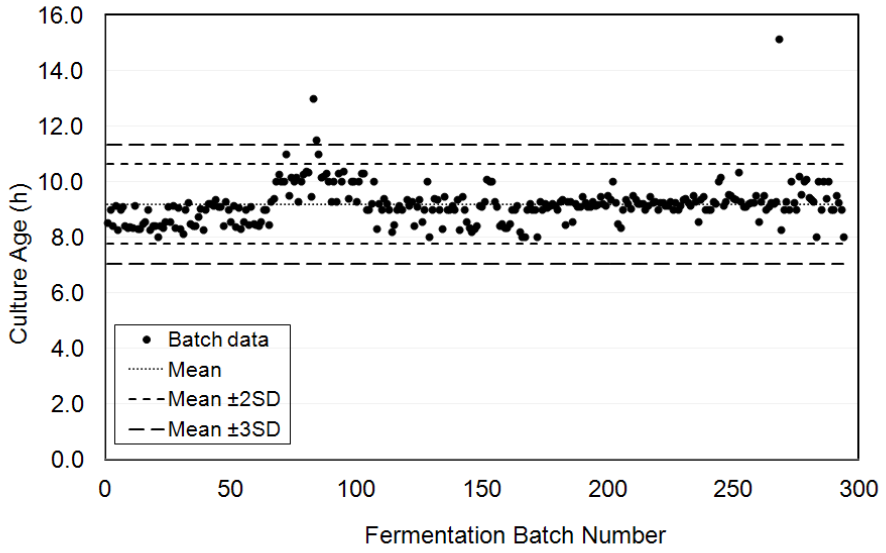
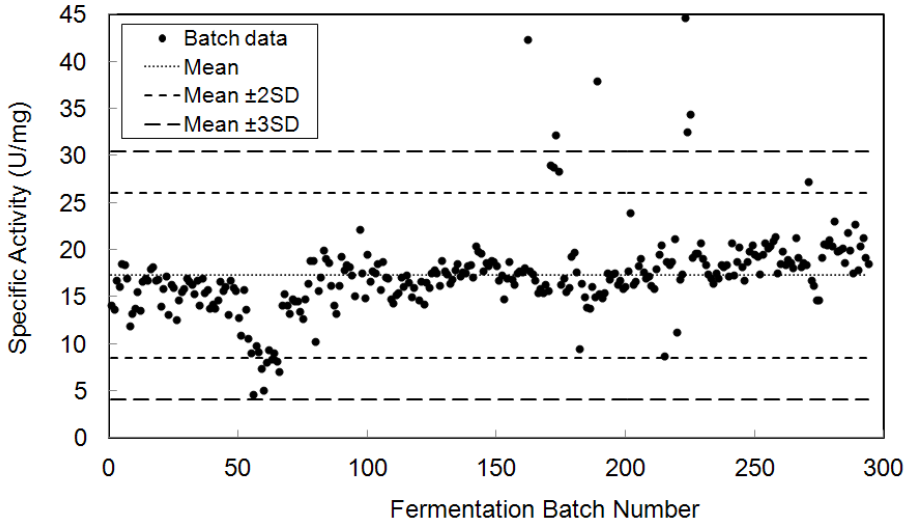


Figure 3. Overview of Process Validation Strategy for *E. chrysanthemi* L-asparaginase.

