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Automated phenylthiocarbamyl amino acid analysis of carboxypeptidase/aminopeptidase digests and acid hydrolysates[a]

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ABSTRACT

A fully automated exopeptidase digestion procedure for the partial determination of N- and C-terminal peptide/protein sequence is described. The digestion of various substrates with aminopeptidase M, carboxypeptidase A, P or Y was accomplished with the Varian 9090 autosampler's robotic automix routines. The released free amino acids, in addition to free amino acids from acid hydrolysates, were derivatized with phenylisothiocyanate in an automated fashion and subsequently chromatographed on a C_{18} column for separation and quantitation. The advantages of automating this precolumn phenylisothiocyanate derivatization are the virtual elimination of sample manipulation errors and very reproducible data due to the precise control of the reaction conditions both of which, facilitate the interpretation of the exopeptidase reaction kinetic data.

INTRODUCTION

Analysis of C- and N-terminal sequences using exopeptidases can provide important information about the primary structure of proteins and peptides. Methods involving the digestion of a protein or peptide with carboxypeptidases and aminopeptidases and subsequent identification of the released amino acids by reversed-phase high-performance liquid chromatography (RP-HPLC) have been employed for such analysis [1,2]. Presently, enzymatic digestion using a carboxypeptidase is the most common method for obtaining C-terminal sequence, although recent work using C-terminal chemical modification appears promising [3,4]. Carboxypeptidase A (CPA) [5,6], P (CPP) [7] and Y (CPY) [8] are all used to obtain C-terminal sequence. The number of amino acids sequenced and the rate at which proteolysis occurs is substrate specific. Each carboxypeptidase has a distinct pH optimum, *i.e.*, pH 7.8 for CPA [6], pH 6.0 for CPY [8] and pH 3.7 for CPP [7]. For all three carboxypeptidases, proteolysis drastically slows when a proline residue is encountered. Classical Edman

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degradation chemistry is the preferred procedure for obtaining N-terminal sequence [9]. Situations arise however, where labile amino acids {*e.g.*, tyrosine-sulfate (Y[SO_3]) and methionine-sulfoxide (M[O])} exhibit poor recovery after exposure to the chemistry of the sequencing reaction. In these cases, exopeptidase digestion can be performed, such as with aminopeptidase M (APM) [10].

All exopeptidase reactions require extensive sample manipulation during the digestion and subsequent residue analysis. Since the rate of the digestion reaction is residue specific, precise timed removal of the aliquots is often crucial and may require 5–10 aliquots to obtain reliable sequence information. For each aliquot then, the reaction must first be quenched and then prepared for HPLC analysis. It is apparent that sample manipulation alone, is a major source of error in manual exopeptidase digestion reactions. Several other potential sources of error associated with amino acid analysis are: (i) the derivatization reaction and the derivative stability; (ii) the procedure used to hydrolyze the samples, *e.g.*, acid hydrolysis or proteolysis; (iii) reagent and sample purity; and (iv) the chromatographic system used for peak identification and quantitation [11,12].

We present data which shows that free amino acids obtained from acid hydrolysates can be reproducibly and quantitatively derivatized with phenylisothiocyanate (PITC) in an automated fashion using a heptane extraction procedure [13] to remove excess PITC. In addition, exopeptidase digests (CPA, CPP, CPY and APM) are automated and the liberated amino acids derivatized as above, thereby minimizing sample manipulation errors frequently encountered with these reactions. Automating this entire procedure aided in the interpretation of the resulting kinetic data. Furthermore, the relevant reaction parameters can be tested, *e.g.*, enzyme/substrate ratio, incubation time, simply by programming the 9090 autosampler for overnight runs thus allowing for "next-day" optimization. Several other researchers have successfully automated the precolumn derivatization procedure primarily for hydrolysates using PITC [14–17], 9-fluorenynmethyl chloroformate (FMOC-Cl) [18–23], *o*-phthaldehyde (OPA) [22,23] and 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) [24].

EXPERIMENTAL

Chemicals and reagents

CPY (lot No. 851101092) and APM (lot No. 840629002) were Pierce (Rockford, IL, U.S.A.) products and Boehringer Mannheim (Indianapolis, IN, U.S.A.) was the supplier for CPA (lot No. 11201822-22) and CPP (lot No. 10109521-01). Peptides and proteins were purchased from Sigma (St. Louis, MO, U.S.A.) or Peninsula Labs. (Belmont, CA, U.S.A.) and used without further purification. Radio-labeled [^{3}H]-proline (30.8 Ci/mmol) and [^{3}H]leucine (153 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). The sources of HPLC columns and solvents, amino acid reagents, water purification and additional materials have been described [25,26].

