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TOMATO EXTENSIN PRECURSORS P1 AND P2 ARE HIGHLY PERIODIC STRUCTURES

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Key Word Index –*Lycopersicon esculentum*; Solanaceae; tomato; extensin precursor; HF deglycosylation; HPLC peptide mapping; Edman degradation; cell suspension cultures; cell wall protein; glycoprotein networks.

Abstract—Intact cultured tomato cells eluted with dilute salt solutions yielded two soluble precursors (P1 and P2) to the bound extensin network. HF-deglycosylation of P1 and P2 (with methanol as scavenger) followed by tryptic degradation and HPLC on Hamilton PRP-1, gave unique tryptic peptide maps. The P1 map consisted predominantly of the deca- and hexadecapeptides, H5 and H20: (P1-H5) Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys, and (H20) Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys; the P2 map consisted almost entirely of the di- and octapeptides, H3 and H4: (P2-H3) Tyr-Lys; (H4) Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys, and small amounts of a closely related decapeptide containing intramolecularly-linked isodityrosine: (H11) Ser-Hyp-Hyp-Hyp-Hyp-Val-1/2IDT-Lys-1/2IDT-Lys. Both P1 and P2 are therefore highly periodic structures: P1 consists to a considerable extent of repeated H5 and H20 peptide blocks, while P2 may consist entirely of a single repeating decapeptide Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys-Tyr-Lys with occasional isoleucine for valine substitutions, and varying only in the extent of intramolecular IDT formation, which could stiffen the molecular rod. In P1, single tyrosine residues occur within the hexapeptide Val-Lys-Pro-Tyr-His-Pro region of H20. This nonglycosylated region is sterically unhindered. Assuming a polyproline II conformation, all the basic residues of H20 lie in the same plane (available for pectic binding), while the hexapeptide tyrosine residue lies out of that plane and is therefore a prime candidate for the postulated *inter*molecular isodityrosine crosslink. Our sequences also reveal fundamental tetrapeptide and tripeptide periodicities of tomato extensin precursors: the contiguous decamers and hexadecamers consist of hydroxyproline tetrapeptides separated by a relatively few tripeptide sequences, (notably Tyr-Lys-Ser, Val-Tyr-Lys, Thr-Hyp-Val, Val-Lys-Pro, and Tyr-His-Pro). Furthermore, the presence of the pentapeptide Ser-Hyp-Hyp-Hyp-Hyp in all the repeat peptides shows that extensin, like many synthetic block copolymers, consists of relatively rigid domains (glycosylated Ser-Hyp-Hyp-Hyp-Hyp) separated by intervening (non-glycosylated) flexible spacers. Thus extensin is well-designed for its suggested role in mechanically coupling the cellulosic load-bearing polymers of the primary cell wall through formation of a network of defined porosity.

INTRODUCTION

Primary cell walls contain the firmly-bound hydroxyproline-rich glycoprotein (HRGP) extensin [1] whose apparent insolubility had limited determination of its primary structure [2–4]. Recent work changes that picture. It is now clear that although the bulk of extensin is firmly bound, and presumably covalently crosslinked [5–7], soluble extensin is present to a greater or lesser extent in the cell wall during growth [8–10]. Furthermore, soluble extensin eluted from the cell wall of intact tomato cell suspensions yielded two components (P1 and P2) displaying kinetic and chemical properties indicating the role of P1 and P2 as precursors to firmly-bound extensin [11].

Availability of the two soluble extensin precursors (P1 and P2) in milligram quantities has allowed us to continue and extend our earlier structural work. Here we report the partial characterization of P1 and P2 by tryptic degradation of the HF-deglycosylated polypeptides, dP1 and dP2, followed by HPLC peptide mapping and subsequent automated Edman degradation of the purified peptides. The tryptic peptide maps were dominated by a very few major peptides, none of them larger than a hexadecapeptide, indicating a repetitive and therefore highly periodic extensin polypeptide backbone. These peptides are the first sequenced for an HF-deglycosylated protein and also provide the first evidence for the location of prolyl residues in extensin. Interestingly, proline occurs in a domain containing a tyrosyl residue tentatively identified as a prime candidate for intermolecular crosslinkage.

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Abbreviations: HA, hydroxyproline arabinoside; HFBA, heptafluorobutyric acid; HRGP, hydroxyproline-rich glycoprotein; P1 and P2, glycosylated extensin precursors; dP1 and dP2, HF-deglycosylated extensin precursors; IDT, isodityrosine; PRP-1, polystyrene reversed-phase; PTH, phenylthiohydantoin; ODS, octadecylsilane; CAPS, chromatography applications package software; tryptide, tryptic peptide.

RESULTS

Precursor isolation, fractionation and composition

We isolated precursors as previously described [11] but with the following modifications: (i) Elution of intact cells with 25 mM aluminium chloride gave higher crude precursor yields than 50 mM calcium chloride although final yields of purified precursors were not significantly different. For example, 15 l. batches of tomato cell suspension gave *ca* 0.6 kg wet weight or 30 g dry weight cells after 7 days growth on M6E medium. Aluminium chloride elution consistently yielded *ca* 200 mg TCA soluble crude precursor (30 mg each pure P1 and P2) while $CaCl_2$ elution yielded *ca* 120 mg crude which gave similar yields of pure P1 and P2.

(ii) We surveyed several weak cation exchangers (notably carboxymethylcellulose (DE-52), BioRex 70, CM-Sepharose and CM-Trisacryl) and optimized precursor separation conditions by changing from CMC to BioRex 70 (100–200 mesh), and using a salt gradient superimposed on a decreasing pH gradient as described in the Experimental. This resolved P1 into two components (P1a and P1b; Fig. 1) of almost identical amino acid composition (Table 1) but distinguished by the slightly higher histidine and lysine content of P1b (consistent with its later elution from the cation exchanger). P2 remained a single peak of composition similar to that previously published [11]. Subsequently we use P1 as an inclusive term when describing features common to both P1a and P1b.

(iii) Gel filtration of each precursor (P1a, P1b and P2) on Sepharose CL-6B gave single retarded symmetrical peaks (at $\sim 1.5\ V_0$) of characteristic composition (Table 1). The presence of threonine, histidine and proline in P1 readily distinguish P1 from P2. P1 contained no isodityrosine but, contrary to our earlier report [11], P2 consistently gave a Tyr:IDT molar ratio of 8:1 (or Hyp:IDT of 20:1). After gel filtration via HPLC on Dupont GF-250 the intact precursors eluted essentially at V_0. Remarkably, even after HF-deglycosylation P1a and P1b eluted at V_0 while P2 remained adsorbed to the column. Succinylation of dP2 overcame this problem; succinylated dP2 eluted at V_0.

