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AN INTRAMOLECULAR LINKAGE INVOLVING ISODITYROSINE IN EXTENSIN

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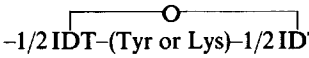
Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; cell-wall glycoprotein; extensin; isodityrosine.

Abstract—We isolated isodityrosine, a diphenyl ether linked amino acid, from cell wall hydrolysates and from two tryptic peptides of extensin. Determination of the molecular weights, net charges and composition of the peptides indicated that isodityrosine (IDT) can form a short *intra*molecular linkage in sequences consisting of:



INTRODUCTION

Primary cell walls of higher plants contain extensin, a hydroxyproline-rich glycoprotein which comprises 2–10% of the primary cell wall [1]; extensin appears important for wall strength [2] and disease resistance [3]. Early work suggested extensin might serve as a covalent crosslink between wall polysaccharides [4]. However, extensin remained insoluble even after anhydrous hydrogen fluoride dissolved all the wall polysaccharides [5]. This implied extensin was cross-linked to itself. Oxidation in acidic sodium chlorite partly solubilized extensin [6], suggesting that chlorite-labile phenolic compounds, such as the 'tyrosine derivative' present in some tryptic peptides, might form the extensin crosslinks [7]. Recently Fry identified isodityrosine (IDT), a new protein amino acid present in cell wall hydrolysates; its properties were similar to those of the 'tyrosine derivative' [8] Consequently, we postulated [9] that extensin is an IDT-crosslinked mesh ('weft') of defined porosity interpenetrated by a cellulose microfibrilar 'warp'. In order to test the 'warp-weft' hypothesis, we need to know if the 'tyrosine derivative' is indeed IDT, and the crosslinking pattern of IDT in extensin.

Here we report that IDT obtained from cell wall hydrolysates has properties identical to those of the 'tyrosine derivative' obtained from extensin peptides. In addition, the IDT of the two peptides S_2A_3 and S_2A_{11} occurs as a short intramolecular linkage separated by one intervening amino acid.

RESULTS

IDT purification and determination of the molar extinction coefficient

Fry [8] isolated microgram amounts of IDT from cell wall hydrolysates via gel filtration, paper chromatography, and paper electrophoresis and utilized ninhydrin and the Folin-Ciocalteau reagent for identification. Here, we used preparative cation exchange chromatography (Aminex A5) to isolate milligram amounts of IDT from sycamore cell wall hydrolysates (Fig. 1) and then further purified the IDT by recrystallization from hot water (Fig. 2). Our preparation matched Fry's characterization

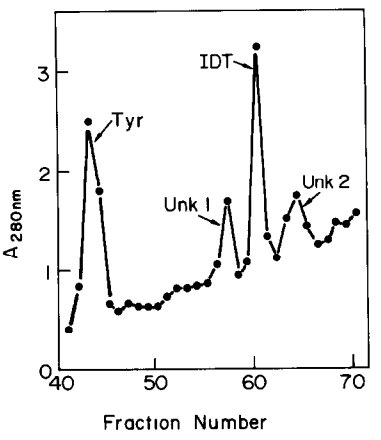


Fig. 1. The purification of IDT from cell wall hydrolysates using preparative cation exchange chromatography. Unk 1 and 2 were unidentified. An Aminex A-5 column was eluted with a pyridine acetate gradient from pH 2.7 to 5.0 using a Technican autograd, where molarities refer to pyridine, as follows: chambers 1–4, 0.07 M, pH 2.7, chambers 5–6, 0.2 M, pH 3.1; chambers 7–8, 2.0 M, pH 5.0. Each chamber contained 75 ml buffer and the size of collected fractions was 6 ml.

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Abbreviations: Tryptides, deglycosylated tryptic peptides; IDT, isodityrosine; kDa, kilodaltons; Ser, serine; Hyp, hydroxyproline; Val, valine; Lys, lysine; Tyr, tyrosine; Asp, aspartic acid; *in muro*, in the cell wall.

