Accelerated high-sensitivity microsequencing of proteins and peptides using a miniature reaction cartridge

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Abstract

Greatly reduced automated protein sequencing degradation times of less than 25 min with concurrent on-line phenylthiohydantoin (PTH) derivative analysis times of less than 16 min have been achieved by using a miniaturized reaction cartridge with optimized chemical and analytical cycles. Using this method a wide range of standard and novel peptides and proteins have been sequenced with reproducibly high initial and repetitive cycle yields. In these accelerated analyses the recovery of the more labile PTH derivatives was markedly improved by using elevated pressure during cleavage steps and temperature programming throughout the Edman cycle.

Keywords: HPLC; microcartridge; sequencing

The major advance in the sensitivity of protein sequence analysis initiated by the development of gas-liquid solid-phase sequencing methods (Hewick et al., 1981) has been followed by the introduction of many refinements that have led to the current range of fully automated and integrated systems. Although these instruments are generally able to determine amino acid sequence using less than 20 pmol of polypeptide with cycle times of approximately 40 min (Shively et al., 1989), there is a continuing need for both greater sensitivity and sample turnover in sequence analysis.

We report here the properties of sequencing methods that employ a microcartridge fitted to a commercially available and widely used automated sequencer. Use of the microcartridge resulted in improved low picomole sequencing yields because the efficiency of reagent and solvent exchange could be increased. Furthermore, we also

describe conditions for the incorporation of temperature programming, pressurized cleavage, and altered reagent consumption and delivery, which allow a substantial decrease in the Edman degradation cycle time, a decrease in the level of by-products, and an increase the recovery of labile PTH-AA derivatives. In order to maintain synchrony of the PTH-AA analyzer with the remainder of the system, we have devised a rapid HPLC cycle that is compatible with the rapid sequencer cycle time.

This report shows that through the use of limited modifications to a commercially available protein sequencer in conjunction with new chemistry cycles improved routine performance at high sensitivity and the capacity to sequence 60 AA residues per 24 h can be achieved.

Results

Construction of the MIC-FAS cycles

The MIC-FAS cartridge and conversion flask cycles shown in Table 1 and the PTH analysis gradient shown in Table 2 were used for all standard sequencing runs. These cycles follow established protocols that have proved widely successful for routine analysis using ABI protein sequencers. The need to integrate the cartridge, conversion, and analysis cycles to achieve optimal sequence identification without compromising any aspect

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Abbreviations: AA, amino acid; ATZ, anilinothiazoline; AUFS, absorbance units full scale; DMPTU, *N*-dimethyl-*N'*-phenylurea; DPTU, *N,N'*-diphenylthiourea; DPU, *N,N'*-diphenylurea; DTT, dithiothreitol; GHRF, growth hormone releasing factor; HPLC, high performance liquid chromatography; PITC, phenylisothiocyanate; PVDF, polyvinylidenedifluoride; PTC, phenylthiocarbonyl; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; TMA, trimethylamine.