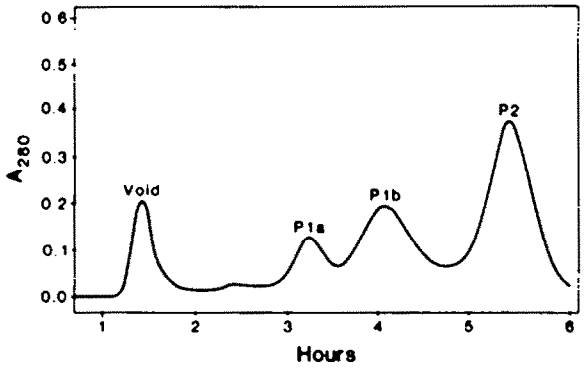

Fig. 1. BioRex 70 cation exchange chromatographic separation of P1a, P1b and P2. Injected 120 mg crude precursor (28 g cells DW, 5 day culture, *ca* 15% PCV, eluted with 4 l. 25 mM $AlCl_3$; see Experimental) in 12 ml 30 mM pH 7.6 NaPi buffer. Sample pH = 7.8, sample conductivity = 4.9 mmhos.

Table 1. Amino acid compositions* of P1a and P1b

Amino acid	P1a	P1b
Hyp	32.7 ± 3.4	30.9 ± 4.9
Asp	1.4 ± 0.5	1.9 ± 0.6
Thr	6.2 ± 0.5	6.5 ± 0.5
Ser	9.8 ± 1.0	9.5 ± 0.9
Glu	1.5 ± 0.6	1.6 ± 0.4
Pro	9.6 ± 2.3	10.2 ± 1.2
Gly	1.7 ± 1.1	2.0 ± 0.6
Ala	2.9 ± 0.5	1.9 ± 0.7
Val	8.3 ± 0.8	7.1 ± 0.8
Ile	1.0 ± 0.2	1.1 ± 0.2
Leu	1.0 ± 0.3	0.8 ± 0.5
Tyr	7.7 ± 1.0	8.0 ± 1.1
Phe	0.0 ± 0.0	0.0 ± 0.0
His	6.1 ± 0.7	7.1 ± 1.1
Lys	9.5 ± 1.3	10.3 ± 0.9
Arg	0.7 ± 0.3	1.2 ± 0.5

*Expressed as mol% ± s.d. (analyses of 10 different preparations).

Sugar analysis. We previously reported hydroxyproline arabinoside profiles of P1 and P2 [11]. A complete quantitative sugar analysis of P1 and P2 showed 90 mole% arabinose, 6–7 mole% galactose and 2 mole% glucose (< 1% mannose, xylose and rhamnose). The Ara:Hyp molar ratio was 2.58:1 for P1 and 2.96:1 for P2. HF-deglycosylation of P1 and P2 followed by dialysis removed > 98% of the sugar and gave a weight loss of 60%.

Precursor tryptic digestion, HPLC peptide mapping and Edman degradation

(i) *Trypsin digestion of glycosylated P1a, P1b and P2.* Incubation with trypsin (48 hr) did not cleave glycosylated P1a as judged by GF-250 HPLC gel filtration. P1b was slightly less resistant to trypsin, degrading within 24 hr to give a minor peak at 1.1 V_0 in addition to the main GF-250 peak at V_0. However, glycosylated P2 gave a single retarded peak at V_e (salt) on GF-250 after 24 hr incubation with trypsin, indicating extensive degradation.

(ii) *Tryptic peptide maps of dP1a and dP1b, and primary structure of the major tryptides.* Trypsin rapidly cleaved HF-deglycosylated P1 (dP1). In a pH-stat the reaction was 50% complete within 20 min and virtually complete by 5 hr (96% theoretical cleavage, excluding Lys-Pro, Table 2). Sephadex G-25 gel filtration resolved the complete tryptic digest into two retarded peaks (S1 and S2; Fig. 2), with two minor shoulders on the high molecular weight side of S1.

Fractionation of the complete tryptic digest via gradient-elution reverse-phase HPLC gave peptide maps of P1a and P1b which were remarkably similar (Fig. 3a and b). DuPont Zorbax ODS (used initially) resolved 20 tryptides (data not shown) while Hamilton PRP-1 improved the resolution of the more hydrophobic peptides, resolving 28 tryptides. However both columns showed two major peptides, H5 and H20 (Fig. 3), which together accounted for 44% of the total absorbance at 273 mm and 33% of the total recoverable peptide weight (70% peptide

Table 2. P1 and P2 tryptic cleavage (after HF-deglycosylation) via the pH stat

Amt. digested* (mg)		Total peptide bonds† (μmole)	% of total cleaved‡			No. cleaved per molecule§			No. cleavable per molecule‖	% of theoretical % cleavage¶		
			20 min	2 hr	5 hr	20 min	2 hr	5 hr		20 min	2 hr	5 hr
dP1	7.3	55	4.5	6.8	7.7	14	20	23	24	56	85	96
dP2	6.5	49	9.2	11.7	14.3	28	35	43	54	51	65	79

*HF-deglycosylated precursors dP1 and dP2 prepared as described in Experimental.

†Calculated from $\frac{\text{total peptide } \mu g}{\text{average residue weight}}$ using 133 as the average residue weight.

‡Calculated from pH-stat delivery of NaOH μmol equivalent to μmol peptide bond cleaved, and expressed as a percentage of the total peptide μmol in second column.

§Best estimate of M_r is *ca* 40 k, an interpolation based on: our SDS-PAGE data [11] of apparent 55 k and 53.5 k for dP1 and dP2 (overestimates), CsCl gradient centrifugation of a similar carrot HRGP [10] giving 35 k, and the cDNA sequence [21] of a carrot HRGP giving 35 k. Taking the average residue weight (derived from the composition) of 133 gives 300 residues/molecule for both P1 and P2, allowing calculation of the number of bonds cleaved/molecule from percent cleaved (third column).

