Control of process-induced asparaginyl deamidation during manufacture of *Erwinia Chrysanthemi* L-Asparaginase


This version is available: 05.08.2016
Available on RADAR: https://radar.brookes.ac.uk/radar/items/459fac79-4d86-4216-93b5-00d7339b97da/1/

Copyright © and Moral Rights are retained by the author(s) and/ or other copyright owners. A copy can be downloaded for personal non-commercial research or study, without prior permission or charge. This item cannot be reproduced or quoted extensively from without first obtaining permission in writing from the copyright holder(s). The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the copyright holders.

This document is the authors’ accepted version.

NB This paper, published as above, is part of a doctoral thesis based on published work. For the body of the thesis please see: David P Gervais, *The effect of degradation on the efficacy and utility of the biopharmaceutical enzyme Erwinia Chrysanthemi L-Asparaginase* (PhD, Oxford Brookes University, 2015)

This version is available: 05.08.2016
Available on RADAR: https://radar.brookes.ac.uk/radar/items/25d16520-90a7-4638-93dd-cd543ecc82fd/1/
PhD by Published Work

Statement of contribution by David Paul Gervais

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


Background: In a separate, unpublished study carried out by D P Gervais, it was observed that the thaw time of Erwinia cells appeared to have an impact on the extent of post-translational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 asparaginase 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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jennifer O’Donnell</td>
<td>[Signature]</td>
<td>16th Dec 13</td>
</tr>
<tr>
<td>Mei-an Sung</td>
<td>[Signature]</td>
<td>21st July 14</td>
</tr>
<tr>
<td>Stuart Smith</td>
<td>[Signature]</td>
<td>24th July 2014</td>
</tr>
</tbody>
</table>

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

This paper is also available on RADAR: https://radar.brookes.ac.uk/radar/items/459fac79-4d86-4216-93b5-00d7339b97da/1/
Control of process-induced asparaginyl deamidation during manufacture of *Erwinia chrysanthemi* L-asparaginase

David Gervais*, Jennifer O’Donnell, Mei-an Sung, Stuart Smith

*Public Health England, Microbiology Services, Development & Production, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom

deltaDot Limited, London BioScience Innovation Centre, 2 Royal College Street, London, NW1 0NH

*Corresponding Author. Tel: +44 1980 619595; fax: +44 1980 612694; email: dave.gervais@phe.gov.uk
Abstract

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

Keywords: deamidation; L-asparaginase; Erwinia; cell freeze/thaw; tetramer; capillary electrophoresis
1. Introduction

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

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

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

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

Deamidation in L-asparaginase was first observed for EcA using isoelectric focusing in the 1970s [12,13], where it was noted that the change in isoelectric point (pI) had no apparent effect on enzyme activity. Deamidation of EcA was also studied by other researchers [14] who investigated deamidated forms using electrophoresis and amino acid analysis. More recently, researchers have used two-dimensional gel electrophoresis to separate post-translationally modified forms (including deamidated forms) of EcA and ErA [15].

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

Cell Thaw Experiments:

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

Protein and Activity Assays:

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

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

WCX Assay:

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

Capillary Electrophoresis Analysis:

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

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

3.0 Results and Discussion

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

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

Due to the freeze-thaw steps in the ErA manufacturing process, the ErA tetramer is subjected to several partial dissociation and reassociation events. The timing and conditions of many of the freeze/thaw steps during the purification process are such that near-complete reassociation into the 140kDa tetramer occurs without issue. However, during the lysis and extraction steps of the process, the thaw of the Erwinia cell paste is immediately followed by an alkaline lysis step mediated by addition of NaOH, a potential cause of protein deamidation.

The susceptibility of individual asparaginyl residues in proteins to deamidation has been shown to be influenced by primary and secondary structure. The amino acid residue C-terminal to Asn has been shown to be particularly important, with proteins and peptides that have the sequence Asn-Gly showing the highest rates of deamidation, up to 70-fold more than other sequences [24]. Furthermore, the secondary structure has been investigated with respect to deamidation rates, with both α-helical and β-sheet structures conferring some protection against nonenzymatic deamidation [25]. NaOH has been shown to be particularly aggressive in peptide deamidation of the Asn-Gly sequence, with increased deamidation rates for 10mM NaOH at pH 12.0 [26]. The sequence of ErA contains one Asn with a C-terminal Gly, at Asn$_{281}$ [27]. Secondary structure analysis of
this region of the ErA molecule using UniProt shows Asn\textsubscript{281} may be in a β-sheet structure, which may confer some protection against alkaline-mediated deamidation. However, deamidation at this site has been previously detected, but not quantified, in lyophilised samples of formulated ErA using two-dimensional electrophoresis and mass spectrometry [15]. Most importantly for Asn\textsubscript{281}, when the quaternary structure of ErA is intact, this residue is buried inside the tetramer complex based on published x-ray structures [28,29].