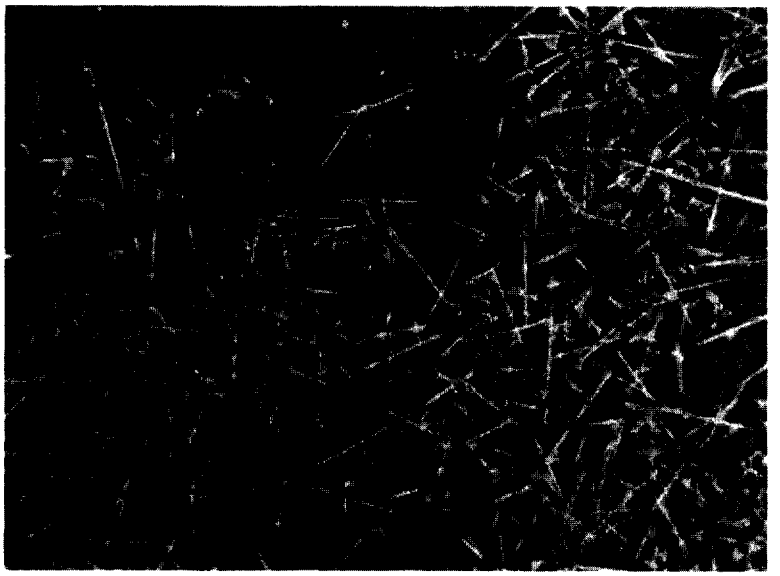

Fig. 2. IDT recrystallized from hot water (× 650)

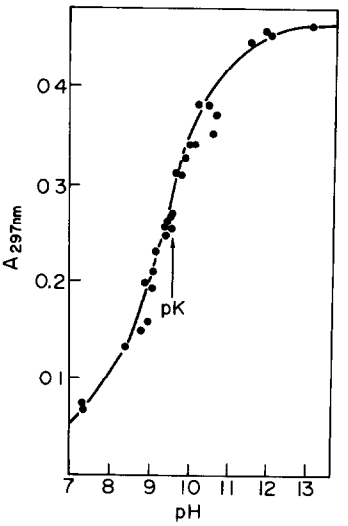

Fig. 3 The determination of the phenolic pK_a of IDT

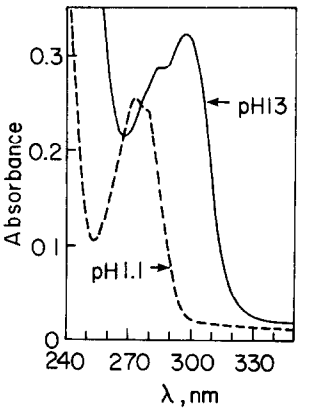

Fig 4. The UV spectra of isodityrosine in 0 1 M HCl (pH 1.1) and 0 1 M NaOH (pH 13).

of IDT's UV maxima at pH 1 and 13, phenolic pK_a = 9.5 (Fig. 3), positive Folin-Ciocalteau and ninhydrin reactions and electrophoretic mobilities at pH 1.9 and 6.5 [9]. For quantitative analysis of IDT we used either a microcolumn amino acid analyser or measured UV absorbance at 297 nm at pH 13 after HPLC purification. We prepared a standard solution from our chromatographically pure recrystallized IDT. Assuming MW = 361 [8], we determined the ninhydrin molar response factor for IDT relative to norleucine = 0.53 (at both 440 nm and 570 nm) via the amino acid analyser where IDT eluted between Lys and ammonia.

The UV molar extinction coefficients are as follows: $\lambda_{max}^{0\,1\,M\,NaOH}$ nm (log ε): 297 (3.63), 284 (3.58) and 225 (4.27); and $\lambda_{max}^{0\,1\,M\,HCl}$ nm (log ε) = 273 (3.53) and 279 (3.51). The ratio of the A at λ_{max} at pH 13 to the A at λ_{max} at pH 1.1 = 1.26. The peak to valley ratio (297 nm/268 nm) at pH 13 = 1.5 and the ratio (273 nm/254 nm) at pH 1.1 = 2.4. The dual peaks and the spectral shift in acid and base make the spectrum qualitatively and quantitatively useful (Fig. 4).

Identification of IDT in tryptides

Of five previously isolated deglycosylated tryptic peptides from tomato cell walls, two of the tryptides (S_2A_3 and S_2A_{11}) contained an unknown tyrosine derivative [10]. To determine if the derivative was IDT, we hydrolysed S_2A_3 and S_2A_{11}, purified the hydrolysate on HPLC, and then examined the UV spectrum at pH 13. The 'tyrosine derivative' from both tryptides had a spectrum characteristic of IDT (see Fig. 4) and co-chromatographed on the amino acid analyser with IDT obtained from wall hydrolysates.