HPLC instrumentation and chromatography

Chromatographic hardware included an Applied Biosystems (Foster City, CA, U.S.A.) 130A separation system (254-nm amino acid analysis) and a Varian (Walnut

Creek, CA, U.S.A.) 9090 autosampler. Data were acquired and analyzed as previously described [25,26]. Radioactivity was determined using a Beckman (Fullerton, CA, U.S.A.) LS7000 scintillation counter.

The solvent program used to effect separation of the phenylthiocarbamyl (PTC) amino acids was a two-step gradient of 7 to 30% B in 10 min followed by 30 to 58% B in the next 10 min at a flow-rate of 0.3 ml/min and column temperature of 37°C with mobile phases 50 m*M* sodium acetate, pH 5.4 (A) and acetonitrile–water (70:30, v/v) (B).

Exopeptidase digestions

For each digest one of the following exopeptidase solutions was prepared: CPA (1.0 μg/μl in 0.025 *M* ammonium bicarbonate, pH 7.8), CPP (0.01 units/μl in 0.1 *M* acetic acid, pH 4.0), CPY (0.1 μg/μl in 0.025 *M* ammonium acetate, pH 6.0) or APM (0.01 μg/μl in 0.1 *M* sodium phosphate, pH 7.0). The reaction was automated using the Varian 9090 autosampler as outlined in Fig. 1B and Table I. Manual digestion reactions were performed under identical conditions to that of the automated procedure. The reaction was started by adding 10 μl of the exopeptidase solution to 90 μl peptide/protein (5–15 μg) solution (same buffer as exopeptidase). Aliquots of 5 μl of the reaction mixture were removed at seven time points (3.5, 6.5, 10.5, 16.5, 25.5, 42.5 and 74.5 min) over 1.5 h. Reactions were quenched by adding 2 μl 1 *M* HCl (CPA, CPY or APM) or 1 *M* NaOH (CPP). The resulting solutions were then subjected to precolumn PITC derivatization and amino acid analysis as described below.

Amino acid analysis

Samples containing norleucine as internal standard were acid hydrolyzed in the vapor phase and derivatized with PITC with slight modifications [25,26] of established procedures [11,27,28]. To a 5–10-μl sample of hydrolysate or exopeptidase digest aliquot was added 40 μl methanol–triethylamine (TEA) (8:1, v/v) followed by 5 μl PITC and incubated for 20 min at room temperature. Excess PITC was extracted with 2 × 15 μl of heptane [13] and the aqueous phase containing the PTC amino acids was diluted with 2 × 40 μl mobile phase A–acetic acid (100:3, v/v) prior to injection (Fig. 1A and Table I).

RESULTS AND DISCUSSION

Automated PITC derivatization of acid hydrolysates

Reagent addition, mixing and extraction with the automix feature of the Varian 9090 autosampler permits the amino acid derivatization steps to be automated with no increase in total analysis time. Fig. 1A and Table I present a schematic of the various automix steps and the autosampler programming, respectively, for amino acid analysis. The derivatization procedure follows standard PITC precolumn reaction conditions as outlined in earlier publications [11,27,28] with two modifications. First, there is no provision to dry the sample to remove excess PITC. Heptane has been reported to efficiently extract excess PITC in a manual procedure [13] thus generating a two-phase system. Analogous procedures have been successfully automated for FMOC-Cl precolumn derivatization to remove excess reagent and by-products [18–21]. In our system, the heptane/PITC extract separates to the top layer after