Table 1. MIC-FAS reaction cartridge and conversion flask cycles

Reaction cycle				Conversion cycle			
Step	Function	Number	Time (s)	Step	Function	Number	Time (s)
1	Prep R2	4	6	1	Block flush	23	6
2	Deliver R2	5	20	2	Load S4	12	3
3	Prep R1	1	6	3	Argon dry	22	6
4	Deliver R1	2	1	4	Ready to receive	13	90
5	Argon dry	29	30	5	Conversion heater	24	75
6	Deliver R2	5	350	6	Argon dry	22	60
7	Deliver R1	2	1	7	Load R4	3	2
8	Argon dry	29	30	8	Argon dry	22	8
9	Deliver R2	5	350	9	Pause	25	350
10	Reaction heater	32	50	10	Argon dry	22	4
11	Argon dry	29	90	11	Pause	25	350
12	Deliver S1	14	10	12	Argon dry	22	120
13	Argon dry	29	5	13	Conversion heater	24	60
14	Deliver S2	17	20	14	Argon dry	22	120
15	Argon dry	29	5	15	Block flush	23	10
16	Deliver S2	17	20	16	Clear injector to waste	14	70
17	Argon dry	29	5	17	Load S4	12	11
18	Deliver S2	17	20	18	Argon dry	22	6
19	Argon dry	29	75	19	Load S4	12	11
20	Prep R3	7	6	20	Argon dry	22	6
21	Load R3	9	1	21	Pause	25	25
22	Argon dry	29	3	22	Argon dry	22	2
23	Pressurize	28	150	23	Pause	25	25
24	Load S2	18	6	24	Argon dry	22	2
25	Block flush	30	6	25	Pause	25	25
26	Argon dry	29	90	26	Load injector	15	14
27	Prep transfer	22	10	27	Inject	16	1
28	Deliver S1	14	5	28	Load S4	12	11
29	Transfer w/ S3	23	15	29	Argon dry	22	6
30	Pause	33	10	30	Load S4	12	11
31	Transfer w/ argon	24	5	31	Argon dry	22	6
32	Transfer w/ S3	23	6	32	Clear injector to		
33	Pause	33	10		fraction collector	17	25
34	Transfer w/ argon	24	25				
35	End transfer	25	1				
36	Reaction heater	32	53				
37	Deliver S3	20	10				
38	Argon dry	29	5				
39	Deliver S3	20	10				
40	Argon dry	29	75				

of the instrument's capability was a primary prerequisite in the design of these cycles.

Cartridge cycle—Coupling

In line with the twofold reduction in the glass fiber filter area and sample loadings of below 20 pmol, the polybrene carrier loading was reduced to 750 μg . The use of lower amounts of carrier led to a reduction in repetitive yields of a 13-residue peptide (TP13), presumably due to increased washout from the filter and increased lag. Lower repetitive yields were also obtained from filters used repeatedly for sample analysis, again presumably due to a reduction of the available carrier on the filter.

In the latter case however background levels were reduced (see Fig. 6).

Efficient modification of the α -amino group while minimizing the formation of the primary by-products DPTU and DMPTU was achieved through the use of two 1-s deliveries of PITC (R1) and a shorter base delivery time of 350 s (Table 1). Each aliquot of R1 (approx. 15 μL) still contained a large molar excess of PITC. Preliminary experiments in which the PITC concentration was reduced to 2.5% in heptane or when a single aliquot of 5% PITC was delivered each resulted in increased lag and negligible reduction in the amount of by-products. Gas-phase delivery of the base (R2, 12.5% TMA in water) at 53 $^{\circ}\text{C}$ required a total coupling time of only 800 s,

Table 2. MIC-FAS PTH-AA analysis gradient^a

Time (min)	Concentration B (%)	Flow (μ L/min)	Event
0.0	10	350	4
0.5	10	350	34
8.5	35	350	4
15.8	36	350	4
15.9	90	450	4
17.1	90	450	0

^a Run time: 17.1 min; maximum pressure: 2,200 psi; minimum pressure: 0 psi; target pressure: 200 psi; target time: 0.5 min; equilibration time: 5.0 min.

which represents a 20% reduction of the current FAST-1 cycle time (ABI, 02.15.89 version). Further reduction of the coupling time caused increased lag, whereas coupling temperatures over 53 °C caused lower repetitive yields, possibly due to partial hydrolysis of PITC (data not shown).

Cartridge cycle—By-product extraction

This critical area in the optimization of efficient cycles initially proved troublesome due to a tendency of the extraction solvents heptane (S1) and ethyl acetate (S2) to channel through the central part of the disc after first diffusing to the perimeter. This was solved by interrupting the 20-s extraction with 5-s bursts of argon, which effected very efficient extraction with minimal sample washout. The level of DPTU remained stable between successive cycles and samples. DMPTU was rarely detected at a level above 10 pmol after the first few cycles. The observed levels of DPU were extremely low and typically less than 1 pmol at the first cycle.