Position of IDT within tryptides

From previous [10] partial sequences of the tryptic peptides and from the identification of the tyrosine derivative as IDT, we knew S_2A_3 was Ser–Hyp_4–Ser–Hyp–Ser–Hyp_4(IDT–Tyr)–Lys and S_2A_{11} was Ser–Hyp_4–Val–(IDT–Lys–Lys); the amino acid sequence within the parentheses was not known because the S_2A_{11} Edman degradation products were detected up to valine but not beyond [9]. These data do not distinguish between an intermolecularly crosslinked peptide dimer (Table 1, no. 2) or an intramolecularly crosslinked monomer (Table 1, no. 1 and 3). We differentiated between these possibilities by determining the empirical formula, the MW, and the net charges of S_2A_3 and S_2A_{11}.

Quantitative determination of IDT tryptides

Using the molar response factor of IDT relative to norleucine (determined above) we calculated the empirical formula for tryptides after varying hydrolysis times under N_2 in constant boiling HCl with 0.05% phenol. Optimum hydrolysis time for maximal IDT release was 22 hr; even after 96 hr there was only a 7% loss of IDT. After normalizing the amino acid concentrations to the moles of Hyp in a monomer, S_2A_{11} was $Ser_{0.8}$ $Hyp_{4.0}$ $Val_{0.9}$ $IDT_{1.0}$ $Lys_{2.4}$ and S_2A_3 was $Ser_{2.4}$ $Hyp_{9.0}$ $IDT_{0.7}$ $Tyr_{0.6}$ $Lys_{1.0}$. The molar ratio data show that the simplest empirical formula contains one IDT residue/peptide chain. This implies a monomeric rather than a dimeric tryptide where a similar empirical formula would have shown only 0.5 IDT/peptide chain.

MW of tryptides via gel filtration

The MWs were reported previously [see Fig. 2, ref. 9]; a carefully calibrated G-25 (fine) Sephadex column eluted with 0.1% TFA or with 0.1% TFA in 0.5 M NaCl gave similar results. Apparent MWs of S_2A_3 and S_2A_{11} were 1776 and 1232, respectively, while the monomeric tryptides have an empirically calculated MW = 2397 and 1398, which includes the O-galactosyl substituted serine residues. The gel filtration data therefore favour a monomeric interpretation.

Determination of tryptide net charge

A dimeric peptide would have twice the number of lysine residues as the monomer and at pH 6.5 dimeric S_2A_3 and S_2A_{11} would have net charges of +2 and +4, respectively, rather than +1 and +2 for monomer. [^{14}C]Acetic anhydride readily acetylates free amino and phenolic hydroxyl groups. We used partial [^{14}C]acetylation followed by paper electrophoresis at pH 6.5 and subsequent autoradiography to determine peptide net positive charge. Stepwise acetylation of amino groups produced new radioactive products of decreasing electrophoretic mobility, while acetylation of the phenolic hydroxyls did not affect tryptide charge or mobility (Table 2). The number of new products and their electrophoretic mobilities indicated a net charge of +1 and +2 for S_2A_3 and S_2A_{11} respectively, which are therefore monomeric peptides. These electrophoretic data also imply that both IDT amino groups are involved in peptide linkage and are therefore unavailable for reaction with amino group reagents such as acrylonitrile [11]. This was confirmed; reaction of intact S_2A_3 and S_2A_{11} with acrylonitrile followed by acid hydrolysis showed complete loss of lysine, while the Hyp/IDT ratio remained constant. This corroborates the involvement of both IDT amino groups in peptide linkage.

Table 1. Conceivable S_2A_{11} sequences

Possible sequence	Comment
1. Ser–[Hyp]$_4$–Val–1/2 IDT(–O–)–Lys–1/2 IDT–Lys	Best fit of peptides MW, charge and composition
2 Ser–[Hyp]$_4$–Val–1/2 IDT–Lys–Lys | O | Ser–[Hyp]$_4$–Val–1/2 IDT–Lys–Lys	Poor fit of peptides MW, charge and composition
3 Ser–[Hyp]$_4$–Val–1/2 IDT(–O–)–1/2 IDT–Lys–Lys	Unacceptable: two consecutive 1/2 IDT residues are not conformationally possible
4. Ser–[Hyp]$_4$–Val–1/2 IDT–Lys | O | 1/2 IDT–Lys	Unacceptable: both amino groups on IDT are blocked

S_2A_{11} was purified from tomato cell walls as described in the text. The *N*-terminal Ser and *C*-terminal Lys and the Ser–[Hyp]$_4$–Val– sequence were determined previously [10]. Compositional analysis indicated IDT and an additional Lys residue were also present in S_2A_{11}.

