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Isoaspartate in peptides and proteins: formation, significance, and analysis☆

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Abstract

Formation of isoaspartyl peptide bonds (isoAsp) is one of the most common forms of non-enzymatic degradation of peptides and proteins under mild conditions. IsoAsp arises when certain Asn–Xaa and Asp–Xaa sites undergo a spontaneous intramolecular rearrangement to form a succinimide which subsequently hydrolyzes to generate a mixture of isoAsp–Xaa and Asp–Xaa linkages in a ratio of ~2:1. This pathway is responsible for the much greater susceptibility of asparagine, compared with glutamine, to deamidation at neutral and alkaline pH. Rearrangement occurs most readily at Asn–Gly, Asn–Ser, and Asp–Gly sequences where the local polypeptide chain flexibility is high. Formation of isoAsp can decrease the biological activity of a protein pharmaceutical, alter its susceptibility to proteolytic degradation, and elicit autoimmunity. The enzyme protein L-isoaspartyl methyltransferase can be used to measure isoAsp sites in the low pmol range with or without the use of radioisotopes.

Keywords: Autoimmunity; β-Aspartyl; Cyclic imide; Deamidation; Degradation; Isoaspartate; Peptides; Proteins; Succinimide

1. Introduction

Formation of atypical isoaspartyl peptide bonds and the related process of asparagine deamidation are generally considered to be among the leading sources of spontaneous protein damage under mild conditions [1–3]. The mechanism of isoAsp formation is shown in Fig. 1. The first step, formation of a cyclic imide (succinimide) by deamidation of –Asn–Xaa– or dehydration of –Asp–Xaa– can occur at pH 7.4, 37°C with half-lives ranging from 6 h to several months [4]. The cyclic imide hydrolyzes with a half-life of several hours, generating a mixture that is typically 60–85% L-isoAsp and 40–15% L-Asp along with small amounts of D-isoAsp and D-Asp [5,6]. The presence of L-isoAsp–Xaa bonds can be inferred by failure of the Edman degradation during sequencing [7] or it can be demonstrated directly

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with the enzyme protein L-isoaspartyl methyltransferase (PIMT) [8–10]. As illustrated in Fig. 2, PIMT catalyzes the *S*-adenosyl-L-methionine (AdoMet)-dependent methylation of the α-carboxyl of L-isoAsp sites, thereby facilitating its conversion back to a cyclic imide with concomitant release of methanol. If [^{3}H]methyl-labeled AdoMet is used as the methyl donor, and the pH, time, and temperature of the reaction are properly controlled, the release of [^{3}H]methanol (assayed by diffusion-trapping into liquid scintillation fluid) can serve as a quantitative measure of isoAsp sites in the analyte peptide [11]. A less complex, non-isotopic method for PIMT-dependent isoAsp analysis, utilizing HPLC to assess *S*-adenosyl-L-homocysteine (AdoHcy) production has recently been developed in our laboratory and is described later in this review.