As previously reported, DPU coelutes with PTH-Trp in the conventional on-line analysis system (Applied Biosystems, 1986b). Consequently the reduction of DPU to consistently low levels, as well as improvements in the recovery of PTH-Trp due to reduced oxidation, has greatly facilitated the identification of this previously elusive residue at low picomole levels.

Cartridge cycle—Cleavage

The process of cyclization of the PTC-polypeptide to form the ATZ derivative of the amino-terminal residue was studied in detail. As previously reported, the rate of this reaction was found to vary considerably with different amino-terminal residue side groups (Tarr, 1986). A twofold reduction in cyclization time at 50 °C could be achieved by pressurization of the reaction chamber following the initial 1-s pulse of TFA solution (R3). This was performed using cartridge cycle function 28 (Pressurize) in place of the normal function 33 (Pause). The result was

a controlled increase in the rate of R3 absorption by the filter, followed by vaporization in the subsequent flow of argon.

Pro-X peptide bonds commonly require dedicated cyclization cycles containing extended cleavage times and elevated temperatures. The MIC-FAS cycle gave a sequence-dependent lag of 15–25% at proline residues (data not shown), similar to that observed with normal liquid pulse 477A operation. A proline cleavage cycle was therefore designed with a 400-s pressure cleavage at 53 °C, which reduced the corresponding lag to less than 10% (Table 3).

Cartridge cycle—Extraction and drying

The ATZ-AA was efficiently transferred to the conversion flask by extraction with a total volume of 150 μ L of butyl chloride (S3) preceded by heptane (S1). The small extraction volume was possible due to the low swept volume of the microcartridge and by pulsing the S3 deliveries with a 5-s burst of argon (Table 1).

Optimization of the drying steps was found to be critical to the construction of a cycle capable of operating at greater than 95% efficiency. Reduction of drying times below those shown in Table 1 invariably led to a drop in repetitive yield (data not shown), and the postcleavage drying step (step 26) in particular proved to be the most important in sample washout and ATZ-AA extraction. The yields of amino acids primarily affected by this part of the cycle were studied using the peptide secretin and are reported below.

Conversion cycle

Temperature programming to optimize the ATZ conversion chemistry was introduced to address the need for faster conversion and efficient dissolution and drying steps, while minimizing the formation of secondary amino acid derivatives. Limitations of the existing conversion flask assembly regarding the rates of heating and cooling restricted the maximum temperature differential to 10 °C. The lower temperature (65 °C) was used prior to ATZ-AA transfer in order to restrict serine and threonine β -elimination (Tarr, 1977) to levels of approximately 15% and 10%, respectively. PTC intermediates were not observed during PTH-AA analyses, which indicated that complete conversion of the ATZ-AA to the PTH-AA had occurred in the time allowed at 75 °C. Partial loop filling by pretransfer conversion aliquots of S4 and R4 was employed to complement the reduction in the S1/S3 transfer volume and to allow shorter drying times. Using this loading method step times of 2–3 s gave reproducible drying times, thereby indicating consistent deliveries to the conversion flask. Efficient dissolution of the PTH-AA following conversion was achieved in 75 s at the higher temperature using 12.5% acetonitrile solution.

Table 3. The 477A microcartridge sequence analysis summary (standards)

