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# Deamidation in ricin studied by capillary zone electrophoresis- and liquid chromatography–mass spectrometry

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ABSTRACT

Deamidation in ricin, a toxin present in castor beans from the plant *Ricinus communis*, was investigated using capillary zone electrophoresis (CZE) and liquid chromatography coupled to high resolution mass spectrometry. Potential sites for deamidation, converting asparagine (Asn) into aspartic or isoaspartic acid (Asp or isoAsp), were identified *in silico* based on the protein sequence motifs and tertiary structure. In parallel, CZE- and LC–MS-based screening were performed on the digested toxin to detect deamidated peptides. The use of CZE–MS was critical for the separation of small native/deamidated peptide pairs. Selected peptides were subjected to a detailed analysis by tandem mass spectrometry to verify the presence of deamidation and determine its exact position. In the ricin preparation studied, deamidation was confirmed and located to three asparagine residues: Asn54 in the A-chain, and Asn42 and Asn60 in the B-chain. Possible *in vitro* deamidation occurring during sample preparation was monitored using a synthetic peptide with a known and rapid rate of deamidation. Finally, we showed that the isoelectric diversity previously reported in ricin is related to the level of deamidation.



## 1. Introduction

Ricin is a highly toxic protein that can be isolated from castor beans, the seeds of the ricin plant *Ricinus communis.* Ricin plants are naturally occurring in tropical climates and castor beans are produced throughout the world as a source of castor oil, which is mainly used in various industrial applications. Annual world production of castor beans exceeds 1 million tons [1]. In addition, ricin plants are widely cultivated as ornamentals due to their attractive colour and shape. The extraction, storage and use of ricin is regulated under the Chemical Weapons Convention (CWC) and must be reported to national authorities [2]. The toxin content in the seed is up to 1% and production of crude toxin from the castor bean is unfortunately fairly simple. Also, the easy access to castor beans has contributed to frequently occurring "white powder" incidents [3–5]. Therefore, an increased need for forensic attribution information relating to the origin of the sample has evolved. Reported forensic markers are sequence information for different varieties of ricin, differences or similarities in the content of co-extracted proteins, variation in the extent of glycosylation and also content of peptide biomarkers [6–12]. Recently, isotope ratio comparison has also been shown to be useful for this purpose [13].

The ricin toxin is a 66 kDa plant lectin of RIP 2-type (ribosome-inactivating protein) that induces toxicity by inhibiting protein synthesis inside a target cell, eventually leading to cell death [14]. Ricin contains two polypeptide chains. The larger B-chain exhibits the lectin properties, thereby mediating binding to a target cell surface and enabling the toxin to be incorporated into the cell. Inside the cell, the B-chain is released and the free A-chain acts as an enzymatic ribosomal inhibitor [14,15].

Deamidation in proteins is a posttranslational process that converts asparagine to aspartic and isoaspartic acid, and glutamine to glutamic acid. These transformations alter the protein charge and affect the pI of the proteins. Resulting conformational changes can lead to protein dysfunction, including enzymatic deactivation, loss or altered affinity for various ligands and reduced stability [16]. However, deamidation can function as a timed signal for protein events such as activity switching and has an important role as a molecular clock in biological systems [17–19]. Deamidation is a commonly occurring process that has been studied in eye lens crystalline proteins [20], proteins extracted from potato tubers [21], antibodies [22], toxicity factors of anthrax [23] and synthetic peptide products [24]. The standard model for nonenzymatic deamidation of asparagine is outlined in Fig. 1. This reaction has been found to be about 100 times faster than glutamine deamidation, mainly because of the larger, less favourable intermediate formed in the latter case [25–28]. The smaller and more flexible the adjacent residues are, especially on the carboxyl side, the more

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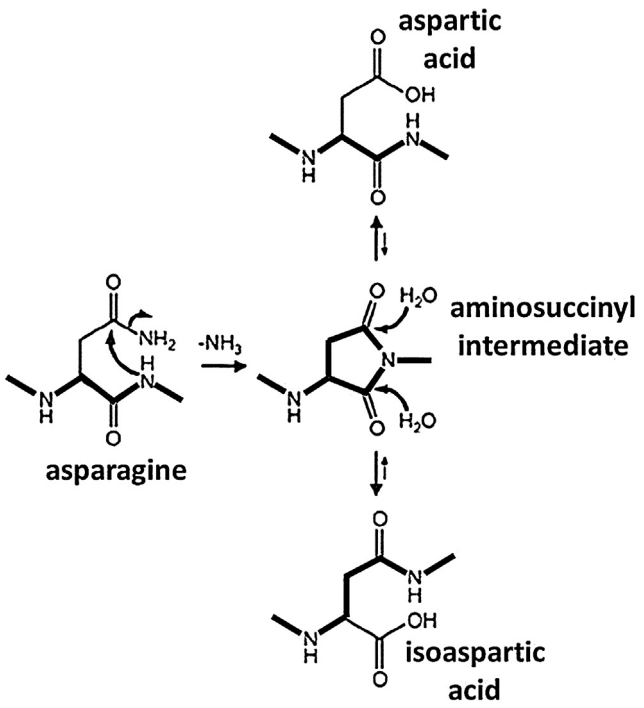


**Fig. 1.** Standard model for nonenzymatic deamidation of asparagine (modified from [68]). The peptide backbone (thick line) is extended by one carbon when isoaspartic acid is formed.

prone asparagines are to undergo spontaneous deamidation. Therefore, an arginine-glycine (NG) motif located in a flexible region of a protein has a high probability of becoming deamidated [29–31]. In such a reaction, both aspartic acid and isoaspartic acid are formed. In neutral or alkaline solutions, formation of isoaspartic acid is favoured with a typical isoAsp:Asp ratio of 3:1, whereas in acidic solutions, only Asp is formed [29]. As displayed in Fig. 1, when isoaspartic acid is formed, the peptide backbone is extended by one carbon. The elongation of the peptide backbone can severely disrupt the protein structure and function, and thus isoAsp formation has been widely studied in the field of protein stability [32,33].