Table 2. Net charge of tryptides S_2A_3 and S_2A_{11} after partial acetylation with [^{14}C]acetic anhydride followed by pH 6.5 paper electrophoresis

Tryptide S_2A_3			Tryptide S_2A_{11}		
Peptide mobility (R_{asp})	No. of amino groups acetylated	Net charge	Peptide mobility (R_{asp})	No. of amino groups acetylated	Net charge
0.16	0	+1	0.36	0	+2
0.06	1	0	0.21	1	+1
−0.10	2	−1	0.03	2	0
			−0.17	3	−1

The partially [^{14}C]acetylated tryptides were run on paper electrophoresis at pH 6 5 concurrently with the unacetylated tryptic peptides and a mixture of neutral amino acids; the locations of the unacetylated compounds indicated the ^{14}C bands with maximum and 0 charge, respectively. The locations of the [^{14}C]acetylated tryptides were determined by autoradiography. Complete acetylation of the tryptides did not produce any additional negatively charged bands

MW of tryptides determined by electrophoretic mobility

Electrophoretic mobility is a function of peptide size and charge. If one is known, the other can generally be deduced using Offord's graphical method [12]. We measured the electrophoretic mobility of S_2A_3 and S_2A_{11} relative to aspartic acid. Knowing the net positive charge as determined above, interpolation of the R_{asp} values gave an S_2A_3 MW = 2050 and an S_2A_{11} MW = 1350. This corroborates the MW determined by gel filtration and confirms a monomeric interpretation.

CPK models of S_2A_{11}

Edman degradation [9] reconfirmed the first six S_2A_{11} residues, but did not sequence the *C*-terminal tetrapeptide containing two lysine residues and one IDT (= two Tyr residues). As S_2A_{11} is a tryptic peptide, one lysine must be *C*-terminal. In fact, Lys can be removed by partial acid hydrolysis. The *C*-terminal tetrapeptide sequence is therefore either (i)–Tyr–Tyr–Lys–Lys–COOH or (ii)–Tyr–Lys–Tyr–Lys–COOH. Our CPK spacefilling models (SchwarzMann, Spring Valley, New York, USA) indicated that contiguous peptidyl diphenyl ether linked tyrosine residues are not conformationally possible; we therefore excluded possibility (i). The remaining possibility, (ii), is feasible and forms a rigid, stable conformation which does not appreciably deviate from a polyproline II rodlike 3 residue/turn helix with a pitch of 9.4 Å (Fig. 5). This structure seems to typify extensin and other proline or hydroxyproline-rich molecules [1].

DISCUSSION

The isolation of isodityrosine (IDT) from purified tryptides unquestionably demonstrates that IDT is a component of extensin. Furthermore, the IDT crystallization allowed us to quantify this newly described amino acid via determination of the UV molar extinction coefficient and a ninhydrin response factor for amino acid analysis. We have also shown that IDT occurs as an intramolecular crosslink in two small extensin tryptides. Molecular models (Fig. 5) show that intramolecular IDT locks the peptide conformation; this would stabilize the protein without significantly changing its overall rod-like shape.

While the identification of intramolecular IDT does not support the 'warp-weft' hypothesis, neither does it disprove it. One must remember that tryptides S_2A_3 and S_2A_{11} contain only a portion of the total cell wall IDT; the linkage pattern of the rest is unknown. However, there is circumstantial evidence that the uncharacterized IDT occurs as *inter*molecular linkages. For example anhydrous hydrogen fluoride, (which does not cleave peptide bonds at 0°), solubilizes all the wall polysaccharide but leaves the hydrophilic extensin insoluble [5]. Only degradative procedures such as chlorite oxidation or proteolysis release fragments of extensin from its firmly bound state in the wall [1,2]. The release by chlorite oxidation suggests the possibility of phenolic crosslinks such as IDT. Significantly, the tyrosine-rich soluble extensin precursors P1 and P2 do not contain detectable IDT; current experiments [13] suggest IDT is formed during crosslinking, i.e. precursor attachment and the appearance of IDT are correlated. We therefore hypothesize that attachment of extensin to the primary cell wall requires *in muro* formation of intermolecular IDT. Whether or not IDT creates a network of defined porosity [9] must await identification of the intermolecular linkage.