Polypeptide	Load (pmol)	Residues identified	Initial yield (%)	Average repetitive yield (%)	Combined repetitive yield (%)	Lag per cycle (%)	Number of runs
β -Lactoglobulin	25	20/20	90	95.5	93.5	0.60	5
β -Lactoglobulin	10	20/20	88	95.2	93.1	0.60	5
β -Lactoglobulin	5	20/20	80	94.8	92.3	0.55	2
β -Lactoglobulin	2.5	19/20	53	94.4	92.0	0.60	2
Ribonuclease	15	30/30	94	93.8	91.2	0.85	2
α -Lactalbumin	5	20/20	65	94.6	91.6	0.80	1
GHRF (1-40)	30	40/40	93	93.5	91.5	0.75	4
GHRF (1-40)	10	29/30	89	92.9	92.8	0.69	2
Secretin	12	26/27	70	94.2	92.5	0.95	4
Insulin B-chain	20	30/30	82	93.5	92.5	1.35	1
β -Endorphin	20	31/32	87	93.7	92.0	1.25	1
Angiotensin II	15	8/8	90	— ^a	84.5	0.85	1
Thyrocaltitonin	25	27/32	81	93.6	91.0	1.35	2
TP13	16	13/13	89	93.5	90.8	0.75	2
TP10	20	10/10	88	93.8	86.5	1.30	2
AIPEFQKLVR	20	10/10	78	— ^a	90.3	33.5	2
AIPEFQKLVR ^b	20	10/10	82	— ^a	93.0	7.83	1

^a No repeating AA residues present.^b Including a proline cycle.

PTH-AA analysis

PTH-AA identification and quantitation at low picomole levels can be most efficiently achieved with on-line reverse-phase HPLC (Rodriguez et al., 1984). Reproducible and accurate gradient formation, temperature control, pregradient equilibration, and postgradient refilling of the syringe pumps are all key areas that we have studied in order to obtain chromatographic data capable of interpretation using detector settings as low as 0.002 AUFS. At this level manual sequence identification involves careful comparison of chromatographic data, whereas quan-

titation and automated sequence identification place extremely high demands upon the accuracy and sensitivity of the system. The high speed of the sequencing cycles described here demanded the use of rapid HPLC solvent flow rates and consequently flow rates of 350 μ L/min for PTH-AA separation and 450 μ L/min for column flushing were used. Although the MIC-FAS gradient (Table 2) used with the corresponding sequencing cycles caused the 120A syringe pumps to run continuously, no detectable decrease in the number of samples that could be analyzed per column was found over a period of many months in comparison to the standard system. Figure 1 shows a 5-

Fig. 1. Separation of PTH-AA standards and sequencing by-products using the MIC-FAS cycles and gradient. The DTT-PITC adduct is indicated by an asterisk (*). The levels of DMPTU and DPTU are higher than in the standard, reflecting typical levels produced by this system.

pmol PTH-AA standard separation including typical by-products of the sequencing chemistry. All derivatives were clearly and reproducibly resolved from each other and from contaminant peaks, which enabled sequences to be determined using the automated data analysis system as well as manually.

Protein sequencing

Results obtained from the sequence analysis of a range of polypeptide standards are shown in Table 3. β -Lactoglobulin is widely used as a sequencing standard because it is available at very high purity and optimal storage conditions have been established; thus, it was chosen as the standard protein for the evaluation of cycle developments. Data from the analysis of 2.5 and 10-pmol of β -lactoglobulin are shown in Figure 2. The data system analysis of a 10-pmol run is also shown (Table 4). Prominent assets of the MIC-FAS system were found to be high theoretical initial yields of 75–90% calculated by least-squares regression analysis and high yields of the first cycle product, PTH-Leu, after background subtraction. The average repetitive yield of all of the amino acid residues that are found more than once in the sequence showed little variation between pairs of residues, and the value of greater than 95% at 10 pmol significantly exceeds results normally obtained at this level using standard systems. The very low lag of less than 0.7%/cycle (Table 4) indicated that near-optimal coupling and cleavage conditions had been obtained. PTH-Trp at cycle 19 was consistently identified by the data system even using a 5-pmol load,