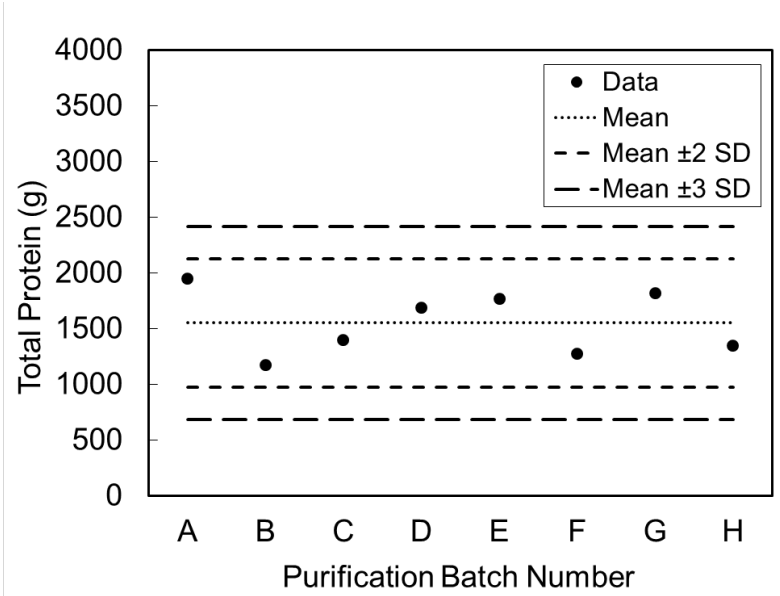


(A)

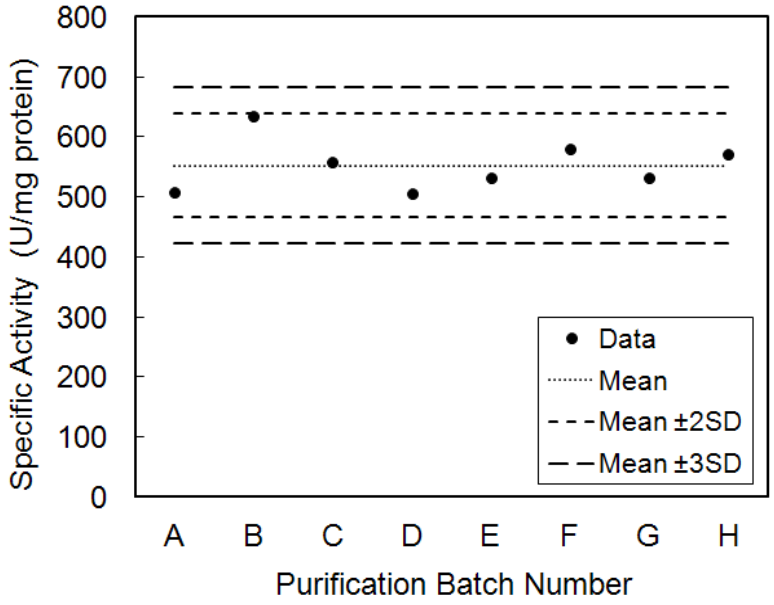


(B)

Figure 4. Historical Data for the Production Fermentation Stage of *Erwinia* L-asparaginase. Each data point represents one fermentation batch out of approximately 300 batches. The mean, mean ± 2 standard deviations (SD) and mean ± 3 SD are also plotted with the data points. (A) Culture Age of Production Fermenter and (B) Specific L-asparaginase activity at harvest.

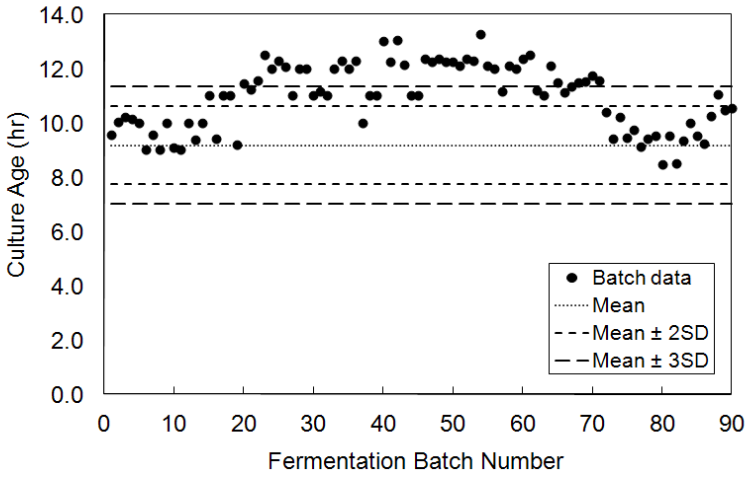


(A)