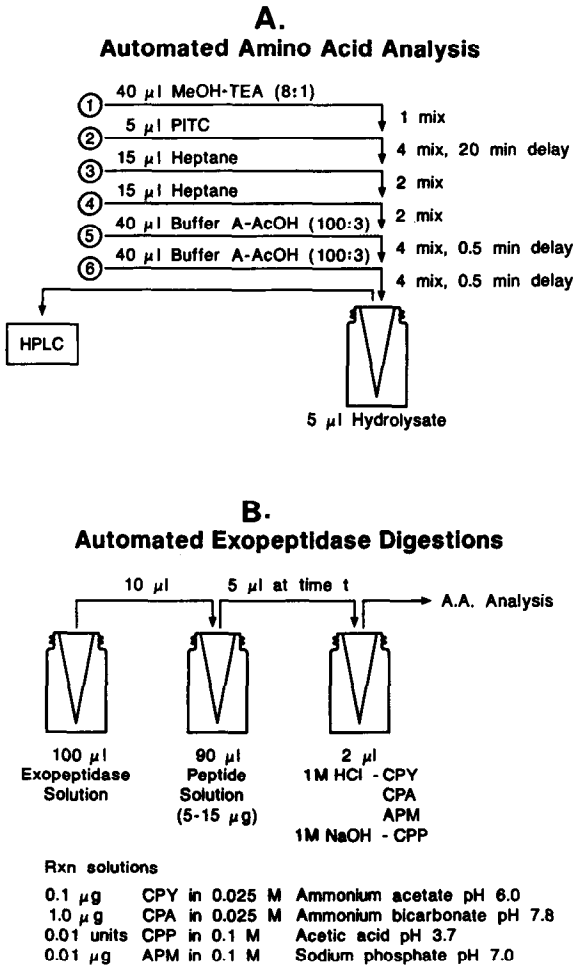

Fig. 1. Schematic layout of the automated derivatization and digestion procedure using the Varian 9090 autosampler. (A) Automated amino acid analysis; (B) automated exopeptidase digestions. See Table I for autosampler programming. MeOH = Methanol; TEA = triethylamine; AcOH = acetic acid; A.A. = amino acid; Rxn = reaction.

thorough mixing with the aqueous solution. This is fortunate since the injection needle adds or removes sample only from the bottom of the vial and thus the PITC in the organic phase will not be injected onto the column. The second modification to the standard PITC reaction conditions required the partial neutralization of TEA and dilution of methanol (Fig. 1A and Table I) which are normally removed from the reaction mixture *in vacuo* [11,27,28]. Failure to dilute these reaction constituents resulted in aberrant chromatography, particularly in the first half of the chromatogram (data not shown). The addition of mobile phase A–acetic acid (Fig. 1A and Table I) completely eliminated this problem and produced an acceptable chromatogram (Fig. 2A). The relative elution order for the PTC amino acids (Fig. 2A) is in

TABLE I

PROGRAMMING THE VARIAN 9090 AUTOSAMPLER

Sequence line	Method	Position	Next sampling time (min)	Automix
Exopeptidase digestion[a]				
1	11	2–1	1.0	D
2	12	1–2	1.0	E
3	13	1–3	2.0	E
4	14	1–4	4.0	E
5	15	1–5	7.0	E
6	16	1–6	15.0	E
7	17	1–7	30.0	E
8	18	1–8	–	E
Amino acid analysis				
9	1	1–1[b]/1–8	9.0	FABBCC
10	0			

	Automix	Position	Delay time (min)	Transfer volume (μl)	Mix cycles
Automix routines[c]					
PITC	A	3–1	20.0	5	4
Heptane	B	3–2	–	15	2
Buffer A–acetic acid (100:3)	C	3–3	0.5	40	4
Exopeptidase	D	2–2	–	10	2
Reaction vial	E	2–1	–	5	0
Methanol–triethylamine (8:1)	F	2–5	–	40	1

[a] The ABI 130 was "double-programmed" so that at the start of the exopeptidase reaction the HPLC was subjected to a 90-min wash cycle. After this mock run, the HPLC system was then ready to accept the first sample (standard H control). Every 43 min thereafter, an exopeptidase aliquot was injected until all seven time points were analyzed. Thus, the total time required to analyze the automated exopeptidase reaction including subsequent PTC-amino acid analysis was about 7 h.

[b] Position 1–1 contains standard H and no enzyme is added to the vial.

[c] The configuration set-up for the autosampler was: tube volume = 0 μl; wash cycle volume = 40 μl; viscosity factor = 1; and air displacement by the syringe was used to mix the liquids.

agreement with previous data [11,27,28] and up to 90 μl out of the 130 μl in the aqueous phase can be chromatographed without a serious loss of resolution.

It was important to demonstrate that the PTC-amino acids were quantitatively recovered in the aqueous phase. To this end, [^{3}H]leucine and [^{3}H]proline were individually added to a standard H mixture and derivatized by the automated procedure. The autosampler was programmed for a 0-μl injection so that the two layers could be independently analyzed for radioactivity. Table II shows that more than 98% of the ^{3}H derivatives was recovered in the aqueous phase. Furthermore, these [^{3}H] PTC-amino acids were found to coelute with their corresponding unlabelled counterparts (data not shown).