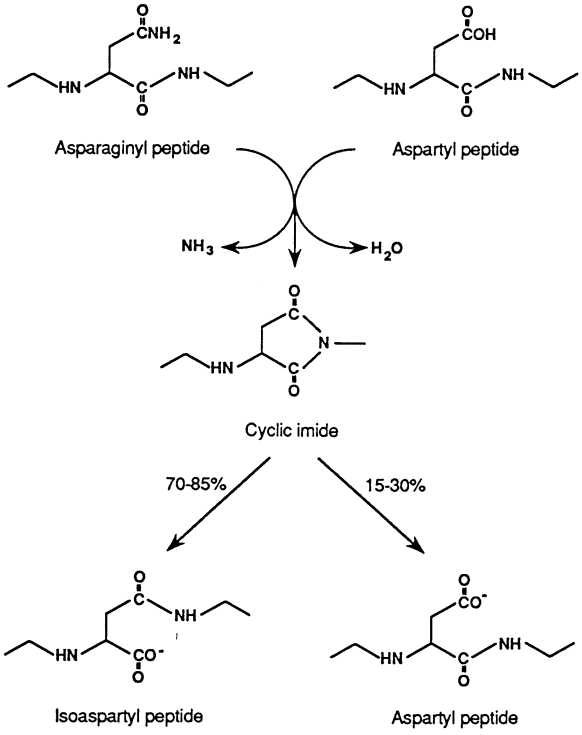

Fig. 1. Mechanism of isoAsp formation at Asn and Asp sites. The α-amino of the residue C-flanking to the Asn or Asp makes a nucleophilic attack on the Asx side chain to form a cyclic imide (succinimide) intermediate. This hydrolyzes to a mixture of isoAsp and Asp in a ratio that ranges from 60:40 to 85:15. In model peptides, deamidation coupled to isomerization of Asn–Xaa bond is favored by alkaline pH, while isomerization of the Asp–Xaa bond is optimal at pH 4–6. Significant levels of isoAsp can accumulate in proteins at both types of sequences during incubation at pH 7.4, 37°C.

2. In vitro aging of recombinant human tissue plasminogen activator (rt-PA): a case study in isoAsp analysis

Recombinant human tissue plasminogen activator (rt-PA), used in the treatment of acute thrombosis, consists of 527 amino acids with three sites of glycosylation and numerous disulfide bonds. In vitro aging of rt-PA at 37°C, pH 7.3, results in a nearly linear increase in isoAsp sites over a 14-day period (Fig. 3). To determine the location of isoAsp sites, the aged protein was subjected to reduction and alkylation of disulfide bonds followed by digestion with trypsin [9]. Reversed-phase HPLC of the digests of control (unaged) and aged rt-PA are compared in Fig. 4 (left panel). The HPLC profiles are nearly identical and differences due to deamidation and/or isoAsp formation are impossible to establish at this point. To focus on peptides containing isoAsp sites, the tryptic digest was methylated by PIMT using [^{3}H]AdoMet and then subjected to the same HPLC separation (Fig. 4, right panel). The difference in profiles is dramatic; while the control shows only minor amounts of isoAsp, the aged sample is found to contain high levels of isoAsp in several peptides. Those peptides making a major contribution to isoAsp in the aged digest were isolated, purified, and further characterized with regard to isoAsp stoichiometry, sequence, and mass. The results allowed us to establish that most of the isoAsp arising during in vitro aging of rt-PA occurs at Asn–177 in the sequence –GNSD– and Asn-58 in the sequence –FNGG– [9].

3. Sequence and flexibility affect rates of isoAsp formation

In vitro aging studies with a wide range of proteins aged under mild conditions have led to