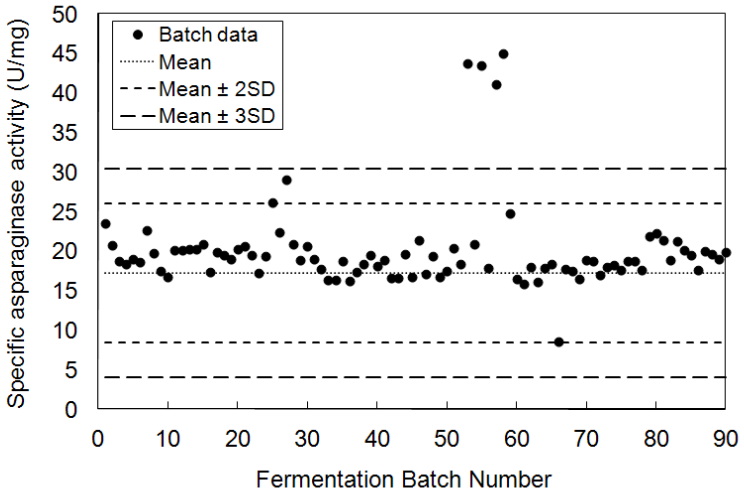


(B)

Figure 5. Historical Data for the Cation-Exchange Chromatography of *Erwinia* L-asparaginase. Each data point represents one purification batch out of eight total batches. The mean, mean ± 2 standard deviations (SD) and mean ± 3 SD are also plotted with the data points. (A) Total Protein Content of Cation-Exchange Product and (B) Specific L-asparaginase activity of Cation-Exchange Product.

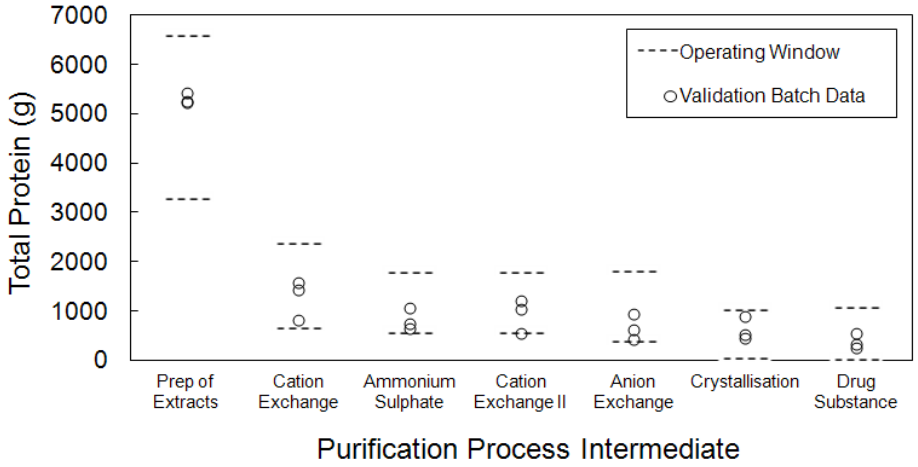


(A)