which cannot be carried out with standard systems. The high signal-to-noise ratio achieved by the microcartridge system was due both to the consistently low level of DPU and to the generally improved yields of the most oxidatively labile residues Ser, Thr, Met, and Trp. β -Elimination at Thr was limited by the reduced cleavage time and led to high yields of PTH-Thr at positions 4, 6, and 18. The inclusion of DTT as an oxidant scavenger improved repetitive yields even at low concentrations as previously reported (Hewick et al., 1981). As DTT reacts with residual PITS to form a UV-absorbing adduct that interfered with PTH-AA identification, its routine use had previously been avoided. However, here we have achieved efficient separation of this adduct by increasing the THF concentration in solvent A, which leads to its elution between PTH-Pro and PTH-Met. The use of 0.001% DTT in S2 caused an adduct signal equivalent to only 5–10 pmol of PTH-AA, which did not interfere with subpicomole PTH-AA identification or quantitation.

Peptide sequencing

The ability to obtain complete sequence information at picomole amounts of 1–5-kDa peptides, typically derived from parent proteins by specific fragmentation methods, is of prime importance in the analysis of protein structure. Although the entrapment of proteins within the polybrene-treated glass fiber matrix is usually very efficient as measured by the amount of sample washout, peptide retention is variable. This often leads to high initial yields from peptides combined with lower peptide repetitive yields than those obtained from proteins. A range of natural and synthetic peptides of varying length, charge, and hydrophobicity were therefore selected to evaluate the MIC-FAS cycle performance.

GHRF (1–40)

Analysis of 10 pmol of this peptide (Fig. 3) showed high initial and combined repetitive yields of 90 and 93%, respectively, which are typical of microcartridge/MIC-FAS performance using this amount of peptide. The average lag was again low at 0.69%/cycle. The retention times were highly reproducible during the run (<0.005% shift), which enabled an automated sequence assignment to be made directly. The total run time for this analysis including precycling and 30 MIC-FAS cycles was 15.0 h.

It should be noted that the yields of PTH-Ser at residues 9, 18, and 28 were greater than 60% of the calculated maximum signals and the major serine by-product, PTH-dehydroserine-DTT, was consistently detected. No significant drop in yield was detected following serine residues, which indicated that N-terminal blocking by O to N migration of acyl groups was very low. Asp 25 was not identified by the data system due to a slight drift in reten-

Fig. 2. Sequence analysis of 10 (solid) and 2.5 (open) pmol of β -lactoglobulin. Note that the high initial yield of 90% using 10 pmol of β -lactoglobulin is reduced to 53% with the 2.5-pmol loading, with the combined repetitive yields dropping from 93.5 to 92.0%.

Table 4. Analysis of 10 pmol of β -lactoglobulin using the MIC-FAS cycles

Position	AA	Retention time (min)	Calibration time (min)	Raw yield (pmol)	Yield – background (pmol)	Yield + lag (pmol)	Ratio (%)
Automated sequence determination							
1	L	15.88	15.90	7.82	7.06	7.22	46.31
2	I	15.28	15.27	9.88	8.00	8.07	51.65
3	V	12.38	12.37	8.49	7.62	7.66	35.26
4	T	5.62	5.63	6.34	5.88	6.02	61.22
5	Q	5.22	5.27	6.03	5.47	5.70	41.15
6	T	5.60	5.63	4.90	4.50	4.70	47.80
7	M	12.13	12.15	6.24	6.10	6.39	77.66
8	K	15.58	15.58	6.33	5.74	6.08	24.15
9	G	5.82	5.82	8.59	6.32	6.73	17.97
10	L	15.90	15.90	6.33	5.26	5.62	36.05
11	D	3.73	3.74	2.98	2.95	3.18	58.85
12	I	15.28	15.27	6.10	4.62	4.99	31.95
13	Q	5.23	5.27	4.64	3.90	4.24	30.61
14	K	15.57	15.57	4.73	4.14	4.52	17.94
15	V	12.37	12.37	4.68	3.94	4.32	19.87
16	A	8.03	8.07	4.57	3.63	3.95	36.91
17	G	5.82	5.85	5.81	3.60	3.60	9.63
18	T	5.60	5.63	2.85	2.44	2.67	27.09
19	W	14.23	14.22	2.70	1.29	1.29	3.61
20	Y	10.03	10.05	2.84	1.44	–	3.51
Amino acid			Positions	Repetitive yield (%)		Variance	
Repetitive yield analysis							
L			1, 10	96.78		1.000	
I			2, 12	94.66		1.000	
V			3, 15	94.65		1.000	
T			4, 6, 18	94.30		0.972	
Q			5, 13	95.88		1.000	
K			8, 14	94.69		1.000	
G			9, 17	93.22		1.000	
Average AA repetitive yield				94.81			
Combined AA repetitive yield				93.00		0.745	
Theoretical initial yield				9.11 pmol (91.08%)