Prior to the exopeptidase experiments, it was necessary to investigate the

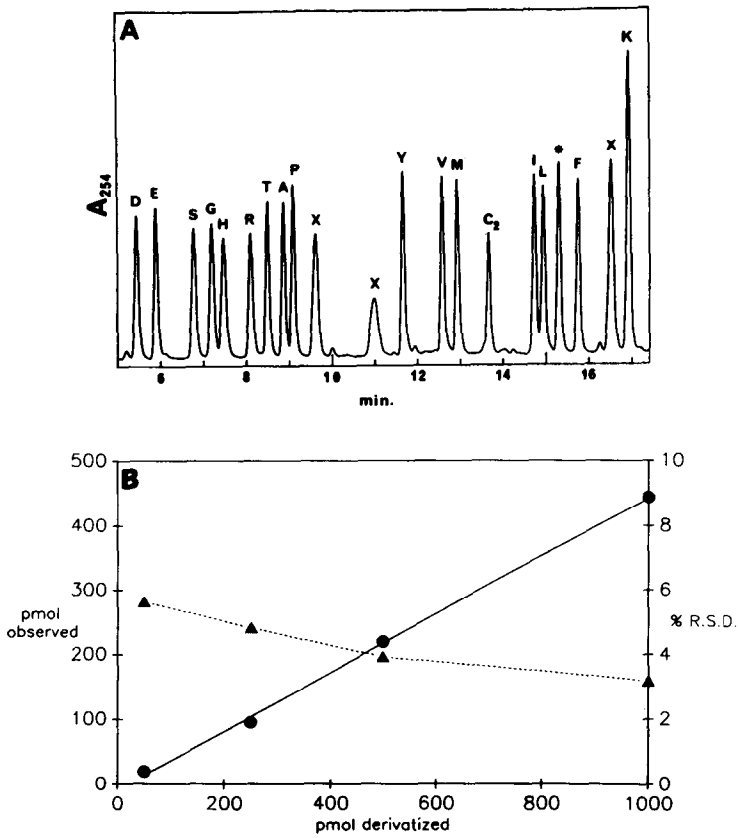

Fig. 2. Chromatogram of PTC-amino acids, and injection linearity and percent relative standard deviation (% R.S.D.) for the automated precolumn PITC derivatization procedure. (A) Chromatogram of PTC-amino acids (Pierce standard H) derivatized at 250 pmol/amino acid and injected at 96 pmol/amino acid monitored at 254 nm (200 mV full scale). The standard single-letter code for the amino acids is used; C_2 = cystine; * = norleucine; X = reagent-associated peaks. (B) Injection linearity and % R.S.D. for four different standard H concentrations (pmol derivatized) of 50, 250, 500 and 1000 pmol/amino acid. Twelve individual analyses were made at each concentration and both the average observed pmol/amino acid and % R.S.D. were then calculated. ●—● = Average pmol/amino acid observed; ▲– – –▲ = % R.S.D. for each standard H concentration.

reproducibility of the automated precolumn PITC derivatization procedure and subsequent chromatography and data quantitation. Twelve separate standard H samples each, at four decreasing concentrations (1000, 500, 250 and 50 pmol/amino acid) were subjected to this procedure. Fig. 2B (filled circles) shows the mean result for all 16 amino acids at each of the four concentrations. All four averaged concentrations fall on the expected line when exactly 50 μl of the aqueous layer is injected which also indicates that the 50-μl loop used in the 9090 autosampler was completely filled. Fig. 2B (filled triangles) also shows the percent relative standard deviation (% R.S.D.) for each of the four concentrations. There was a slightly greater variation from run to run when less material was taken for analysis, 5.63% R.S.D. (50 pmol) *vs.* 3.18%

TABLE II

RECOVERY OF TWO PTC-AMINO ACIDS AFTER HEPTANE EXTRACTION

Extraction with heptane (and radioactivity analysis of each phase) as described in Experimental and as illustrated in Fig. 1A and Table I. Five independent samples (100 000 cpm) were analyzed and the average % cpm for each phase calculated, with the range of all five determinations shown in parentheses for the aqueous phase.

	% cpm	
	Aqueous	Organic
[^{3}H]Leucine	98.8 (97.3–99.6)	1.2
[^{3}H]Proline	98.9 (98.5–99.6)	1.1

R.S.D. (1000 pmol). These values are in the range reported for other automated precolumn derivatization procedures [17,19–23]. The % R.S.D. for each of the individual 16 amino acids were determined at each concentration and two of these (50 pmol/amino acid and 1000 pmol/amino acid) are shown in Table III. Not

TABLE III

PERCENT RELATIVE STANDARD DEVIATION (% R.S.D.) FOR 50 AND 1000 PMOL DERIVATIZED STANDARDS

Twelve separate standard H samples at the indicated concentration were derivatized as described in Experimental and as illustrated in Fig. 1A and Table I. The standard single-letter code for the amino acids is used and the % R.S.D. was calculated for an individual amino acid at a given concentration from these twelve analyses. See also Fig. 2B (filled triangles) for a global average % R.S.D. for other standard H concentrations.