We used the previously-solved structure (ID 1HG1) from the worldwide Protein Data Bank (PDB) to show that when the enzyme dissociates into monomers, Asn\textsubscript{281} becomes solvent accessible, rendering this site available for deamidation to Asp (Figure 2).

Therefore, prior to alkaline lysis of ErA it is important to allow sufficient time post freeze-thaw, for dissociated monomer to reassociate into tetramer. Allowing sufficient time for tetramer reassociation protects Asn\textsubscript{281}, an extremely labile residue, from deamidation. This effect was tested by performing ErA extraction and purification at both 1/50\textsuperscript{th} and 1/2900\textsuperscript{th} of full manufacturing scale. Each 1/50\textsuperscript{th}-scale purification batch was made from an extract lot which had been thawed pre-lysis for either 24 or 36h, representing the upper and lower limits of the manufacturing operating range for this parameter. In the 1/2900\textsuperscript{th}-scale experiments, a portion of cell paste was thawed, and subdivided in half. The first portion was lysed immediately (0h thaw time) while the remaining paste was held at 2 – 8°C for 24h before alkaline lysis. The 1/2900\textsuperscript{th}-scale lysates (0h and 24h) were then processed normally, stopping at the crude extract stage (just prior to cation-exchange chromatography in the full-scale process).

The samples from both the 1/50\textsuperscript{th} and 1/2900\textsuperscript{th} scale experiments were analysed using a variety of techniques. WCX HPLC can be used to monitor the content of the acidic species in ErA process intermediates. The WCX results (Figure 3) show that longer cell paste thaw times result in lower ErA deamidated species content. The WCX data are
expressed as a ratio (in %) of the sum of the area of the peaks representing acidic ErA species (referred to as peaks C, D and E) over the area of the peak representing the unmodified ErA (peak F). Peaks C, D and E are known by LC-MS to consist of ErA species which have been deamidated at various sites, as well as other modifications (data not shown). Due to the additional impurity peaks observed in WCX for crude extract samples, retention times for the WCX peaks C, D, E and F in the crude materials produced from 1/2900\textsuperscript{th}-scale cell-thaw experiments were verified using purified ErA which had an acidic species content of approximately 10%. It is important to note that the difference in thawing times between the laboratory-scale (0 – 24h) and full-scale (24 – 36h) data are solely due to the increased time necessary to thaw larger blocks of frozen materials during the generation of production-scale data.

The WCX HPLC data were further confirmed by using CZE which separates proteins based on the mass-to-charge ratio. This technique is useful for monitoring deamidation in protein products as has been discussed by other investigators [30]. The CZE data (Figure 4) for both the laboratory-scale and full-scale manufacturing data show the effect of cell paste hold time on the appearance of acidic species, with longer hold times resulting in reduced acidic species content. The time values in the figures are presented as relative migration times (RMT), calculated by dividing each time value by the migration time of the main ErA peak (analogous to relative retention times in HPLC). The deamidated forms of ErA migrate within the capillary just after the main band, at RMT between 1.02 and 1.09. The RMT behaviour of the deamidated forms of ErA in the CZE assay was confirmed (Figure 4C) using purified ErA and the method of DiDonato \textit{et al.} [31] with incubation in 1% ammonium bicarbonate at 37°C for 24h. The location and appearance of several more acidic species in the RMT 1.02 – 1.09 region after NH\textsubscript{4}HCO\textsubscript{3} treatment is highly indicative of deamidation.
Although both WCX and CZE showed a link between cell thaw time and acidic species content, neither technique was capable of confirming the acidic species were due to deamidation, or the specific involvement of Asn$_{281}$. Therefore, peptide mapping was used to confirm the location of the affected residue(s) and provide a more definitive link between deamidation and the acidic species results from CZE and WCX. Tryptic digest of ErA results in a number of fragments including tryptic fragment T33, which has the sequence TGNGIVPPDEALPGLVADSLNPAHAR. The peptide map of a tryptic digest of pure ErA results in a peak corresponding T33 at approximately 34min retention time closely followed by two shoulder peaks. Although the peptide map in this work has been run only using UV detection of the various fragments, previous work on this validated ErA method using mass spectrometry (LC-MS) detection has shown the 34min peak to correspond to the mass of T33, and the two shoulder peaks corresponding to +1 and +2Da variants of T33, consistent with deamidation at one or two sites (data not shown). T33 contains two Asn residues, with Asn$_{281}$ closest to the N-terminus of this peptide fragment. The other Asn residue, Asn$_{299}$, is also buried inside the tetramer structure in native ErA, and is only solvent-accessible upon tetramer dissociation, like Asn$_{281}$. According to the literature, Asn$_{299}$ should be much less susceptible to deamidation than Asn$_{281}$, as the residue immediately C-terminal to Asn$_{299}$ is Pro [24].