‖P1 is 10 mol% lysine and thus would have 30 bonds available for tryptic cleavage, except for the four uncleavable Lys-Pro bonds. Peptide compositions suggest more as peptides H21-H28 contain more than one lysine (Table 3). Therefore we corrected for six trypsin-resistant lysyl bonds. Edman degradation showed that P2 also contained trypsin-resistant lysyl bonds (lysine surrounded by IDT in H11 and H12). If Tyr:IDT = 8:1, there are six such bonds in P2.

¶Expresses no. cleaved/molecule as a percentage of no. cleavable/molecule.

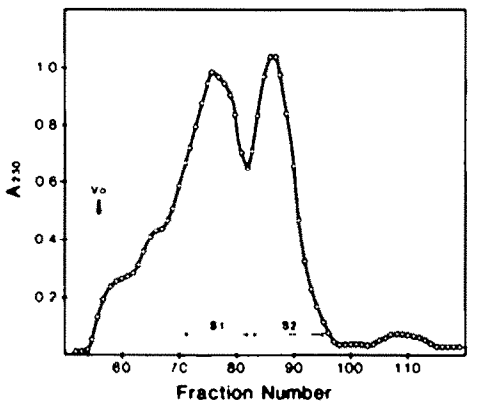

Fig. 2. Sephadex G-25 gel filtration of dP1 tryptic digest. Injected 9 mg freeze dried dP1 tryptides (dissolved in 0.5 ml 0.1 M HOAc) and collected 120 2-ml fractions.

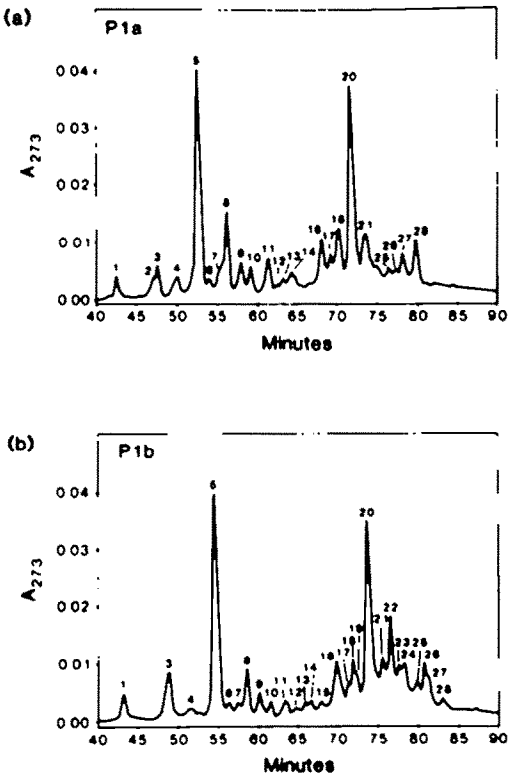

Fig. 3. HPLC peptide maps of P1a and P1b. (a) 10 μl of a trypsin digest containing 100 μg dP1a tryptides. (b) As (a) except 10 μl reaction mix contained 100 μg dP1b tryptides. No peptides eluted before 40 min although the void contained minor amounts of serine and glycine.

recovery from PRP-1; Table 3). Amino acid analysis (Table 3) and sequencing via automated Edman degradation (Table 4) showed that H5 and H20 were decapeptide and hexadecapeptide respectively (in accord with their G-25 elution behaviour: H5 in peak S2 and H20 in S1) with sequences as follows: H5—H_2N-Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys-COOH, and H20—H_2N-Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys-COOH (note trypsin did not cleave Lys-Pro [12]). Verification of H20 via chymotryptic cleavage yielded the two predicted peptides C1 and C2 (Tables 3 and 4) with sequences as follows: C1—H_2N-Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys-Pro-Tyr-COOH and C2—H_2N-His-Pro-Thr-Hyp-Val-Tyr-COOH.

Table 3. Amino acid compositions* of major P1 tryptic peptides

Amino acid	H3	H5	H8	H9	H10	H11	H16	H20	H20/C1	H20/C2 before cyanoethylation	H20/C2 after cyanoethylation	H28
Hyp	10.0 (10)	5.0 (5)	5.0 (5)	5.0 (5)	5.0 (5)	4.8 (5)	5.0 (5)	4.3 (5)	4.1 (4)	1.0 (1)	1.1 (1)	4.2 (4)
Asp												
Thr	1.2 (1)	1.1 (1)	1.1 (1)	1.1 (1)	0.5	0.8 (1)	1.0 (1)	1.0 (1)		0.5 (1)	0.6 (1)	0.8 (1)
Ser	3.2 (3)	1.3 (1)	1.1 (1)	1.3 (1)	1.7 (2)	1.5 (2)	1.4 (1)	1.3 (1)	1.0 (1)			1.1 (1)
Glu												
Pro				1.5 (2)	2.2 (2)	0.8 (1)	2.6 (3)	2.0 (2)	1.1 (1)	1.1 (1)	0.9 (1)	2.1 (2)
Gly												
Ala												
Val	1.2 (1)	1.0 (1)		1.0 (1)	1.3 (1)	0.8 (1)	1.8 (2)	1.6 (2)	0.9 (1)	0.6 (1)	1.0 (1)	1.2 (1)
Ile			0.8 (1)									
Leu				0.8 (1)		0.6 (1)						
Tyr	1.4 (2)	0.5 (1)	1.2 (1)	1.3 (1)	1.4 (1)	1.0 (1)	1.9 (2)	1.4 (2)	0.8 (1)	0.6 (1)	0.5 (1)	1.9 (2)
Phe												
His	1.0 (1)					0.6 (1)	2.3 (2)	1.4 (1)	0.4	1.0 (1)	0.1	2.7 (3)
Lys	2.0 (2)	1.1 (1)	1.0 (1)	2.0 (2)	1.6 (2)	1.0 (1)	3.1 (3)	1.7 (2)	1.0 (1)	0.3		1.0 (1)
Arg												
% (w/w)†		16.6						15.7				
%A273		21.2						21.8				

*Expressed as molar ratios.

† % of total weight recovered from PRP-1 HPLC column.

Note: Although we were unable to completely separate peptides H16 H19 and H21 H27 in high enough yield for compositions of pure peptides, we did determine compositions across these areas of the HPLC maps. Most of the histidine and proline of P1 is in these areas. H1–H8, which size in the decamer range on G-25 (S2; except H3) contain no proline or histidine (except H3). H16–H28 are all in the G-25 S1 fraction.