Fig. 3. MIC-FAS sequence analysis of 10 pmol of GHRF (1–40).

tion time, but a 500-fmol peak of PTH-Asp was observed in the chart recorder output. The recovery of PTH-Arg 29 was only 370-fmol above background but was still clearly identified by the data system.

Secretin

This 27-residue peptide hormone was used to evaluate the recovery of the more problematic residues. Extraction of ATZ-His from the polybrene matrix was found to be poor using butyl chloride (S3) as previously reported (Applied Biosystems, 1984). However, reduction of the post-cleavage drying at step 26 from 90 to 70 s dramatically improved the recovery of PTH-His, as well as PTH-Asp, Ser, Thr, and Arg to a lower degree (Fig. 4). The recovery of PTH-Asp 3 and 15 improved by up to 40%, but as found with Asp residues in other peptides the yield was

Fig. 4. Optimization of postcleavage drying time. The effects of changing the postcleavage drying time at 56 °C from 90 s (hatched) to 70 s (solid) are shown using secretin. Reduction of this time improved the yields of PTH-His, Asp, Ser, Thr, and Arg.

quite variable within individual runs. Although the yields of PTH-Ser and Thr were improved using shorter drying times, the levels of the corresponding β -elimination products were unaffected.

Novel peptides

We have successfully used the microcartridge to determine the sequences of peptides derived from intracellular signalling proteins following proteolytic digestion and separation by reverse-phase HPLC. The methods of Kawasaki and Suzuki (1990) have been used to generate peptides from a putative bovine phosphatidylinositol 3-kinase using lysyl endopeptidase. Results from these peptides were all obtained at low picomole levels (Figs. 5, 6). Figure 5 shows the analysis of an estimated 7 pmol of a 24-residue fragment from a partial digest of a 110-kDa protein that initially copurified with the PI 3-kinase. All 24 residues were identified with a combined repetitive yield of 94.3%. Figure 6 shows the primary chromatographs obtained from the analysis of residues 1–20 of a peptide that was derived from the 110-kDa PI 3-kinase subunit. Nineteen residues were identified from an initial yield of 2.8 pmol of PTH-Thr and an average repetitive yield of 92.9%.

Discussion

In this report we have described the advantages of the use of a rapid Edman chemistry cycle that employs limited hardware changes on an ABI 477A Protein Sequencer.

While retaining the standard method of sample entrapment within a glass fiber matrix, a 50% reduction in reaction chamber dimensions accompanied by lower amounts of carrier and the use of smaller reagent and solvent volumes have made it possible to carry out routine sequence analysis at sample levels below 5 pmol. Identification of all PTH-AA below 250 fmol was achieved using a cycle time of 25 min. Although the glass fiber matrix capacity was reduced by 50%, the decreased load volume of 15 μ L, which had to be used was consistent with the manipulation of nanogram quantities of samples. The size of the microcartridge chamber was adequate to accommodate electroblotted samples, but the development of a dedicated microblot cartridge using tangential reagent flow for the analysis of PVDF filters (Sheer et al., 1991) led us to focus on the analysis of samples in solution using methods that were particularly applicable to peptides in microbore HPLC fractions.