Peptide maps were conducted on tryptic digests of four purified ErA samples which had undergone various cell thaw times during their manufacture, and therefore had differences in WCX acidic species content. The ErA samples were confirmed to be pure by SDS-PAGE prior to peptide mapping (data not shown). The UV peaks corresponding to T33 and the +1 and +2Da variants were integrated and the results expressed as the ratio of peak areas of deamidated T33 species to unaffected T33. The results (Figure 5) correlate well with the WCX acidic species for these samples. These results confirm that deamidation at
Asn\textsubscript{281} occurs and the extent of deamidation at this site is related to the *Erwinia* cell thaw time during processing.

Although the acidic ErA species content varied in laboratory-produced purification batches, there was no correlation with specific activity (Table 1). Although no specific activity data are available in the literature for acidic ErA species, this finding is consistent with the findings for EcA [13] where the acidic species observed did not affect the overall activity of the enzyme preparations. It should be noted that even those preparations which have a long cell thaw time result in a degree of acidic species in the product as observed by WCX, CZE and peptide mapping, and this may be due to deamidation of Asn which are on the tetramer solvent-exposed surface (e.g. Asn\textsubscript{41}) or other side reactions with ErA residues.

**4. Conclusions**

ErA is a tetrameric enzyme which contains multiple Asn residues. A small fraction of the total amount of tetramer dissociates into monomeric subunits upon freezing (at -20\(^\circ\)C) and thawing. However, given approximately 24h after thawing, the dissociated 35kDa monomers reassemble back into 140kDa tetramers. The dissociation and subsequent reassociation is observed using purified enzyme and size-based analytical techniques such as Native PAGE and SEC.

Analysis of ErA structure using published results and molecular structure viewing software shows that one Asn residue (Asn\textsubscript{281}) is theoretically very susceptible to deamidation due to the ErA sequence (Asn\textsubscript{281}Gly\textsubscript{282}). This part of the ErA monomer is normally protected from solvent exposure due to its internal location in the tetrameric
quaternary structure. However, upon freeze-thaw, this site is exposed due to tetramer dissociation.

Through a set of repeated, controlled experiments and analytical characterisation, we have shown that deamidation of Asn_{281} can be easily controlled by lengthening the time utilised to thaw the *Erwinia* cells immediately prior to the alkaline lysis step. The results of this study may be used to reduce the amount of process-related acidic ErA variants appearing in the final purified product. Allowing a longer time for the cell thaw step allows the small fraction of dissociated enzyme to reassemble into tetramers, protecting Asn_{281}. The long cell-thaw times result in lower ErA acidic species content as measured by several analytical techniques, including WCX-HPLC, CZE, and peptide mapping.

This study shows a direct link between deamidation and processing conditions at the very early, crude stage of manufacture. Deamidation is typically studied in pure protein samples, due to the difficulty of analysing this slight change in the presence of contaminating proteins. Often, the specific process conditions that cause deamidation and other degradation pathways during processing, such as oxidation, are poorly understood. This kind of structural approach, linking processing conditions to analytical measurements far downstream, may be of interest in other protein purification processes.
Contributions

D.G. designed the research, performed the research, analysed the data and wrote the manuscript; S.S., M.S. and J.O. contributed analytical methods and supported data evaluation.