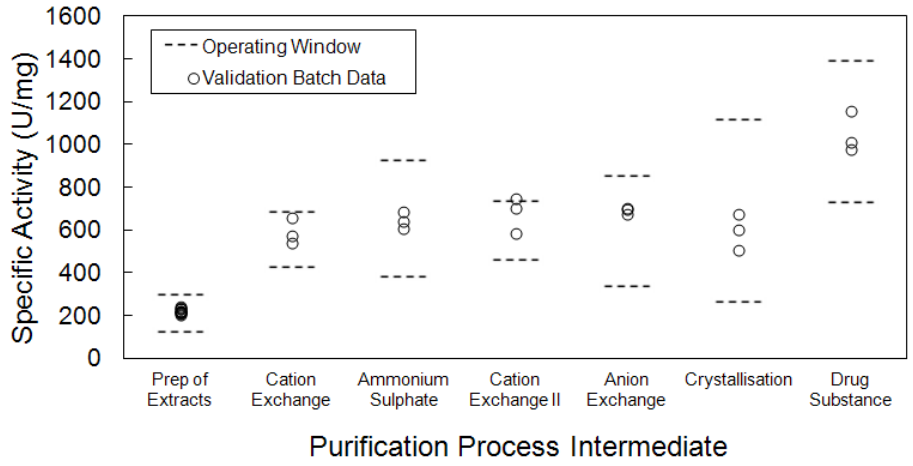


(B)

Figure 6. Selected Fermentation Process Validation Data. The data presented are from the 90 fermentation batches executed in order to make the 3 process validation purification batches. Data in the figures are compared to the statistical operating windows (depicted by horizontal lines) established for the historical database. Data are presented for (A) production fermentation culture age and (B) specific activity at fermentation harvest.



(A)



(B)

Figure 7. Example Purification Process Validation Data. Data are shown for each of the process validation purification batches, at each intermediate stage of the process. Data in the figures are compared to the ± 3 standard deviation operating windows (depicted by horizontal lines) established for the historical database. Data are presented for (A) total protein and (B) specific activity.