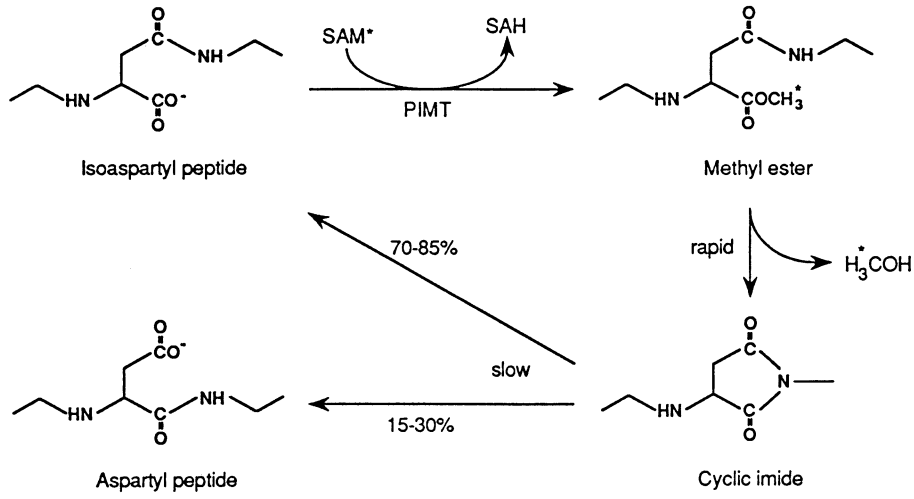

Fig. 2. Enzymatic methylation of isoAsp sites. PIMT catalyzes transfer of the active methyl group from AdoMet onto the atypical α-carboxyl of an isoAsp site. The methyl ester rapidly decomposes to the same succinimide intermediate observed during formation of isoAsp. Hydrolysis of the succinimide generates a mixture of the original isoAsp–Xaa peptide along with a significant portion of 'normal' Asp–Xaa. The isoAsp product can reenter the methylation/demethylation cycle so that eventually nearly all of the isoAsp is converted to Asp. This is the basis for the hypothesis that PIMT serves a repair function in vivo. If [^{3}H]methyl-labeled AdoMet is used in the reaction, and conditions are carefully controlled to prevent recycling of the isoAsp, the evolution of [^{3}H]methanol in step 2 can be used to quantitate isoAsp sites in proteins [11]. Alternatively, isoAsp can be quantitated with equal sensitivity by measuring SAH (S-adenosyl-L-homocysteine) production using narrow bore HPLC with UV detection (Fig. 6).

two important generalizations regarding the roles of sequence and structure in determining sites of isoAsp formation. First, isoAsp tends to form preferentially at Asn–Gly, Asn–Ser, Asp–Gly, and possibly Asn–His sites [3,12–15]. Second, isoAsp tends to form preferentially in regions where the polypeptide chain is highly flexible [3,8,16,17]. The dramatic effect of the C-flanking amino acid (Xaa) on the propensity of an Asn–Xaa or Asp–Xaa bond to isomerize has been known for peptides for several years and has been attributed, at least in part, to the lack of side-chain steric hindrance with Gly and Ser combined with favorable electron withdrawing effects to maximize the probability of a nucleophilic attack on the Asn or Asp side-chain carbonyl by the amide nitrogen of Xaa [18]. It has been pointed out that the structural constraints characteristic of folded proteins can override sequence effects and thereby lessen the influence of the C-flanking amino acid [19–21]. In practice, however, a significant majority of isoAsp prone sites in struc-

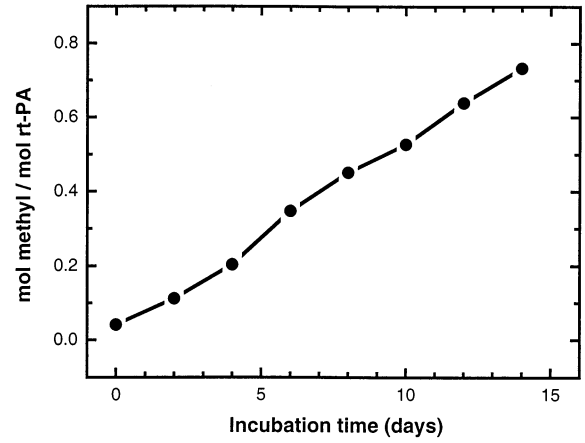

Fig. 3. IsoAsp accumulation during in vitro aging of rt-PA. Samples of rt-PA were incubated at 37°C in 200 mM arginine-phosphate, pH 7.3, 5% glycerol and 0.02% sodium azide for varying times as indicated on the abscissa. IsoAsp was determined by the methanol release assay after PIMT-dependent methylation using [methyl-^{3}H]AdoMet.