The MIC-FAS system used 1-s deliveries of R1 and R3 and partial loop filling for the delivery of R4 and S4 to the conversion flask. The delivery of all of these reagents was found to be highly reproducible and allowed stringent cycle comparisons to be made. Use of the minimum reagent volumes possible with the current hardware design did not cause any sequencing failures, and each aliquot of R1 (15 μ L) still contained a large molar excess of PITC over the peptides or proteins analyzed. A more versatile method of microcartridge operation may be obtained by the use of integrated micropathways for fluid delivery, as has been recently described for a new compact sequencer by Calaycay et al. (1991).

Fig. 5. MIC-FAS sequence analysis of an estimated 7 pmol of a 24-residue fragment of a 110-kDa putative PI 3-kinase. The sequence was determined to be SLINKNDLQRLKFDQLNIQRTQK for which the combined repetitive yield was 94.3%.

Fig. 6. MIC-FAS analysis of a novel 22-residue peptide from a 110-kDa protein. A 5-pmol PTH-AA standard and 19 primary chromatographs are shown. The peptide sequence was determined to be TVSQRFGLLESYCRA-GMY. The background-corrected yield of PTH-Thr at cycle 1 was 2.8 pmol, and the combined repetitive yield was 92.9%. Cycle 14 was tentatively assigned as a cysteine residue. Residue 17 was not identified and is not shown. Subsequently, complementary DNA analysis identified a cysteine residue at both these positions.

The presentation of samples on a reduced surface area was found to improve the initial yields relative to the standard system. Such changes have increased the PTH-AA signal-to-noise ratios and allowed extended low picomole sequence analyses; however, the reason for this effect is not clear. Problems arising from use of a polybrene/glass fiber matrix previously included high amino acid backgrounds and the retention of by-products, such as DPTU and DPU. Furthermore the need to condition the matrix by precycling was time consuming. These problems have been reduced by the changes described here to the matrix area and the amount of polybrene carrier, as well as by the use of shorter precycle times. It was found to be possible to decrease the carrier concentration used on the microcartridge filter still further to carry out very high-sensitivity analysis using this system, but a small drop in repetitive yield was observed.

The pulsed liquid delivery of TFA was modified by the introduction of a forced delivery step, which we have termed "pressure cleavage." This step enhanced the absorption of TFA by the filter after the initial pulse and therefore accelerated the cleavage reaction. Although any standard degradation cycle chemistry is necessarily a compromise of different optimal conditions required for each amino acid residue, and although problems arise inherently from the use of noncovalent sample attachment (Pappin et al., 1990), the pressure cleavage method (150 s) has allowed substantial progress to be made from the previous gas-phase (900 s) and subsequent liquid-pulse (300 s) approaches.

Use of the modified cartridge procedure greatly improved the recovery of the more labile amino acid derivatives, whereas accelerated conversion at temperatures over 70 °C did not compromise sequence determination. Optimal performance of the standard 120A integrated PTH-AA analysis system was essential for low picomole sample analyses and was achieved using much increased flow rates. The analyzer cycle was synchronized with sequencer cycle times of 25 min, which is the probable limit of the speed and resolution of current hardware. The future development of PTH-analyzer sensitivity may exploit radically different types of instrumentation such as capillary electrophoresis or mass spectrometry.

The revised operating programs described here have improved performance, productivity, and chemical consumption. Extensive analysis of a wide range of standard samples has revealed no inherent loss in the ability of the original sequencer to analyze both peptides and proteins with no obvious sequence-specific restrictions, and we have used the system routinely to determine the sequence of many novel peptides and proteins (Brooks et al., 1990; Kozma et al., 1990; Abo et al., 1991; Diekmann et al., 1991; Otsu et al., 1991). The increased sample turnover and sensitivity obtainable using these cycles now justifies the development of systems for multiple sample loading and very low picomole to femtomole analysis.