Amino acid	% R.S.D.	
	50 pmol	1000 pmol
D	5.32	2.58
E	4.27	2.28
S	5.05	2.97
G	5.09	2.91
H	4.35	3.43
R	5.50	2.83
T	5.25	3.44
A	5.26	2.67
P	5.51	2.91
Y	5.02	3.95
V	5.36	3.58
M	5.16	2.90
I	6.20	3.47
L	7.98	3.09
F	6.15	3.13
K	8.61	4.66
Average	5.63	3.18

surprisingly, the % R.S.D. for a given amino acid at the 50-pmol level was greater than at 1000-pmol level. The lysine % R.S.D. was noticeably higher for both, which may be due to incomplete reaction at the two potential reactant sites or a recovery problem in the heptane extraction step. The leucine % R.S.D. at the 50-pmol level was appreciably higher than the average. It is unlikely however, that this is the result of the heptane extraction step (Table I).

Peptides and proteins were acid hydrolyzed and the resulting free amino acids derivatized by the automated procedure. Table IV shows the amino acid analysis for three peptides, Leu-enkephalin, bradykinin and angiotensin I and three proteins, myoglobin, concanavalin A and lysozyme. No major diferences between the observed

TABLE IV

AUTOMATED PTC-AMINO ACID ANALYSIS OF PEPTIDE AND PROTEIN ACID HYDROLYSATES

Samples were hydrolyzed as described in Experimental and derivatized as described in the legend to Table III. The amount of sample hydrolyzed was 0.9 μg Leu-enkephalin; 2.0 μg bradykinin; 2.1 μg angiotensin I; 2.7 μg myoglobin; 4.2 μg concanavalin A; and 2.3 μg lysozyme. The standard single-letter code for the amino acids is used and the values corresponding to the observed number of residues are given in parentheses. C and W were not determined. The peptide hydrolysates were diluted/injected such that 1 residue corresponds to about 250 pmol and for the proteins, 1 residue corresponds to about 25 pmol.

Leu-enkephalin			Bradykinin			Angiotensin I		
G	2.0	(2)	S	0.88	(1)	D	1.1	(1)
Y	1.0	(1)	G	0.98	(1)	H	2.1	(2)
L	0.98	(1)	R	2.2	(2)	R	1.1	(1)
F	0.99	(1)	P	2.9	(3)	P	0.97	(1)
			F	2.0	(2)	Y	0.90	(1)
						V	0.99	(1)
						I	0.94	(1)
						L	1.0	(1)
						F	1.0	(1)

Myoglobin			Concanavalin A		Lysozyme	
D/N	10.5	(10)	31.1	(32)	22.1	(21)
E/Q	19.6	(19)	12.4	(12)	5.6	(5)
S	4.8	(5)	28.0	(31)	8.6	(10)
G	14.9	(15)	16.1	(16)	11.4	(12)
H	10.9	(11)	6.1	(6)	1.1	(1)
R	2.3	(2)	6.5	(6)	11.7	(12)
T	6.6	(7)	17.5	(19)	6.5	(7)
A	15.3	(15)	18.4	(19)	11.9	(12)
P	4.3	(4)	10.3	(11)	2.0	(2)
Y	1.8	(2)	6.6	(7)	2.7	(3)
V	6.8	(7)	16.2	(16)	5.9	(6)
M	2.3	(2)	1.8	(2)	2.2	(2)
I	7.6	(9)	13.2	(15)	5.5	(6)
L	16.6	(17)	18.8	(18)	8.0	(8)
F	6.8	(7)	10.7	(11)	3.1	(3)
K	17.5	(19)	12.2	(12)	6.0	(6)

and expected values were found for any of the six samples. The 9090 autosampler was able to perform all the necessary manipulations for the analysis of acid hydrolysates except for the hydrolysis step [14,17].