Table 4. Amino acid sequence of major P1 tryptic peptides

Cycle	Residue	% Yield of PTH-amino acid
P1/H5 (50 nmol in cup)		
1	Ser	47.6
2	Hyp	50.4
3	Hyp	73.3
4	Hyp	63.7
5	Hyp	68.4
6	Thr	46.3 (O 29.3)
7	Hyp	39.0
8	Val	54.2 (O 13.5)
9	Tyr	38.1 (V 36.9)
10	Lys	40.6 (Y 18.3)
P1/H8 (50 nmol in cup)		
1	Ser	4.0
2	Hyp	8.0
3	Hyp	22.4
4	Hyp	20.4
5	Hyp	22.4
6	Thr	2.6 (O 6.0)
7	Hyp	13.4
8	Ile	17.0 (O 6.6)
9	Tyr	13.6 (I 4.4)
10	Lys	7.4
11	—	
P1/H20 (20 nmol in cup)		
1	Ser*	
2	Hyp	25.0
3	Hyp	25.5
4	Hyp	27.0
5	Hyp	45.5
6	Val	74.5 (O 31.5)
7	Lys	33.0 (V 25.5)
8	Pro	57.5 (K 14.0)
9	Tyr	15.0 (P 13.0)
10	—	(Y 17.0)
11	Pro	35.5 (Y 9.5)
12	Thr*	(P 24.0)
13	Hyp	16.5
14	Val	27.5 (O 13.5)
15	Tyr	26.5 (V 29.5)
16	Lys	6.0 (Y 14.5)
P1a/H20 (10 nmol in cup)		
1	Ser*	—
2	Hyp	35.0
3	Hyp	59.0
4	Hyp	45.0
5	Hyp	42.0
6	Val	49.0 (O (16.0)
7	Lys	29.0
8	Pro	54.0
9	Tyr	51.0
10		—
11	Pro	39.0
12	Thr*	—
13	Hyp	10.0
14	Val	33.0
15	Tyr	30.0
16	Lys	7.0
17	—	
P1b/H20 (10 nmol in cup)		
1	Ser*	
2	Hyp	19.0
3	Hyp	100.0
4	Hyp	62.0
5	Hyp	41.0
6	Val	74.0 (O 25.0)
7	Lys	5.0
8	Pro	30.0
9	Tyr	52.0 (P 32.0)
10	His†	20.0
11	Pro	65.0
12	Thr*	
13	Hyp	27.0
14	Val	28.0
15	Tyr	26.0
16	—	
P1/H20/C1 (50 nmol in cup)		
1	Ser	4.0
2	Hyp	12.0
3	Hyp	3.4
4	Hyp	11.6
5	Hyp	9.0
6	Val	10.8 (O 2.6)
7	Lys	7.8 (V 2.4)
8	Pro	8.0 (K 0.6)
9	Tyr	2.8 (P 1.0)
10	—	(Y 1.8)
P1/H20/C2 (12 nmol in cup)		
1	—	—
2	His†	81.0
3	Pro	50.0
4	Thr	21.0 (P 10.0)
5	Hyp	28.0
6	Val	75.0 (O 38.0)
7	Tyr	18.0 (V 5.0)
8	—	(Y 8.0)

*Qualitatively identified by the presence of unique degradation peaks from serine and threonine.

†Quantitated using pH 5.5 buffer.

‡Other amino acids indicated by the standard single letter abbreviations (O = Hyp).

(iii) *Tryptic peptide map of P2, and primary structure of the major tryptides.* Trypsin also rapidly cleaved HF-deglycosylated P2 (dP2) and cleaved 79% of the theoretically cleavable bonds (1/2IDT-Lys-1/2IDT-Lys not cleaved; Table 6) in the pH-stat within 5 hr (Table 2). Gel filtration of the dP2 tryptic digest on Sephadex G-25 gave two major retarded fractions (S1 and S2; Fig. 4) but no large fragments and little or no trypsin resistant core. The first peak (S1) had shoulders on both the higher molecular weight side (S1a) and the lower molecular weight side (S1c). The highly retarded second peak (S2) was chromatographically homogeneous on Zorbax ODS, contained equimolar tyrosine and lysine, and was identified as Tyr-Lys by cochromatography with authentic tyrosyllysine and by Edman degradation (Tables 5 and 6). Estimation of Tyr-Lys in tryptic digests after HPLC (using authentic Tyr-Lys as a standard) showed that [Lys]-Tyr-Lys occurred 15 times in P2 (assuming a polypeptide of M_r 40 k, Table 2).

Fractionation of the complete dP2 tryptic digest on Zorbax ODS or Hamilton PRP-1 resolved 12 tryptides

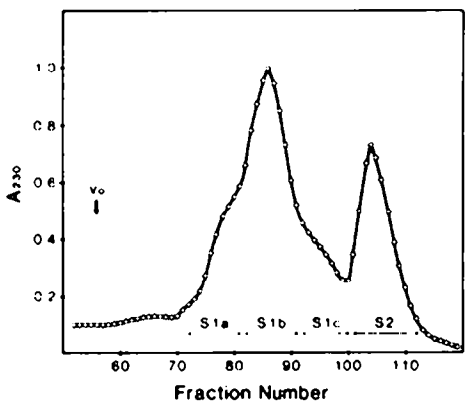

Fig. 4. Sephadex G-25 gel filtration of dP2 tryptic digest injected 5 mg freeze dried dP2 tryptides (dissolved in 0.5 ml 0.1 M HOAc) and collected 120 2-ml fractions. S1a contained peptide H2, S1 contained H4, H5(6) and H9, S1c contained H1, and S2 contained H3 (see Fig. 5).

(Fig. 5). The two major peptides (H3 and H4) accounted for 60% of the total absorbance at 280 nm and 55% of the total recoverable peptides by weight (Table 5) while a third (H11) accounted for 15% of the total absorbance and 10% of the total weight. The remaining minor peptides accounted for 25% of the absorbance and 35% of the weight (probably a high estimate due to inclusion of major peptides from overlap of pooled fractions).

Amino acid analysis (Table 5) and Edman degradation (Table 6) showed that H3, H4 and H11 were dipeptide, octapeptide and decapeptide respectively (corroborated by G-25 elution data) with sequences as follows: H3—H_2N-Tyr-Lys-COOH; H4—H_2N-Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys-COOH; H11—H_2N-Ser-Hyp-Hyp-Hyp-Hyp-Val-1/2IDT-Lys-1/2IDT-Lys-COOH.