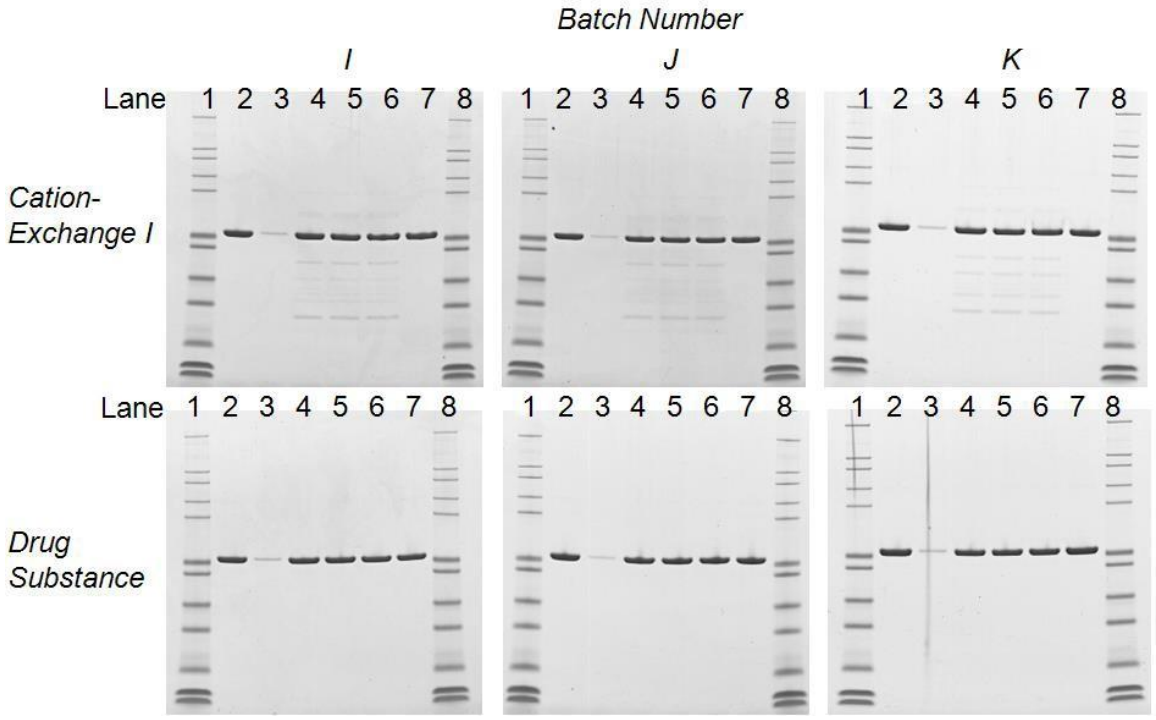


Figure 8. Coomassie-stained SDS-PAGE images from the *Erwinia* L-asparaginase Purification Process Validation campaign. The product is shown after the cation-exchange I step and at final drug substance. In all gel images, lanes 1 and 8 (reading from left to right) are the molecular weight markers, lanes 2 and 7 are the reference standard (ERS), lane 3 is the intensity standard (representing a 2% or 20 ng load) and lanes 4 – 6 (marked with 'test') are the sample run in triplicate. The loading of each standard and test well is 1 µg. The data show the reproducibility and robustness of the purification.

Table 1. Historical Process Parameters and Quality Attributes for the Fermentation, Cation Exchange I Chromatography, and Ammonium Sulphate Precipitation steps in the manufacture of *E. chrysanthemi* L-asparaginase.

Process Step	**Process Parameters**	**Quality Attributes**
Production Fermentation	• Media Composition • Fermentation pH • Dissolved Oxygen Content • Temperature • Agitation Rate • Airflow • Antifoam Feed Rate • Evolved CO_2 Profile	• 600 nm Absorbance at Start • 600 nm Absorbance at Harvest • Fermentation Duration • Growth rate • Doubling time • Asparaginase Activity at Harvest • Asparaginase Specific Activity • Cell dry weight at Harvest • Viable Cell Count
Cation Exchange Chromatography	• Flow Rate • Bed Height • Column Load (g protein/L resin) • Buffer pH • Temperature	For Column Eluate: • Asparaginase Activity • Protein concentration • Specific activity • Total activity • Endotoxin • Bioburden
Ammonium Sulphate Precipitation	• Amount of $(NH_4)_2SO_4$ added • Precipitation Time • Centrifuge Speed • Redissolution Volume • pH	• Asparaginase Activity • Protein concentration • Specific activity • Total activity • Endotoxin • Bioburden

Table 2. New Analytical Techniques Developed for Product Quality Assessment During Process Validation. The same assays were applied to purified Drug Substance as well as other product intermediate stages.

Process Step	**Technique**	**Purpose**
Cation Exchange Chromatography	Reversed-Phase UPLC	Product Oxidation, Impurities
	Weak Cation-Exchange HPLC	Product Charge Variants
	Size-Exclusion HPLC	Product Aggregation
	SDS-PAGE	Impurities
	DNA Content by Pico-GreenTM	Host Cell DNA Contamination
	Host-Cell Protein ELISA	Non -L-asparaginase *Erwinia* protein detection

Table 3. Selected Profiling Batch Historical and Process Validation Data using New Analytical Methods. The data show the area percent of the main L-asparaginase peak. The Reversed-Phase method characterises the product with respect to oxidised species and impurities. The Size-Exclusion method characterises the product with respect to aggregates. The tetrameric form of the L-asparaginase is the active species. N/m indicates that the method is not suitable for product at this stage of processing.

Process Intermediate	**Batch Series**	**L-asparaginase Purity by Reversed-Phase HPLC**	**Percent Tetrameric L-asparaginase by Size Exclusion**
Extract	Historical Profiling (G – H)	36 – 40%	N/m
	Process Validation (I – K)	38 – 47%	N/m
Cation-Exchange I Product	Historical Profiling (G – H)	87 – 91%	N/m
	Process Validation (I – K)	89 – 93%	N/m
Cation-Exchange II Product	Historical Profiling (G – H)	92 – 95%	98 – 99%
	Process Validation (I – K)	94 – 96%	100% (n=3)
Anion-Exchange Product	Historical Profiling (G – H)	93 – 94%	99% (n=2)
	Process Validation (I – K)	94 – 95%	100% (n=3)
Drug Substance	Historical Profiling (G – H)	94 – 95%	100% (n=2)
	Process Validation (I – K)	94 – 95%	100% (n=3)