Automated exopeptidase digestions

Automated exopeptidase digestions were performed using the automix feature of the Varian 9090 autosampler. Fig. 1B and Table I present a schematic of the automix steps prior to amino acid analysis and the autosampler programming, respectively. The 9090 transfers 10 μl of the exopeptidase solution (CPA, CPP, CPY or APM) to another vial containing the substrate to start the reaction. Samples (5 μl) are then removed at seven different time points and the reaction was quenched with 2 μl 1 *M* HCl (CPA, CPY or APM) or 2 μl 1 *M* NaOH (CPP). Programming steps for the 9090 autosampler are sequential so that the next sampling time (min) of 1.0, 1.0, 2.0, 4.0, 7.0, 15.0 and 30.0 (Table I) translates to 3.5, 6.5, 10.5, 16.5, 25.5, 42.5 and 74.5-min aliquots in real time. A feature of the 9090 autosampler is that transfers must be directed to a single target vial and at the end of all transfers an injection must occur. For the exopeptidase digests, the reaction is quenched by transferring aliquots from the reaction vial to a third vial containing the quenching agent. We defeated the injection command, which would have resulted in sample loss, by programming 0-μl injections after the addition of exopeptidase to the substrate and after the reaction aliquot was transferred to the quenching solution. Each timed aliquot remained in the presence of acid or base until all seven were sampled after which, PITC derivatization proceeded as above. Since each aliquot was derivatized and then immediately chromatographed, the stability of the PTC derivatives was not a concern [11,12].

Examples of several peptide digests are presented to demonstrate the utility of the 9090 autosampler for exopeptidase digests (Figs. 3 and 4). All digests were designed so that the release of one amino acid would be equivalent to 100–400 pmol. Fig. 3A shows a typical CPY digestion of angiotensin I. From these kinetic data, one would predict a C-terminal sequence of F–H–L–COOH, in perfect agreement with this peptide's known sequence. In this example all three amino acids were cleaved at a steady rate resulting in simple interpretation of the kinetic data. Often, C-terminal predictions become more difficult as the number of liberated residues increases. The CPY digestion of renin substrate tetradecapeptide (Fig. 3B) provides such an example by showing the rapid cleavage for the first five C-terminal amino acids. S, Y, V and two residues of L all appear to be cleaved almost simultaneously from the peptide. Careful inspection of the first 20 min of the digestion profile indicated that S is the first amino acid cleaved (data not shown). After S however, no additional sequence could be unambiguously assigned. Decreasing the CPY concentration did not improve this situation.

Fig. 3 C–F shows more examples of peptide digests using other carboxypeptidases. CPA digested two amino acids (I–V–COOH) for gonadotropin releasing hormone (14–26) as illustrated in Fig. 3C and two amino acids (F–M–COOH) for Met-enkephalin (Fig. 3D). In both cases, further digestion was impeded when the enzyme encountered an unfavorable residue [5]. Examples for CPP digestion are shown in Fig. 3E for angiotensin I (F–H–L–COOH) and Fig. 3F for dynorphin A (1–6) (F–L–R–COOH). Again, the rate of cleavage was affected by specific residues [7]. For all the examples presented in Fig. 3 C–F, increasing the enzyme concentration did not materially change the results.