To study deamidation at the amino acid level, one of the most feasible methods is to digest the protein of interest into peptides and then separate and analyse the peptides by liquid chromatography coupled to mass spectrometry (LC–MS) [34]. However, deamidation induces only a small shift in peptide hydrophobicity and size, hampering separation of the peptide pairs (native and deamidated) by reversed phase chromatography systems. In addition, deamidation is detected by mass spectrometry as a ~1 Da shift in the peptide mass. More accurately, the mass shift is +0.9840 Da, making the mass difference between the $(M+H)^+$ monoisotopic ion of the deamidated peptide and the $[(M+\text{neutron})+H]^+$ isotopologue ion of the native peptide to be only 0.0193 Da (based on the $^{13}C$ contribution). Thus, the isotope clusters of the native and deamidated peptide pairs overlap and co-elution makes detection and identification of the deamidated peptide difficult.

As a complementary technique for the analysis of proteins, peptides and small molecules, on-line capillary zone electrophoresis-mass spectrometry (CZE–MS) has gained increasing attention [35–40]. The separation principle of CZE is based on the charge-to-size ratio of the analytes, making it a complement to the hydrophobicity-based separations in reversed phase-LC. However, the coupling of CZE with MS limits the choice of available buffer systems due to the requirement for volatile components. Furthermore, stability problems are usually worse for CZE–MS than for LC–MS. On the other hand, if the drawbacks can be controlled, CZE–MS is a powerful analytical tool with high separation efficiency that has proven useful in various applications. One example is the separation of small peptides, i.e. shorter than five amino acids, as these are difficult to retain in reversed phase-LC [41]. In addition, the technique is useful for resolving the native and deamidated peptide pairs of short peptides, as both the difference in charge and mobility significantly contribute to their separation. The migration order in both CZE–MS and LC–MS is usually Asn→Asp→isoAsp. The deamidated, aspartic/isoaspartic acid-containing peptides have lower mobility and slightly higher hydrophobicity than the corresponding native peptide and the effect is strongest for the isoaspartic acid-peptide due to the induced extension of the peptide backbone by one carbon. Protein deamidation has been studied at the peptide level for various proteins. Zhang et al. [42] determined the deamidation positions in recombinant human interleukin-11 after digestion, separation and Edman degradation. Terreaux et al. used N-terminal sequencing to identify sites of deamidation in synthetic gliadin peptide [43]. In addition, LC–MS/MS has been used to locate deamidation in IgG1 antibodies [44–46] and *Bacillus anthracis* protective antigen [23].

The aim of this study was to investigate deamidation in ricin for potential use in forensic applications. The complete workflow from prediction of sites in intact ricin to verification and exact determination of the position of these sites using CZE–MS/MS and LC–MS/MS in both tryptic and chymotryptic ricin peptides is reported. Finally, the effect of deamidation on the pI pattern of the toxin was demonstrated.

## 2. Experimentals

### 2.1. Safety considerations

Ricin is listed in Schedule 1 of the Chemical Weapons Convention (CWC), which states that countries that have ratified the CWC are required to declare the production (extraction), possession and usage of ricin to the Organisation for the Prohibition of Chemical Weapons, OPCW [2,47]. Ricin is a toxin that inhibits protein synthesis in cells. Due to its high toxicity, it is recommended that all work performed with intact toxin is carried out in specially designated laboratories. All contact with the active toxin should be avoided. In this study, all the laboratory materials used, including single-use materials, were decontaminated by submersion in 2 M NaOH.

### 2.2. Chemicals

Methanol and acetonitrile (LC–MS grade) were obtained from Merck (Germany). Formic acid (MS grade) was obtained from Fluka (Switzerland), NaOH (CE grade) and $H_2O$ (ultra-pure) from Agilent (USA). CE-capillaries of bare fused silica type were obtained from Genetec (Sweden). Porcine trypsin (MS grade) was obtained from Promega (USA), and bovine pancreas chymotrypsin (TLCK treated) and ammonium bicarbonate were obtained from Sigma-Aldrich (USA). Peptides and proteins used for developing the CE and LC methods were also obtained from Sigma-Aldrich (USA). The CIEF ampholyte solution (pH range 3–10), CIEF gel, anolyte phosphoric acid and catholyte NaOH were all obtained from Beckman Coulter (USA).

### 2.3. Preparation of ricin

The toxin of isoform D was isolated from *Ricinus communis* of zanzibariensis variety (Rara Växter, Stockholm, Sweden) and purified in-house according to previously described methods [7]. Sequence information on ricin (isoform D) was obtained from Uniprot [48]. The three-dimensional structure of ricin was obtained