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DUAL-DETECTOR METHODS FOR SELECTIVE IDENTIFICATION OF PROLYL RESIDUES AND AMIDE-BLOCKED N-TERMINAL GROUPS IN CHROMATOGRAPHICALLY SEPARATED PEPTIDES

TIMOTHY D. SCHLABACH
Varian Instrument Group, Walnut Creek Instrument Division, Walnut Creek, CA 94598 (U.S.A.)

SUMMARY

On-line methods have been developed to screen for peptides containing proline residues or for the presence of an N-terminal blocking group such as an amide. Both methods combine UV detection with post-column reactions, producing a fluorescent product. The fluorescamine post-column reaction is used to detect peptides lacking a free amino terminus. Common blocking groups that can be detected are acetyl, formyl and internally cyclized amino acids such as pyroglutamic. Such groups prevent coupling of phenyl isothiocyanate to the peptide and prevent Edman sequence analysis. The two step, hypochlorite–*o*-phthalaldehyde post-column reaction detects the presence of proline in the peptide. Proline cannot be readily detected in peptide hydrolyzates by some popular methods of amino acid analysis. These techniques should enhance the success of sequence and composition analysis of collected peptides.

INTRODUCTION

Peptide separations by high-performance liquid chromatography (HPLC) have become routine of late[1,2], but peptide identification remains a problem for complex samples because retention time is not an adequate index to establish peptide sequence. The presence of certain, key amino acid residues can be identified from the ratio of the output of a selective detector or reaction detector to the absorbance at 215 nm. For example, because tryptophan and tyrosine fluoresce strongly, these residues are readily identified in a peptide separation by addition of a fluorescence detector in-series with a UV detector[3]. Although such techniques help confirm peptide identity, sequence information or at least composition data are required to firmly establish peptide identity. The latter are off-line techniques that work best with volatile mobile phases that are easily removed from the collected fractions.

Peptide sequencing is accomplished by the well established method of Edman degradation[4]. This technique has been fully automated and can readily sequence peptides of at least fifty residues. The method is based on the coupling of phenyl isothiocyanate to the N-terminal amino group of the peptide. If this group is blocked by an acetyl group or a cyclic pyroglutamyl group, then coupling cannot occur. To

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avoid abortive attempts at Edman degradation because of blocked peptides, on-line techniques were investigated for the rapid identification of peptides with a blocked amino terminus.

Amino acid composition data usually suffice to identify peptides from a known source, such as a tryptic digest of a larger polypeptide, produced by recombinant DNA techniques. The peptide is hydrolyzed with acid, and the hydrolyzate is analyzed by conventional amino acid analysis or pre-column derivatization methods[5,6]. Conventional amino acid analysis usually employs *o*-phthalaldehyde (OPA) in the post-column reaction because it is substantially more sensitive than ninhydrin[7]. Its only drawback is the inability to detect proline. Similarly, OPA is used to form pre-column derivatives because the derivatives fluoresce intensely and can be readily separated on conventional, reversed-phase columns[6]. Proline detection is again a problem. Proline can be detected as the nitrobenzofurazan derivative at the pmol level[8], but the nitrobenzofurazan derivatives of the primary amino acid fluoresce much more weakly than the secondary amino acid derivatives[9], making this method a supplementary technique.

An on-line method was developed to screen for peptides containing proline so that appropriate pre- or post-column derivatization methods could be chosen for off-line amino acid analysis. Two methods are described: one for detecting peptides blocked by an amide bond at the N-terminus, such as the pyroglutamyl group, and another one for detecting peptides containing proline at any position. The presence of a free α-amino group can be confirmed with a single injection by the first method. Proline residues can be distinguished by two repetitive injections with the second method. One injection is required to establish the presence of proline or lysine, and a second is required to identify peptides with proline but without lysine. Because these techniques yield results immediately after completion of the chromatographic separation, they should provide useful information for subsequent off-line analyses within an acceptable time frame.