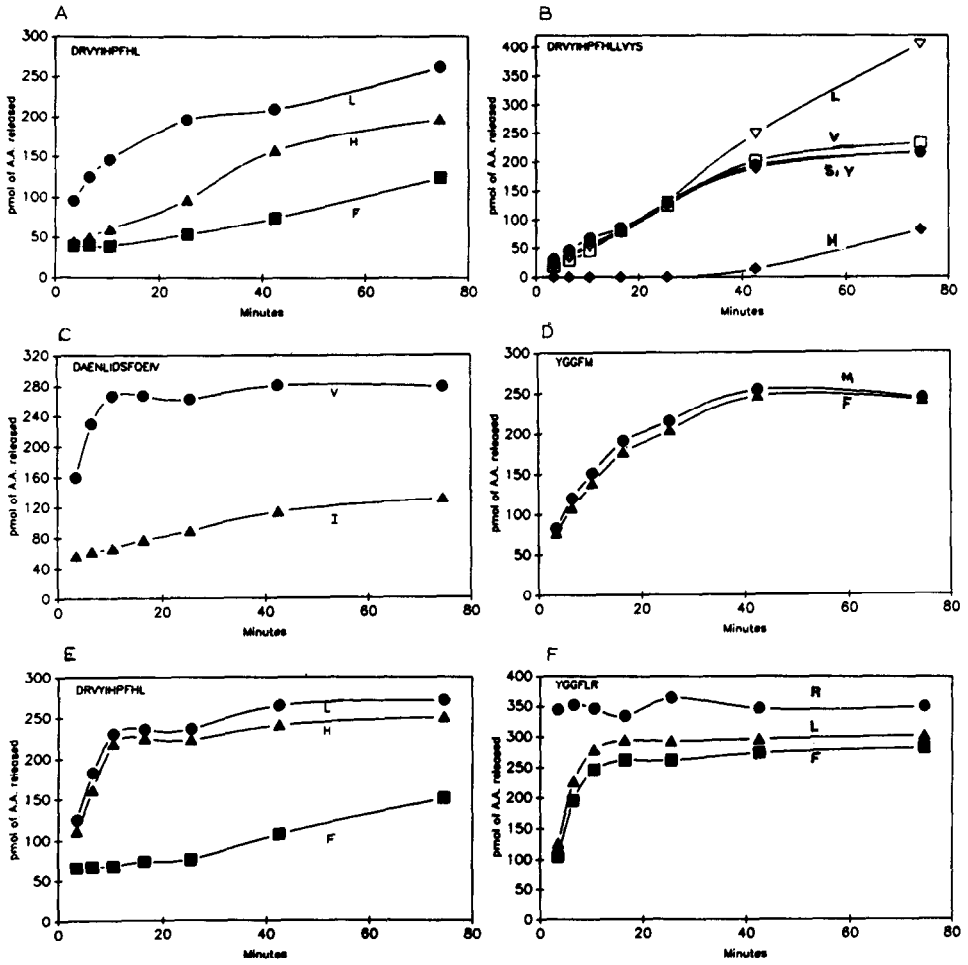

Fig. 3. Kinetic analysis of released amino acids from automated carboxypeptidase digestion of six peptides. (A) Angiotensin I (CPY); (B) renin substrate tetradecapeptide (CPY); (C) human pre-pro-gonadotropin releasing hormone (14–26) (CPA); (D) Met-enkephalin (CPA); (E) angiotensin I (CPP); (F) dynorphin A (1–6) (CPP). The order of released amino acids is indicated by the following symbols: ● = 1st; ▲ = 2nd; ■ = 3r; ▼ = 4th; ◆ = 5th. Filled symbols indicate a positive sequence assignment while open symbols indicate that this residue could not be assigned in the sequence. The sequence of the peptide, using the standard single-letter code, is located in the top left corner of each plot.

N-Terminal sequencing was accomplished using APM. Fig. 4A presents the kinetic plot of an APM digest of the peptide adrenocorticotropic hormone (1–10). For this reaction, eight amino acids were released yielding a tentative prediction of NH_2–S–(S, Y)–M–(E, H, R, F). It becomes increasingly difficult to assign sequence when either multiples of a given residue are released or the total number of liberated residues exceeds about 5.

Peptides with modified amino acids, *e.g.*, Y[SO_3] and M[O] are known to quantitate poorly, if at all, after standard acid hydrolyis [29]. Exopeptidase digestions

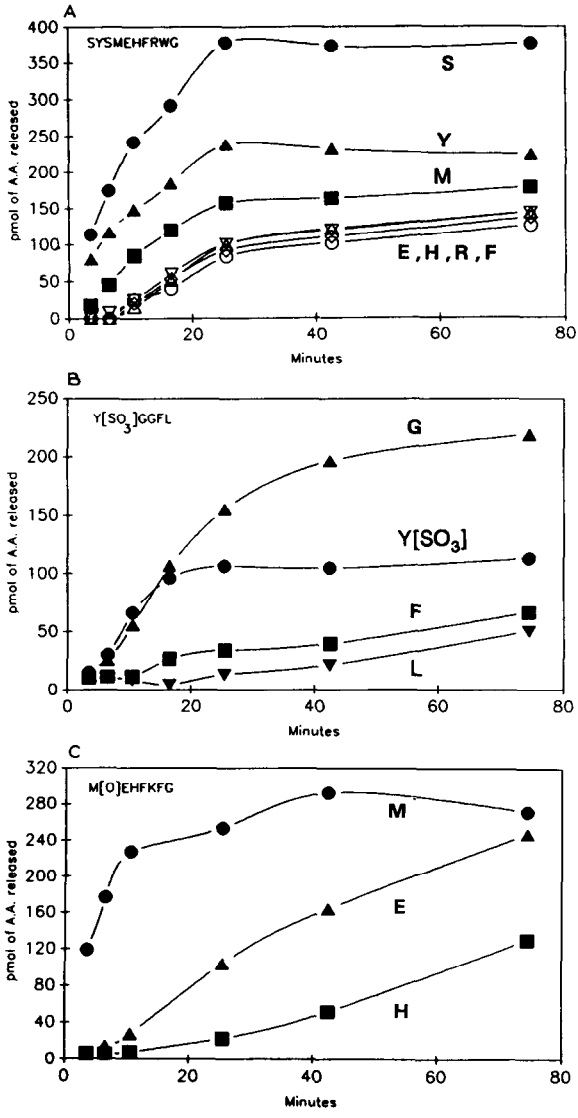

Fig. 4. Kinetic analysis of released amino acids from automated aminopeptidase digestion of three peptides. (A) Adrenocorticotropic hormone (1–10) (APM); (B) Leu-enkephalin (Y[SO_3]) (APM); (C) adrenocorticotropic hormone (4–10) (M[O]) (APM). See Fig. 3 for symbol designation.