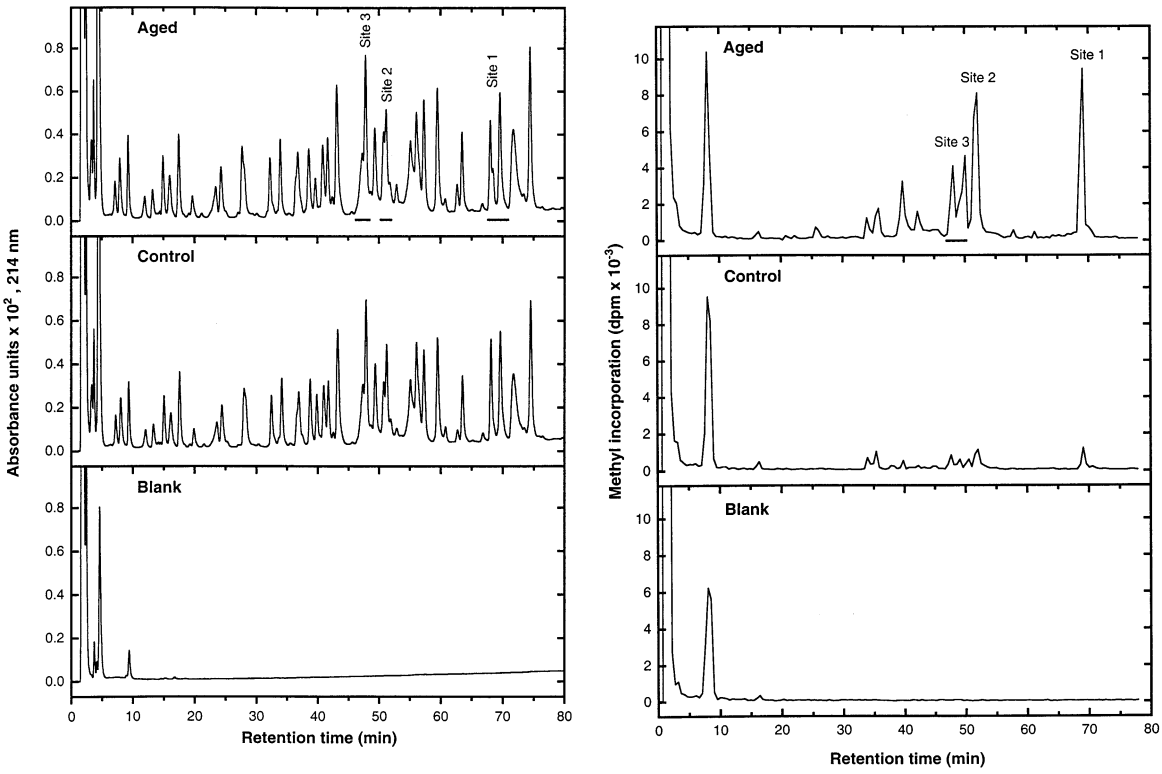

Fig. 4. Reversed-phase HPLC of tryptic digests of aged and control rt-PA with or without prior methylation by PIMT. Reduced and *S*-carboxymethylated samples of aged and control rt-PA were digested for 2 h at 37°C at a rt-PA:trypsin ratio of 100:1. Fresh trypsin was added to bring the ratio to 50:1 and the digestion was then continued for an additional 2 h. Reactions destined for HPLC with UV detection were stopped by adding 0.1 vol. of 85% phosphoric acid. Reactions destined for [^{3}H]methyl analysis were stopped by addition of phenyl-methylsulfonyl fluoride to a final concentration of 4 mM. Reversed-phase HPLC was carried out on a C-8 column using a linear gradient of acetonitrile in a base solvent of 0.1% trifluoroacetic acid in water. The left panels show the profiles of absorbance at 214 nm for tryptic digests of the aged and control rt-PA along with the absorbance for a blank trypsin digest lacking the substrate rt-PA. The right panels show the radioactivity profiles obtained after PIMT-dependent methylation of the digests using [methyl-^{3}H]AdoMet. The numbered peaks in the top right panel indicate the presence of peptides which accumulated significant amounts of isoAsp during in vitro aging. These were collected for further analysis by Edman degradation and mass spectrometry to identify the exact sites of isoAsp formation [9].

tured proteins have been found to occur in the same sequences that favor their formation in oligopeptides [3,15,27]. As might be expected, isoAsp formation in proteins seems to be most common in susceptible sequences that also occur in highly flexible regions of the polypeptide chain. Strong evidence of both an experimental and theoretical nature indicates that elements of well-formed secondary structure, especially α-helices and the β-turns, dramatically reduce the rate of succinimide formation for a given sequence compared with the same sequence in a random coil [19,22–24].