Materials and methods

Materials

All solvents and reagents were of sequencing grade (Applied Biosystems, Inc. [ABI]) unless stated otherwise. DTT was added to the ethyl acetate (S2) to a final concentration of 0.001% (w/v) immediately prior to installation on the sequencer. HPLC-grade water was from Rathburn Chemicals (UK). Ribonuclease, lysozyme, α -lactalbumin, GHRF (1–40), secretin, insulin, angiotensin II, and β -endorphin were from Sigma, and β -lactoglobulin was from ABI. Fresh dilutions of β -lactoglobulin were made weekly and stored according to the manufacturer's instructions. All polypeptide loadings were verified in duplicate by fully automated amino acid analysis (ABI model 420H). Peptides TP10 and TP13 were synthesized using Fmoc chemistry (ABI model 430A) and purified by reverse-phase HPLC.

Instrumentation

Polypeptides were sequenced using an ABI model 477A, and on-line PTH analysis was performed using an ABI model 120A. Gas regulator settings were within the manufacturer's normal limits, as were all other parameters likely to influence instrument performance with the following exceptions.

The standard cartridge block (12.0 mm) was replaced by a microcartridge of reduced chamber diameter (8.0 mm) but retaining the external dimensions of a standard cartridge. Glass fiber discs (TFA-treated, ABI) were cut to a diameter of 8.5 mm using a cork borer. The cartridge inlet delivery line was reduced in length from 33 cm to 20 cm to facilitate the precise delivery of reduced reagent volumes.

Glass fiber discs were spotted with 15 μ L of Biobrene (750 μ g of polybrene and 60 μ g of NaCl in water) and conditioned using two MIC-FIL precycles (available on request from the author) before samples were applied. Sequencing runs consisted of one MIC-BGN begin cycle (available on request from the author), a series of solvent washes using S1, S2, and S3, and a truncated PITC coupling step prior to the required number of fast degradation (MIC-FAS) cycles (Table 1). Precoupling treatment with TFA (R3) was found to be superfluous using these methods (data not shown).

The 120A PTH analyzer was fitted with a standard PTH C18 2.1 $\times$ 220-mm reverse-phase HPLC column (ABI). Fresh PTH standards (R5, 66.6 fmol/ μ L in acetonitrile) were prepared from a stock solution every 24 h for optimal quantitation. The standard flask loop (75 μ L) was used to deliver 5 pmol of R5 to the conversion flask. The standard injector loop (50 μ L) was replaced by a 100 μ L loop and optimal separation was achieved by re-

ducing the acetonitrile concentration of the dissolution and transfer solvent (S4) from 20% to 12.5%. PTH-AA yields were corrected to allow for partial sample injection and thus represent 100% of the transferred PTH-AA. Results were calculated using the inbuilt data system (software revision of 02.15.89), which allowed direct comparisons to be made with results obtained by us and by others using the manufacturer's recommended sequencing methods. Chromatographic data were routinely recorded using the filtered data mode and a strip chart recorder to allow further interpretation of sequence information at the subpicomole level. Calibration tables were also used in the filtered data mode to allow the determination of PTH-AA yields. Such tables were based on measurement of peak height and were updated according to the manufacturer's instructions.

Modifications to the PTH analyzer solvent A (ABI) were as follows. The tetrahydrofuran concentration was increased to 5.5% to improve the resolution of PTH-proline from the DTT-PITC adduct. Tryptophan (Sigma) was added to a final concentration of 1 μ M to offset UV-absorbing scavengers present in solvent B (Tempst & Riviere, 1989), and TMA was added to a final concentration of 0.01% to improve the peak shape of PTH-histidine and PTH-arginine (Applied Biosystems, 1986a). Due to increased sample turnover, solvent A was prepared in 2-L batches, which was sufficient for 10 days continuous operation; however, the final 100 mL was discarded due to the increasing tendency of the aged solvent mixture to give drifting retention times and poor PTH-lysine recovery. Solvent B (ABI) contained 0.5 μ M DMPTU to act as scavenger and increase the yield of the more hydrophobic PTH-AA at the low picomole level.

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