The minor tryptides H5 (or H6) and H12 are isoleucine for valine substitution analogs of H4 and H11 respectively. The proportion of tryptide H9 decreased drastically during digestion (Fig. 5) with a concomitant increase in H3 and H4. H9, isolated from a 90 min digest (Fig. 5), has the provisional sequence: H9– H_2N-Tyr-Lys-Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys-COOH (Edman degradation showed Lys and Hyp in the correct positions, but Val and Tyr were identified equivocally).

H1 and H2 (Table 5) had compositions similar to H3 and H4 respectively, except H1 and H2 contained no tyrosine, dityrosine or isodityrosine despite the absorptivity at 280 nm. Sephadex G-25 gel filtration showed H1 in S1c and H2 in S1a while H3 and H4 appeared in S2 and S1b respectively. Eliminating light and maintaining a low temperature during the precursor preparation virtually eliminated H1 and H2 from the peptide map.

Partial tryptic digests of dP2 (4 min) followed by gel filtration gave a void peak and a retarded fraction (SO) between the void peak and S1a. Complete digestion of SO gave typical P2 peptide maps except for an enrichment in H1 and H2. Edman degradation of H1 and H2 yielded an unknown PTH-amino acid at residue 1 in H1 and residue 7 in H2 (Table 6).

DISCUSSION

Although the primary structure of extensin is incomplete, the data here, representing the first peptides sequenced from HF-deglycosylated material, permit the following conclusions:

Tryptic peptide maps of precursors P1a, P1b and P2 showed remarkably few major tryptides; these occurred in molar ratios very much greater than one compared to the minor peptides. As tryptic digestion was essentially com-

Table 5. Amino acid compositions of P2 tryptic peptides-molar ratios

Amino acid	H1	H2	H3	H4	H5(6)	H9	H11*	H12*
Hyp		4.2 (4)		3.9 (4)	3.6 (4)	4.1 (4)	3.6 (4)	3.0 (4)
Asp								
Thr								
Ser		1.0 (1)		1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Glu								
Pro								
Gly								
Ala								
Val		0.9 (1)		0.9 (1)	0.2	0.7 (1)	0.9 (1)	0.6
Ile					0.7 (1)			0.4 (1)
Leu								
Tyr			0.7 (1)	1.0 (1)	0.5 (1)	1.2 (2)		
Phe								
His								
Lys	1.0 (1)	1.3 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.6 (2)	1.7 (2)	2.2 (2)
Arg								
% (w/w)†			15.0	39.4			(9.6)	
$\%A_{273}$	5.4	6.5	29.9	29.8	7.1	1.0	14.3	1.8

*Also contain IDT as determined by (1) cochromatography with S_2A_{11} [7], (2) Edman degradation of H11 and H12 (Table 6), and (3) UV spectra characteristic of IDT [7].

† % of total weight recovered from PRP-1 HPLC column.

plete, these major peptides represent repeating units along the extensin polypeptide backbone.

The tryptide maps allow us to determine the relatedness of P1a and P1b, and the periodicities of P1 and P2. P1a contains H2 but lacks the minor peptides H15, 19, 22, 23 and 24 of P1b. P1a and P1b also gave small differences in relative peptide yields (Fig. 3). Despite these differences, the overall peptide maps of P1a and P1b are remarkably similar, and indicate a periodic structure for both P1a and P1b based on repeats of H5 and H20 which occur in a 2:1 molar ratio.

Table 6. Amino acid sequences of P2 tryptic peptides

Cycle	Residue	% Yield of PTH-amino acid
P2/H1 (50 nmol in cup)		
1	Unk A	101.4*
2	Lys	52.4 (U 21.2)†
3		(K 1.44, U 17.8)
P2/H2 (200 nmol in cup)		
1	Ser	21.9
2	Hyp	22.8
3	Hyp	40.6
4	Hyp	20.0
5	Hyp	20.4
6	Val	17.4 (O 6.9)
7	Unk A	8.9* (V 6.1, O 3.3)
8	Lys	1.1 (U 1.5, V 0.6)
9	Unk A	4.4* (K 2.7, V 1.2)
P2/H5(6) (120 nmol in cup)		
1	Ser	19.5
2	Hyp	37.9
3	Hyp	53.9
4	Hyp	45.9
5	Hyp	39.8
6	Ile	22.4 (O15.7)
7	Tyr	26.9 (I 7.6, O11.5)
8	Lys	10.6 (Y 5.9, I 5.0)
9	—	(K 4.1, Y 7.2)
P2/H12 (10 nmol in cup)		
1	Ser	< 10.0
2	Hyp	21.0
3	Hyp	38.0
4	Hyp	39.0
5	Hyp	46.0
6	Ile	32.0 (O15.0)
7	-	(I 6.0)
8	Lys	17.0
9	Unk B	6.0*
10	Lys	14.0
P2/H3 (64.5 nmol in cup)		
1	Tyr	75.1
2	Lys	31.3 (Y < 1%)
3	—	(K 11.9)
P2/H4 (240 nmol in cup)		
1	Ser	10.5
2	Hyp	75.4
3	Hyp	49.5
4	Hyp	27.8
5	Hyp	57.8
6	Val	44.8 (O 8.8)
7	Tyr	45.0 (V 14.0)
8	Lys	8.4 (Y 3.8)
9	—	
P2/H11 (60 nmol in cup)		
1	Ser	39.8
2	Hyp	98.2
3	Hyp	118.0
4	Hyp	68.0
5	Hyp	81.3
6	Val	64.0 (O21.7)
7	-	(V 17.7, O 8.7)
8	Lys	17.7 (U 2.2, V 4.7)
9	Unk B	18.2* (K 18.3)

*Used PTH RF = 1 for unknowns; unknown A had a PTH retention time of *ca* 0.1 relative to Norleu. Unknown B had a PTH retention time of *ca* 1.1 relative to Norleu and is probably PTH-IDT.

†Other amino acids indicated by the standard single letter abbreviations (O = Hyp, U = unknown).