The importance of both sequence and flexibility is aptly demonstrated by superimposing known sites of isoAsp formation on the flexibility plot of Ragone et al. [25]. Such a plot for recombinant human growth hormone (hGH) is shown in Fig. 5. The three circles indicate positions of the leading candidates for isoAsp formation based solely on sequence: Asp130–Gly131, Asn149–Ser150, and Asn99–Ser100. In vitro aging of hGH at 37°C, pH 7.4 for 2 weeks generates 0.8 mol of isoAsp per mol hGH with ~60% occurring at Asn149–Ser150 and 40% occurring at Asp130–Gly131, while little or no isoAsp was detected at

Asn99–Ser100 [10]. Thus, of the three sites whose sequences constitute potential 'hot spots' for succinimide formation, only the two that fall in regions predicted to be highly flexible actually make a significant contribution to isoAsp formation. Subsequent to the prediction that these sites would fall in flexible regions, the 3-D structure of hGH was determined [26], confirming that Asp130 and Asn149 both reside in flexible loops. Studies in my own laboratory on in vitro aging of calmodulin [8] and human recombinant tissue plasminogen activator [9] have led to similar conclusions regarding the effect of sequence and flexibility on the propensity of Asn and Asp sites to generate isoAsp. A review of this topic has been published [27].

4. Significance of isoAsp formation

The degradation of Asn and Asp sites via the succinimide pathway can have profound effects on protein activity. Loss of function associated with isoAsp formation has been found with a variety of proteins including calmodulin, epidermal growth factor, and ribonuclease, while the activities of other proteins such as human growth hormone, appear to be largely unaffected [28]. For proteins such as serine hydroxymethyltransferase [29], and triosephosphate isomerase [30], deamidation may serve as a 'marking step' that triggers proteolytic degradation. Since isoAsp formation involves the addition of extra carbons to the backbone of a polypeptide, it is not surprising that its presence in a functional protein domain or enzyme active site will often lead to at least partial loss of function.

Experiments with PIMT-deficient mice have demonstrated rather dramatically that widespread accumulation of isoAsp in cellular proteins leads to early death between 28 and 60 days after birth [31,32]. Death is preceded by an epileptic seizure, perhaps reflecting a disruption of normal synaptic transmission at inhibitory synapses. Prior to death, the homozygous PIMT knockouts accumulate isoAsp in all tissues tested at levels 2.4 to six times that of the normal low level. This accumulation is predicted by, and is consistent with, the idea that PIMT functions as a repair enzyme to keep isoAsp levels low in cells. In the absence of