Acknowledgements

The authors would like to thank Roger Hinton, Head of Development & Production, for facilitating these studies, Nigel Allison for advice and critical review of the manuscript, Trevor Marks, Ros Brehm, Patrick Kanda, and Alan Jennings for helpful discussions, Lucy Elliott for performing activity assays, and Darryl King for conducting the peptide mapping UPLC analysis. Further thanks go to the entire Development & Production team at HPA Porton, and the team at EUSA Pharma, a division of Jazz Pharmaceuticals plc, without all of whom this work would not have been possible.
References


Figure 1. Effect of time on freeze-thaw induced ErA tetramer dissociation. The main frame shows an SEC trace of purified ErA at 0h and 24h post-thaw time, with the aggregate (A), tetramer (B) and monomer subunit (C) marked on the trace. Inset figure shows a NativePAGE gel image of the same experiment, with the location of monomeric (bottom arrow), tetrameric (middle arrow) and higher-order aggregate (top two arrows) species indicated. Lane 1- 1µg/well at 0h post-thaw, Lane 2- 1µg/well at 24h post-thaw, Lane 3- 5µg/well at 0h post-thaw, and Lane 4- 5µg/well at 24h post-thaw.
Figure 2. Three-dimensional ErA structures created using Jmol and the published PDB structure 1HG1 (Lubkowski, 2003). One of the four 35kDa subunits is coloured in blue, the other three are coloured in grey. The location of the four Asn281 residues is shown in orange (CPK style). Clockwise from top left: front view, left view, top view and view showing one subunit only.
Figure 3. Effect of Erwinia cell paste hold time on ErA acidic species content (WCX HPLC assay). Data are mean values with error bars indicating the range of values in the data set. Longer thaw times allow dissociated ErA subunits to reassemble into tetramer, protecting the labile residue Asn\textsubscript{281} from deamidation during the subsequent alkaline lysis step. (A) Acidic species content of cell extracts from laboratory-scale cell lysis experiments. (B) Acidic species content of purified protein obtained from full-scale cell lysis. (C) Representative WCX chromatogram for laboratory-scale data showing acidic species peaks (C, D and E) and the main (undegraded) species (F).
Figure 4. CZE Electropherogram Traces Showing Effect of *Erwinia* cell paste hold time on ErA acidic species content. The absorbance data have been normalised to the maximum absorbance for the main ErA peak, and the time axis normalised relative to the migration of the main peak. The region of interest of acidic ErA variants, verified in panel C, is between relative migration times of 1.02 and 1.09, and is marked in panels A and B. (A) CZE comparison of laboratory-scale cell extracts held pre-lysis for 0h and 24h. (B) CZE comparison of full-scale cell extracts held pre-lysis at 24h and 36h. (C) Effect of 24h 1% NH$_4$HCO$_3$ treatment at 37°C on CZE profile. The post-treatment appearance of acidic variants in the relative migration region of interest (1.02 – 1.09) is marked with arrows.
Figure 5. Correlation of WCX acidic species content with deamidation content in the peptide fragment T33 (corresponding to amino acid sequence TGNGIVPPDEALPGLVADSLNPAHAR and containing the residue Asn\textsubscript{281}) using peptide mapping HPLC. The analyses were carried out on ErA samples which were verified to be free from other protein contaminants using SDS-PAGE.
Table 1. Comparison of Acidic Species Content and Specific Activity of Purified ErA Produced at 1/50\textsuperscript{th} manufacturing scale.

<table>
<thead>
<tr>
<th>Batch ID</th>
<th>% Acidic Species by WCX HPLC</th>
<th>Specific Activity of Purified Enzyme (U/mg Lowry protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9%</td>
<td>971</td>
</tr>
<tr>
<td>2</td>
<td>18%</td>
<td>1302</td>
</tr>
<tr>
<td>3</td>
<td>18%</td>
<td>884</td>
</tr>
<tr>
<td>4</td>
<td>17%</td>
<td>1218</td>
</tr>
<tr>
<td>5</td>
<td>17%</td>
<td>1405</td>
</tr>
<tr>
<td>6</td>
<td>11%</td>
<td>978</td>
</tr>
</tbody>
</table>