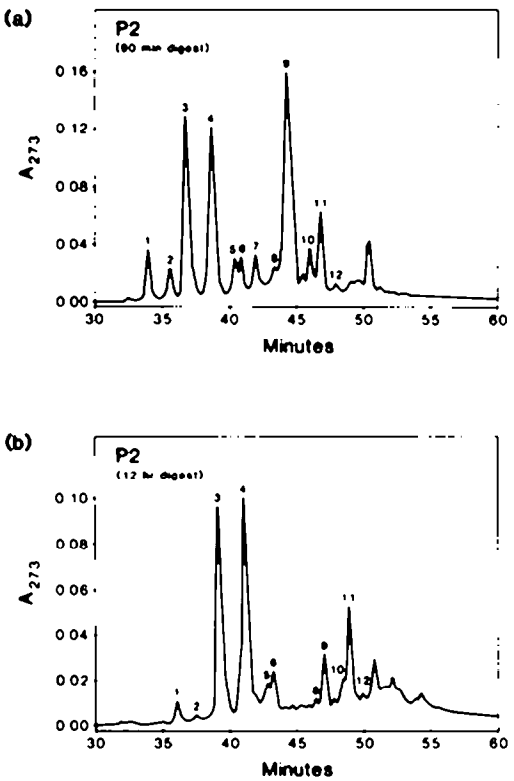

Fig. 5. HPLC peptide maps of dP2. (a) 10 μl of a trypsin digest containing 100 μg dP2 tryptides after 90 min reaction time. Note dominance of H9 peak. (b) 10 μl of a dP2 tryptic digest (50 μg tryptides) after 12 hr reaction. All peaks beyond H12 disappear after 24 hr reaction (not shown). Note reduction of H9 peak. Maps in (a) and (b) are from different batches. No peptides eluted before 30 min although the void contained minor amounts of serine, glycine and alanine.

Tryptide H20 is particularly significant; its sequence is quite unlike any others obtained earlier from tomato [13] and it contains the interesting hexapeptide 'insert' Val-Lys-Pro-Tyr-His-Pro as an obvious putative site for intermolecular (IDT) crosslinkage (accounting for its absence from earlier tryptic digests of wall-bound extensin?). We estimate (Table 2) that H20 occurs about six times in P1 which, assuming the 300 residue polypeptide exists in a polyproline II conformation as an extended rod of 80–100 nm [14], yields an average crosslink separation of 13–17 nm.

The P2 tryptide map was much simpler than those of P1a and P1b. It consisted essentially of two major tryptides (H3 and H4) with a third minor but quite noticeable tryptide (H11) containing IDT. P2/H11 is identical to the decapeptide S2A11 isolated earlier [5, 13] from wall-bound extensin. Because the octapeptide H4 and the dipeptide H3 are equimolar, and because we were able to isolate the IDT-linked decapeptide H11, Ser-Hyp-Hyp-Hyp-Hyp-Val-1/2IDT-Lys-1/2IDT-Lys, we suspect that the entire structure of P2 consists of the repeated decapeptide H11, with occasional isoleucine for valine substitutions and varying only in the extent of intramolecular IDT formation. An alternative model consisting of contiguous octapeptides (H4) separated by runs of contiguous dipeptides (H3) is feasible, but inconsistent with short-term tryptic digests of dP2 which gave the IDT-free tryptide H9 (Tyr-Lys-Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys, Fig. 5) but no Tyr-Lys multimers. H9 generation reflects the differing stabilities of peptide bonds to tryptic cleavage: Lys-Ser is more polar and hence more stable than Lys-Tyr [12].

It is now possible to discuss the role of IDT and putative crosslink domains in more detail: IDT, originally observed in cell wall glycopeptides [13], and then identified in cell wall hydrolysates [5, 6] and tryptic peptides of bound extensin [7], appears to be correlated with progressive insolubilization of extensin monomers during the incubation of isolated cell walls [15]. These results, together with the identification here of the putative crosslink sites Val-Lys-Pro-Tyr-His-Pro (hexapeptide domain in tryptide P1/H20), Thr-Hyp-Val-Tyr-Lys (from P1/H5 and H20), and Val-Tyr-Lys-Tyr-Lys (from P2), make IDT the prime *inter*molecular crosslink candidate for extensin.

If extensin precursors have polyproline II helical conformations as reported for glycopeptides isolated from the cell wall [13] and soluble extensin from carrot [14], then the lysine and histidine side groups of tryptide P1/H20 lie in one plane while two potential tyrosine crosslinks of P1/H20 are aligned 120 degrees out of that plane. We predict that these coplanar positively charged clusters of H20 interact strongly with pectic carboxylate ions (possibly assisting mutual molecular orientation) thereby facilitating (peroxidatic? [16, 17]) crosslinkage reactions of the coplanar tyrosine residues oriented 120 degrees out of that plane.

The tryptide sequences also reveal fundamental structural similarities and differences between the P1 and P2 polypeptides. What are the underlying periodicities of the extensin precursors P1 and P2? All major tryptides sequenced containing hydroxyproline-tetrapeptides have N-terminal serine, and C-terminal lysine. (Compositional data for other peptides imply similar N- and C-termini.) Thus Lys-Ser occurs frequently because most sequences prior to tryptic cleavage are Lys-tryptide-Ser, i.e. Lys-[Ser Lys]-Ser. Therefore the contiguous decamers and hexadecamers of P1 and P2 consists of hydroxyproline tetrapeptides generally separated by relatively few different tripeptide sequences (Fig. 6). Tyr-Lys-Ser is common to both P1 and P2. Val-Lys-Pro, Tyr-His-Pro and Thr-Hyp-Val are characteristic of P1, while Val-Tyr-Lys is characteristic of P2 (note: replacing P1/H5s Thr-Hyp-Val with Val-Tyr-Lys gives the P2 'core' decapeptide).

The tetrapeptide and tripeptide periodicities of P1 and P2 combined with three-fold helical symmetry emphasize macromolecular orderliness and suggest fine control of crosslink frequency, network topology and ultimately wall rheology. Regularly repeated crosslinks would create an extensin network of defined porosity quite possibly penetrated by cellulose microfibrils [18, 19]. Such mechanical coupling of the load-bearing polymers in the growing fabric of the wall could be as important a determinant of cell morphology as the initial orientation of cellulose microfibrils [20]. Our peptide sequencing strategy is nicely complemented by genomic cloning and sequencing data from the carrot root phloem disc system [21, 22]. Carrot clone pDC5A1 contains the sequence Ser-Pro-Pro-Pro-Pro-Thr-Pro-Val-Tyr-Lys eight times (the unhydroxylated analog of P1/H5), and the sequence Tyr-Lys-Tyr-Lys eleven times [21] (note relation to P2). This similarity of extensins from two distant plant families (Umbelliferae and Solanaceae), although in related orders (Umbellales and Solanales), is quite remarkable.