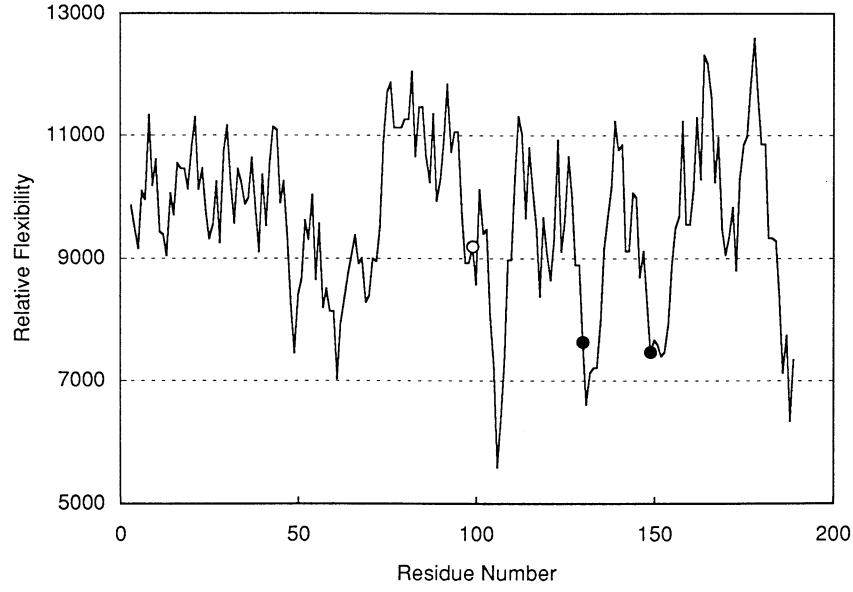

Fig. 5. Flexibility plot of recombinant human growth hormone. The location of three potential hot spots (two Asn–Ser and one Asn–Gly) for isoAsp accumulation are indicated by the superimposed circles. The circles are filled for the two sites (Asp-130 and Asn-148) that most rapidly accumulate isoAsp during in vitro aging [10].

proper repair, isoAsp bearing proteins may disrupt cell function by overloading protein degradation systems and/or by competitively displacing normal proteins in protein-protein or protein-ligand interactions, thereby disrupting a wide range of important biochemical pathways. It seems unlikely that the presence of small amounts of isoAsp in a protein pharmaceutical would be harmful; nevertheless, it is conceivable that a dysfunctional isoAsp-bearing subpopulation could compete with the normal molecules in binding to a ligand or receptor and thus block or disrupt the activity of the receptor. In such cases, the isoAsp forms of the protein would be acting as unwanted antagonists. The presence of isoAsp may affect not only the activity of a protein, but also its clearance.

Recent findings in the laboratory of Mark Mamula at Yale University suggest that isoAsp may greatly increase the immunogenicity of certain proteins [33,34]. A peptide derived from mouse cytochrome C became highly immunogenic in mice when a critical Asp residue was replaced with an isoAsp residue. In exploring the possibility that isoAsp may play a role in the autoimmune disease lupus erythematosus, Mamula made synthetic isoAsp and Asp versions of a peptide derived from the D protein of the murine small nuclear ribonuclear protein complex (snRNP). The snRNP is a major target of autoantibodies in lupus. When immunized with the isoAsp peptide, mice produced antibodies that cross-reacted with numerous nuclear proteins in a human cell line as visualized by immunofluorescent staining. In contrast, serum from mice immunized with the normal peptide produced little or no anti-nuclear Ab. By the process of epitope spreading, immunization with a single isoAsp-containing peptide elicited a wide spectrum of Abs against numerous normal proteins. The same mice that produce Ab in response to the isoAsp-antigen also produce T-cells whose proliferation could be stimulated in vitro by the isoAsp peptide. Interestingly, these T-cells were not stimulated by the normal peptide, indicating that the T-cell response (unlike the Ab response) does not generalize to other antigens. The ability of isoAsp to render a self-protein immunogenic is clearly of great concern. Pharmaceutical and cosmetic injectibles such as botulinum toxin and collagen contain numerous sites that are predicted hot spots for isoAsp formation. If autoimmune disease were to arise in patients receiving these or other biopharmaceutical agents, the role of isoAsp should be considered. At the very least, it would seem prudent to monitor isoAsp levels widely in protein pharmaceuticals until more is known about the role of isoAsp in autoimmunity.

5. Enzymatic isoAsp analysis without radioactivity

Methods for qualitative and quantitative assessment of isoAsp in peptides and proteins have recently been reviewed [35]. Once the presence of isoAsp is demonstrated in a protein, numerous physical methods can be used to monitor its levels provided that the isoAsp variant can be specifically detected as, for example, a specific chromatographic, electrophoretic, or mass variant. The initial identification and characterization of isoAsp variants can be challenging, however, especially in those cases where the isoAsp arises from isomerization of Asp. Such a subtle change, involving only a rearrangement of the peptide bond, will not necessarily be detectable or quantifiable by mass analysis, electrophoresis, or chromatography. The complete failure of Edman degradation at an isoAsp–Xaa bond is a good indicator, but suffers from being 'negative evidence', and from ambiguity in cases where there is a mixture of normal and isoAsp forms.