provide an alternate means of sequencing and potentially quantitating these residues. Fig. 4B demonstrates an almost complete digest of Leu-enkephalin (Y[SO_3]) with APM yielding a predicted sequence of NH_2–Y[SO_3]–G–G–F–L. In this instance, the concentration of peptide was approximately 100 pmol. Fig. 4C shows the analysis of the modified amino acid M[O] in the peptide adrenocorticotropic hormone (4–10) (M[O]) after digestion with APM. Sequence prediction for this reaction was NH_2–M[O]–E–H.

A total of six peptides were digested with each of the four exopeptidases CPA, CPP, CPY and APM by either the fully automated procedure (both digestion and amino acid analysis) or in the totally manual mode (both digestion and amino acid analysis). Table V presents these results for the total number of released amino acids for a specific substrate–enzyme pair and the number of residues unambiguously assigned to the sequence is given in parentheses for each mode of analysis. The number of amino acids cleaved per peptide varied from 0 to 10 and the number of positive assignments ranged from 0 to 4 residues. The results derived from any automated procedure must, as a minimum, be the equal of those obtained from a manual analysis if the automated procedure is to gain acceptance. Furthermore, the automated procedure should be less labor intensive than the manual counterpart and also allow for both rapid analysis and optimization. The data listed in Table V clearly show that the automated exopeptidase procedure (97 total released residues and 36 total positive sequence assignments) is comparable to the manual procedure (89 total released residues and 33 total positive sequence assignments). We interpret this to mean that the automated procedure performs as well as the more labor intensive manual procedure.

It should be emphasized that in the course of this investigation, no attempt was made to use "ideal" substrates which may yield extended sequence information. Rather, we surveyed a broad spectrum of substrates and exopeptidases to evaluate the general utility of the automated procedure. In this regard, our preliminary experiments with automated protein–exopeptidase reactions were not very successful. Few if any residues were liberated for the six proteins tested (myoglobin, β-casein, cytochrome *c*, concanavalin A, β-lactoglobulin and lysozyme) when using the above four exopeptidases. This may be due to poorly accessible termini and future experiments will include the use of denaturants in an attempt to force the reaction. Lastly, in this initial investigation we decided to analyze samples at what we considered to be a comfortable minimum level of 50 to 100 pmol. This amount of material is in the range reported [11,27,28] for successful, routine PITC derivatization. It is fully anticipated that if

TABLE V

NUMBER OF AMINO ACIDS CLEAVED COMPARING MANUAL (M) *VERSUS* AUTOMATED (A) EXOPEPTIDASE SEQUENCING

The six peptides were digested with the four exopeptidases as described in Experimental either in the totally automated mode (both digestion and amino acid analysis) or totally manual mode (both digestion and amino acid analysis). The total number of released residues for a specific substrate/enzyme pair is listed and the values in parentheses correspond to the number of correct sequence assignments from this total number of released residues.

Peptide	CPA		CPP		CPY		APM	
	M	A	M	A	M	A	M	A
Renin substrate tetradecapeptide	8(1)	7(0)	6(1)	7(2)	7(1)	7(3)	6(0)	6(0)
Angiotensin I	3(3)	3(3)	3(3)	4(2)	3(3)	3(3)	10(0)	10(0)
Adrenocorticotropic hormone (1–10)	2(2)	4(1)	8(2)	8(1)	2(0)	2(2)	8(2)	8(1)
Dynorphin A (1–6)	0(0)	0(0)	4(2)	3(1)	3(3)	3(3)	6(2)	6(2)
Bradykinin	0(0)	0(0)	2(2)	4(4)	2(2)	3(3)	1(0)	2(0)
Gonadotropin releasing hormone	3(2)	2(2)	0(0)	0(0)	2(2)	5(3)	0(0)	0(0)

greater sensitivity is required, *e.g.*, FMOC-Cl [14–17], OPA [22,23] and Dns-Cl [24], these derivatization procedures could be incorporated into the automated exopeptidase protocol.

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