We note that IDT may be analogous to cystine, which stabilizes peptide conformation via *intra*molecular crosslinks, as for example in the insulin A chain. Cystine also enables covalent subunit interaction via *inter*molecular linkages, for example between the insulin A and B chains. Extensin lacks cystine, but the far more stable IDT crosslink may have advantages in the harsh extracellular environment.

Finally, we suggest two general principles for the extracellular assembly of the primary cell wall: first, diphenyl phenolics crosslink wall polymer subunits, be they lignin [14], polysaccharide [15] or protein; and second, specific peroxidases catalyse the formation of these diphenyl crosslinkages *in muro*. Thus peroxidases may play a major role in the regulation of growth and development, through catalysis of supramolecular assembly.

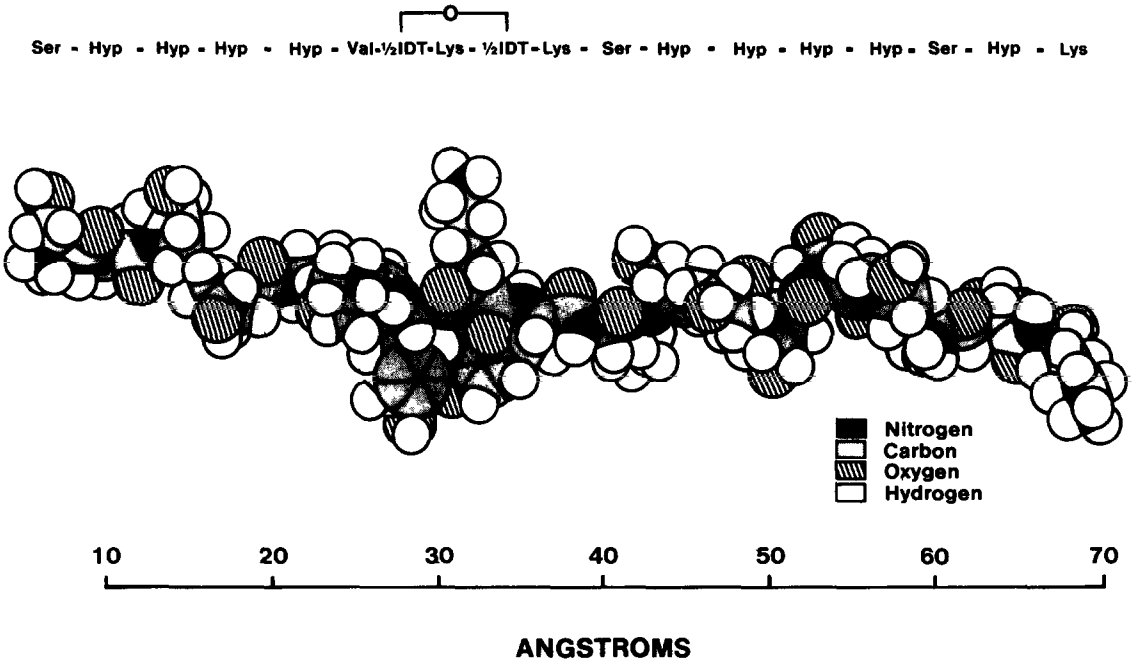

Fig. 5. A molecular model of two extensin tryptides in a polyproline II type conformation. The sequence of S_2A_{11} coupled with S_2A_7 is Ser–[Hyp]$_4$–Val–1/2IDT–Lys–1/2IDT–Lys–Ser–[Hyp]$_4$–Ser–Hyp–Lys. The short intramolecular linkage does not significantly affect the helical conformation. Model constructed by James J. Smith.

EXPERIMENTAL

Cell walls. Cell walls were prepared from suspension cultures of either tomato (*Lycopersicon esculentum* Mill.) or sycamore (*Acer pseudoplatanus* L.) as previously described [2].

Purification of IDT from wall hydrolysates. Twelve g sycamore cell walls were refluxed in 6 N HCl for 24 hr, followed by filtration, and rotary evaporation. A preliminary clean up by adsorption on Dowex 50 (H^+) followed by 2 N NH_4OH elution yielded total amino acids including IDT. We then purified the IDT on Aminex A-5 (0.5 × 9 cm) eluted by a pyridine acetate gradient from pH 2.7 to pH 5.0 (Fig. 1) and recovered 61 mg crude IDT after buffer removal by repeated evaporation under high vacuum. Further purification by recrystallization (× 4) from hot water gave white needle-like crystals. The purified IDT crystals were lyophilized, dried over P_2O_5 *in vacuo* at 40°, and then weighed on a Cahn microbalance (yield 176 μg).