Developmental regulation of extensin networks is

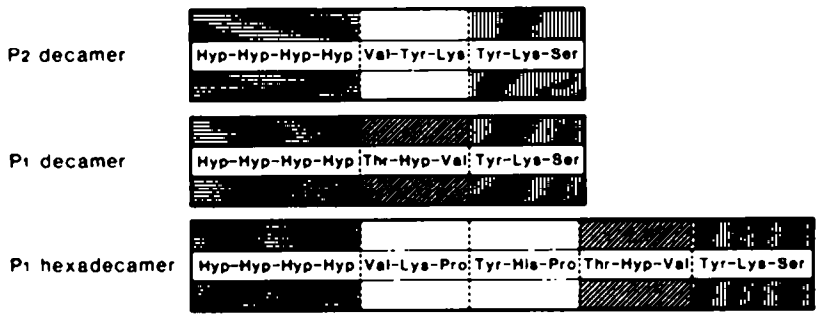

Fig. 6. Tri- and tetrapeptide periodicities of P1 and P2. P1 and P2 major peptides consist of hydroxyproline tetrapeptides separated by tripeptides. The different tripeptides of P1 and P2 are indicated by their shading.

suggested by the existence of at least two extensin precursors (P1 and P2) whose levels depend on growth phase [11] and culture medium. The resolution of P1 into P1a and P1b (Fig. 1) suggests variants of P1 due to peptide microheterogeneity (Fig. 3) rather than the major differences in peptide sequence seen in P2. Indirect evidence for a third precursor ('P3') exists in the form of three major extensin tryptides, Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Lys, Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-1/2IDT-Tyr-1/2IDT-Lys, and Ser-Hyp-Hyp-Hyp-Hyp-Lys previously isolated from bound extensin [7, 13] but not present in tryptic peptide maps of either P1 or P2. The absence of 'P3' from salt eluates, while puzzling, is paralleled by the absence of P2 from cells grown on the MET medium.

In bean cultures the hydroxyproline arabinoside profile varies significantly during the growth cycle [23]. As each precursor has a characteristic HA profile [11] the levels of different extensin precursors in bean probably fluctuate during growth as they do in tomato [11]. Assuming the feasibility of different types of extensin network, cells may build different primary cell wall networks as a function of position, environment and age. Presumably such developmental regulation would involve differential expression of an extensin multigene family and the suppression of P2 on MET-grown cells may be one such example.

We assume tentatively that in tomato cell cultures, extensin precursors P1 and P2 create a heteromultimeric network, perhaps with lateral braces of 'P3' whose 'non-elution' might be attributed to a periclinal (tangential) rather than anticlinal (radial) orientation. On the other hand secretion of a single major extensin precursor by wounded carrot slices [10] suggests the possibility of very tightly crosslinked homopolymeric extensin networks induced as a specific response to stress such as mechanical wounding or infection [24, 25]. The entire situation is reminiscent of the analogous metazoan matrix, where the hydroxyproline-rich glycoprotein collagen is quite definitely tailored to the tissue [26, 27]!

EXPERIMENTAL

Suspension cultures. We grew tomato cell suspension cultures (derived from a callus culture of the variety 'Bonnie Best' donated to us by Dr. H. Murakishi in 1967) in 1 l. flasks containing 550–600 ml M6E medium at 28° as described previously [11]. For some experiments cells were grown as described but on MET medium.

The MET medium consisted of sucrose and salts as follows (all as mg/l. of medium): sucrose (18000); $Ca(NO_3)_2 \cdot 4H_2O$ (242); KNO_3 (85); KCl (61); $MgSO_4 \cdot 7H_2O$ (210); NH_4NO_3 (83); KH_2PO_4 (170); NaFeEDTA (37); H_3BO_3 (6.2); $MnSO_4 \cdot H_2O$ (22); $ZnSO_4 \cdot 7H_2O$ (8.6); KI (0.83); $Na_2MoO_4 \cdot 2H_2O$ (0.25); $CuSO_4 \cdot 5H_2O$ (0.025); $CoCl_2 \cdot 6H_2O$ (0.025); and myo-inositol (60); Thiamine · HCl (3); and 2,4-dichlorophenoxyacetic acid (1). In addition, each litre of medium contained the 70% EtOH soluble fraction of 1.25 g Difco yeast extract (dissolved in H_2O).

Isolation of crude precursors. We prepared the crude precursors from cultures of desired age (4–6 days for high P1 yield, 7 days or later for high P2 yield, when grown on M6E) by rapid filtration of the cultures (15–30 1 l. flasks) on a large 120 μm polypropylene filter followed by a brief water wash (2 l.), suspension of the cell pad in 2 l. 25 mM $AlCl_3 \cdot 6H_2O$ (or 50 mM $CaCl_2 \cdot 2H_2O$) for 5 min and then suction (×2). The 4 l. eluate was reduced in vol. to *ca* 100 ml at 40°. Addition of TCA to a final concn of 10% (w/v) in the concd eluate yielded a ppt after 18 hr at 4°. Centrifugation of the TCA treated eluate (at 9000 rpm, 90 min) yielded a hydroxyproline-poor pellet (discarded) and a hydroxyproline-rich supernate which was dialysed 48 hr at 4° and then freeze-dried. The yield of crude precursor was 80–200 mg/30 g dry wt cells (avg. batch size) depending upon the eluting salt.

Ion exchange chromatography. We routinely fractionated 100–200 mg crude TCA-soluble precursors (10 mg/ml in 30 mM pH 7.6 NaPi buffer) via gradient elution of a 90 × 1.5 cm i.d. column of BioRex 70 (100–200 mesh); the mixing chamber contained 300 ml 30 mM NaPi buffer (pH 7.6) and the reservoir contained 300 ml pH 6.1 NaPi buffer (containing 1 M NaCl). The flow was 60 ml/hr (6 ml fractions), and monitored at 280 nm (ISCO Model UA-4).

HF deglycosylation. We deglycosylated 5–50 mg glycosylated P1a, P1b or P2 in a micro apparatus (described previously [28]) containing 2–4 ml anhydrous HF and 5–10% anhydrous MeOH for 1 hr at 0°. The reaction was quenched by pouring into stirred H_2O at 2° to a final concn 5–10% (v/v) HF, and then dialysed for 16–24 hr at 4° and freeze dried. Yields were typically 35–40% of the original wt for P1 and P2.