PIMT has proven to be useful in quantifying isoAsp levels in proteins and in identifying sites of isoAsp formation in conjunction with peptide mapping. A commercial kit, IsoQuant™, based on purified recombinant PIMT is available from Promega, Madison, WI. IsoAsp peptides are methylated with [^{3}H]AdoMet and the [^{3}H]methanol produced (Fig. 2) is recovered by diffusion into scintillation fluid for counting. Because of the concerns and expenses associated with the use and disposal of radio-isotopes, my laboratory has recently modified the PIMT-based method to eliminate the use of radiolabeled AdoMet (B. Schurter and D. Aswad, in prepara-

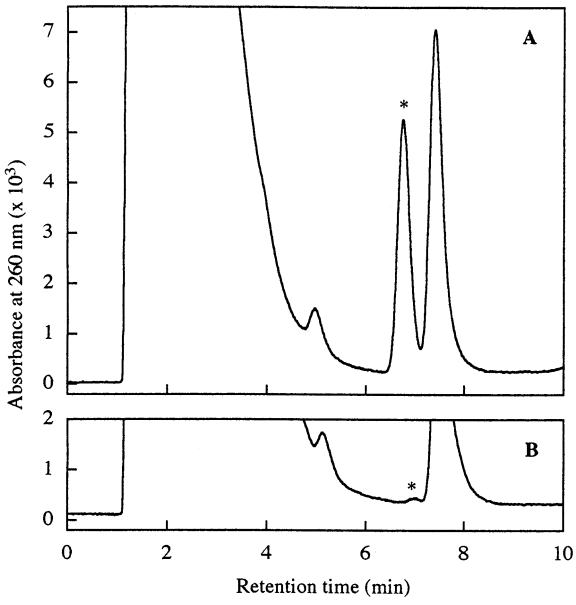

Fig. 6. Non-radioactive assay for isoAsp. (A) A total of 45 pmol of an isoAsp-containing peptide was methylated by PIMT and two-thirds of the reaction mixture was then subjected to reversed-phase HPLC. The *S*-adenosyl-L-homocysteine produced elutes as a peak at 7 min (*). (B) Chromatogram of a blank reaction (no peptide) showing only a trace peak in this area. The large peak eluting at 7.5 min in both A and B represents UV absorbing material in the PIMT preparation. The large trailing peak at 1–4 min is mostly buffer and unreacted AdoMet. The latter is present in great molar excess over isoAsp sites in order to promote their complete methylation.

tion). This new method uses HPLC in conjunction with UV detection to monitor production of AdoHcy instead of methanol. The AdoHcy method is just as sensitive as the [^{3}H]AdoMet method, takes less time, and can be done with the same set of reagents present in the IsoQuant™ kit. Eliminating the isotope significantly reduces the cost of the assay. An example of a typical isoAsp analysis by this new method is shown in Fig. 6. To carry out this analysis, 45 pmol of a commercial isoAsp decapeptide (isoAsp-DSIP; BACHEM California) was incubated with PIMT and unlabeled AdoMet for 40 min at 30°C. The reaction was stopped with dilute phosphoric acid and two-thirds was injected onto a reversed-phase column under conditions that separate AdoHcy from other components of the reaction. Using isoAsp-DSIP as a standard, we have found a highly reproducible and linear relationship between the amount of isoAsp present and the AdoHcy peak area over a range of 5–250 pmol of isoAsp. The sensitivity of the assay is limited by the amount of AdoHcy produced in a blank reaction containing everything except the analyte. The blank typically produces 0.5–2 pmol of AdoHcy that apparently is produced by automethylation of the PIMT. Mammalian PIMT contains an Asn–Gly sequence near its N-terminus, and this apparently leads to small amounts of isoAsp in the enzyme.

6. Conclusions

The formation of isoAsp is a major source of protein heterogeneity that can arise in vivo, or during purification, analysis and storage. Recent findings raising a possible connection between isoAsp and autoimmune disease emphasize the need for monitoring isoAsp levels in pharmaceutical proteins. The recent development of a sensitive assay for isoAsp that does not use radioisotopes should greatly facilitate the assessment of this widespread protein modification.

Acknowledgements

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