UV absorbance of IDT. The UV absorbance from 210 to 400 nm of the recrystallized IDT in 0.1 M HCl (pH 1.1) and 0.1 M NaOH (pH 13) was measured on a Hitachi 110 spectrophotometer. The molar extinction coefficient $\varepsilon = A/CL$ where A is the absorbance at a given λ, C is the concentration in mmol/ml and L is a path length = 1. We determined the phenolic pK_a of IDT by measuring the A at 297 nm as a function of increasing the pH from 7.1 to 13.1 with NaOH.

Purification of tryptides S_2A_3 and S_2A_{11}. The crude dearabinosylated tryptides [10] were further purified by pH 6.5 paper electrophoresis [12]. The amino acid composition and partial sequence of S_2A_{11} were the same as reported previously [10].

Isolation of IDT from tryptides via HPLC. Tryptides S_2A_3 and S_2A_{11} were hydrolysed under N_2 in conc HCl–TFA (2 : 1) at 166° for 25 min [16]. The hydrolysates were evaporated to dryness, redissolved in 0.1% TFA and then injected onto a Dupont Zorbax column (4.6 mm × 25 cm) eluted with 0.7 ml/min of a gradient made from (I) 0.1%TFA and (II) 0.1% TFA in iso-PrOH. The gradient ranged from 100% (I) at zero time to 70% (I) and 30% (II) after 60 min. We monitored the eluate at 273 nm and confirmed IDT elution from the column by the determination of the UV spectrum in 0.1 N NaOH and by co-chromatography on the amino acid analyser with the IDT obtained from wall hydrolysates.

Amino acid analysis. Tryptides were hydrolysed under N_2 in either conc HCl–TFA (2 : 1) with 0.05% phenol at 166° for 25 min [16] or, for critical analyses, in constant boiling HCl with 0.05% phenol at 110° for 22 hr. After evaporation, we redissolved the hydrolysates in the appropriate column running buffer and injected aliquots onto a DC5A microcolumn of a Dionex amino acid analyser attached to a SP4100 computing integrator; the buffer pHs were 3.05 and 4.25 (0.2 N, Dionex) and 7.29 (1.0 N, Benson). Ninhydrin positive compounds were monitored at 440 and 570 nm. IDT was quantitatively identified using either the regular 3 buffer, 45–65° program which required 72 min or only the pH 7.29 buffer at 65° which required 30 min. We used recrystallized IDT to prepare a standard solution with norleucine as an internal standard, and calculated a response factor (RF) for IDT = 0.53, where RF = area integrated for 1 nmol IDT/area integrated for 1 nmol norleucine.

Net positive charge of tryptides at pH 6.5. We partially acetylated 5.5 nmol of either S_2A_3 or S_2A_{11} peptide in 100 μl 0.2 M pyridine at 2° for 1 hr with 0.7 μCi [^{14}C]acetic anhydride (sp. act. = 18.8 μCi/mmole), and then evaporated the mixture to dryness at 45° with N_2 (while collecting the ^{14}C-waste in 1 M NaOH and toluene traps). The ^{14}C-labelled peptides were electrophoresed on Whatman #52 paper at pH 6.5 for 4 hr at either 4 kV or 3 kV for S_2A_3 and S_2A_{11}, respectively. Adjacent lanes contained untreated peptide, Asp and a neutral amino acid standard; after electrophoresis these controls were stained with

0.3% ninhydrin in acetone containing 1% pyridine and 1% HOAc. We determined peptide mobility (R_{asp}) by the method of Offord [12]. Autoradiographs of the labelled peptides were developed after 4 days on X-ray film. Longer development did not reveal additional anodic bands. For complete acetylation, the peptide was treated as above, except after 15 min reaction with [^{14}C]acetic anhydride, 1 μl acetic anhydride in 20 μl *N*-ethylmorpholine was added, and then reacted for an additional 45 min.

Cyanoethylation. Tryptides containing *ca* 7 μg Hyp were reacted with 25 μl acrylonitrile in 100 μl 0.1 M NEt_3 at 37° overnight [11], dried under N_2 and then hydrolysed as described above. We used amino acid analyses to compare Hyp/IDT ratios before and after cyanoethylation

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