Amino acid analyses. We used a highly modified Dionex microcolumn system with B-X8 resin (Benson Co.) eluted by Pickering Buffers A and B, and Benson's buffer C. Fluorometric detection after NaOCl oxidation and OPA coupling allowed Hyp and Pro detection [29]. Data capture was by IBM 9001 computer with IBM CAPS software.

We estimated the Tyr/IDT ratio in dP2 by reverse phase HPLC on Hamilton PRP-1 (150 × 4.1 mm) in an SP8000 liquid chromatograph. Solvent A was 0.13% HFBA and solvent B was 0.13% HFBA in 80% MeCN (aq). Programmed gradient elution involved 0–30% Solvent B for the first 15 min, then a 5 min hold at 30% B followed by a return to 100% A in the next 5 min. We monitored the absorbance at 273 nm (IDT max in acid) and used IDT previously purified [7] and reagent grade L-Tyrosine as standards. Data capture was via the IBM 9001 and CAPS. Deglycosylation was imperative, otherwise sugar degradation products appeared in the chromatograms preventing accurate Tyr and IDT estimation.

Sugar analyses. We analysed sugars as their alditol acetates [30] by GC using a 6 ft × 2 mm i.d. PEGS 224 column programmed from 130 to 180° at 1°/min and using an SP4100 computing integrator for data capture.

Sepharose CL-6B filtration. We injected 1–5 mg P1, P2, dP1 or dP2 (10 mg/ml in 1 M NaCl) onto a 100 cm × 1.25 cm i.d. column of Sepharose CL6B-200, eluted with 1 M NaCl at 13 ml/hr using a Sigmamotor peristaltic pump, and monitored absorbance at 280 nm.

Zorbax GF-250 HPLC gel filtration. We injected 200 μg P1, P2, dP1 or dP2 (10 mg/ml in 0.2 M pH 7 NaPi buffer containing 0.005% NaN_3) onto a 250 mm × 9.4 mm i.d. column of DuPont GF-250, eluted at 1 ml/min with the same buffer using an LDC Model I Constametric Pump (Milton Roy Co.), monitored absorbance at 254 nm.

Tryptic digest. We incubated 0.2–20 mg HF-deglycosylated extensin precursors (10 mg/ml) in freshly prepared 2% (w/v) NH_4HCO_3 (aq.) containing 10 mM $CaCl_2$ (minimum vol. 100 μl) with TPCK-trypsin (Worthington) (substrate:enzyme ratio 100:1) at 30–35° with constant stirring.

pH-Stat. In some experiments we followed the time course of tryptic cleavage under unbuffered conditions at room temp. (23°), titrating to pH 8 with M/100 NaOH in a pH-stat using a Corning model 135 pH/ion meter interfaced to a Radiometer Automatic Burette Unit.

HPLC peptide mapping. We obtained tryptic peptide maps via reverse phase HPLC of tryptic digests on either DuPont Zorbax

ODS (250 × 4.6 mm i.d.) or Hamilton PRP-1 (150 × 4.1 mm i.d.) columns, using programmed gradient elution (0.5 ml/min) with the following mobile phase solvents: A = 0.13% HFBA, and B = 0.13% HFBA in 80% (v/v) MeCN (aq.). For resolution of dP1 tryptides, the gradient began at 100% A and increased (0.5%/min) from 0 to 50% B in 100 min. For dP2 tryptide resolution, solvent B increased (1%/min) from 0 to 60% in 60 min. Absorbancy was monitored at 273 nm. We manually collected fractions for analyses allowing for the 200 μl dead volume between the detector and 'fraction collector'.

Sephadex G-25 gel filtration. We injected 1–10 mg freeze-dried tryptides (in 0.5 ml 0.1 M HOAc) onto 2 × 100 × 1.25 cm i.d. columns of Sephadex G-25-80 (fine), eluted with 0.1 M HOAc at 10 ml/hr using a syringe pump (Harvard Apparatus Co. Model 2201), collected 2 ml fractions and read their absorbance at 230 (or 280) nm.

Automated Edman degradation. We sequenced 5–200 nmol HPLC-purified peptides (5 mg polybrene added as a carrier [31] without precycling) using a Beckman 890C Spinning Cup Sequencer plus cold trap (0.1 M quadrol program [32]), in conjunction with a Sequemat P-6 Autoconverter. After Sequemat conversion of anilinothiazolinone derivatives to the corresponding phenylthiohydantoins (PTH) in methanolic HCl (6.25 ml AcCl, 43.75 ml MeOH) for 6 min at 65° we dissolved the PTH derivatives in 100 μl pH 4.4 Zorbax buffer containing 10 nmol PTH-norleucine as internal standard [pH 4.4 Zorbax buffer was 29 ml, pH 4,4, 0.2 M NaOAc, 551 ml H_2O, 420 ml MeCN (Spectral Grade, Burdick & Jackson Laboratories, Muskegon, MI 49442) per l.], and chromatographed them on DuPont Zorbax ODS (250 × 4.6 mm i.d.) isocratically eluted at 0.5 ml/min with pH 4.4 Zorbax buffer or pH 5.0 Zorbax buffer (500 ml pH 5.0 0.01 M NaOAc and 500 ml MeCN per l.) for PTH-histidine identification. We monitored the eluate at 269 nm and integrated the peak areas with an SP4100 computing integrator.

More recently we have used an IBM cyano column (250 × 4.5 mm i.d.) with an SP8000 liquid chromatograph at 40° using the following ternary solvent program at a flow rate of 0.5 ml/min:

	Time	%A	%B	%C
Solvent A: 0.015 M NaOAc (pH 5.8)	0	85	15	0
Solvent B: MeCN	10	58	30	12
Solvent C: MeOH	14	67	15	18
	30	50	25	25
	40	40	30	30
	44	85	15	0

Detection was at 254 nm using the SP770 Model.

Spectrophotometric Detector and data capture was via the IBM 9001 computer running CAPS software. This column and solvent program gave better separations of all PTH-amino acids and allowed identification of histidine and arginine without using a separate solvent system [33].

PTH-serine and PTH-threonine were sometimes identified by the presence of distinctive peaks resulting from their degradation during conversion [34], but most often these degradation peaks merely verified identification.

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