EXPERIMENTAL

Chromatography was performed with a Varian Model 5060 ternary high-performance liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a Varian UV-100 detector. The Varian System II post-column reaction system was used along with the reaction coil heater accessory for controlling the reaction temperature. Peptides were obtained from Peninsula Labs. (San Carlos, CA, U.S.A.) and separated on a MicroPak-SP-C18 3-μm column. A 4.6-mm I.D. column was used, except where a 2-mm column is specified. Solvent A was 0.1% trifluoroacetic acid (TFA) in water, and solvent B was 80% acetonitrile in 0.1% TFA. The gradient for the 2-mm column started at 5% B and progressed to 45% B in 10 min and 60% B by 25 min at a flow-rate of 0.4 ml/min. For the 4.6-mm column, the concentration of B started at 2% and rose quickly to 20% in 5 min and then to 55% after 20 min; the flow-rate was 0.7 ml/min. All gradient segments were linear.

The post-column reagents for the fluorescamine reaction were obtained from Varian. Fluorescamine was dissolved in acetone to a concentration of *ca.* 0.3 mg/ml. The 1.0 *M* borate buffer was diluted ten-fold with distilled water. This buffer was added first at a rate of 0.2 ml/min for the 2-mm column and 0.4 ml/min for the 4.6-

mm column. The fluorescamine solution was added with the second pump at an identical flow-rate. The standard, stainless-steel reaction coil was used (30 ft. × 0.01 in. I.D.). Fluorescence was detected with excitation around 370 nm and emission collected with a 500 nm interference filter.

The post-column reagents for the hypochlorite–OPA reaction were also obtained from Varian. A 5% standard hypochlorite solution was diluted with distilled water and 1 *M* sodium hydroxide to make a 0.5% solution of hypochlorite in 0.1 *M* sodium hydroxide. The hypochlorite was added first at a flow-rate of 0.4 ml/min. The first reaction coil was the standard, stainless-steel coil. The reaction temperature was 40°C unless indicated otherwise. The OPA reagent contained 1 mg/ml OPA and 1.0% mercaptoethanol in 0.1 *M* borate buffer. This reagent was introduced second at a rate of 0.4 ml/min. The second reaction coil was 7 m × 0.3 mm I.D. PTFE tubing, also supplied by Varian. Fluorescence with the OPA reaction was detected with excitation around 350 and emission around 450 nm.

RESULTS AND DISCUSSION

Fluorescamine has been reported to be a sensitive reagent for detecting peptides and amino acids in alkaline solution[10]. The optimal pH of the fluorescamine reaction differs for each group, peptides being more reactive around pH 7 and amino acids around pH 9[11]. Fluorescamine has been reported to react with peptide amino groups at the N-terminus but not to react appreciably with the ε-amino group in lysine residues, when the reaction was carried out at pH 8.5[12]. The poor reactivity of the ε-amino group appears at odds with the broad reactivity of fluorescamine with primary amines[13].

The reactivity of two small peptides with fluorescamine was studied by separating the peptides on a C_{18} reversed-phase column and detecting them by the fluorescamine reaction at varying pH by adjusting the first buffer. The peptides glycylglycylglycine and serum thymic factor were readily separated with 10% acetonitrile in 0.1% TFA. Serum thymic factor has the amino acid sequence pEAKSQYGGSN. The one letter abbreviations for the amino acids can be found in Lehninger[14]. The pE designation is of particular importance and refers to pyroglutamic acid. The injected sample contained *ca.* 5 μg of each peptide. The peptides were detected by a UV detector as they left the column. The alkaline buffer was added next, followed by the fluorescamine reagent. The ratio of the fluorescence (F) to absorbance (A) response

TABLE I

EFFECT OF pH ON DETECTION OF PEPTIDES BY FLUORESCAMINE

Reagent pH	*Peptide*	*Fluorescence (mV)*	*Absorption, 210 nm (a.u.f.s.)*	*Fluorescence/absorption (mV)/(a.u.f.s.)*
10.4	GGG	127	0.170	747
10.4	pEAKSQGGSN	25.8	0.143	180
9.4	GGG	131	0.146	897
9.4	pEAKSQGGSN	16	0.143	112
8.4	GGG	129	0.129	1000
8.4	pEAKSQGGSN	3.6	0.187	19

for these peptides is shown in Table I. The F/A ratio for glycylglycylglycine is *ca.* 1.3 times greater at the lower pH, whereas the ratio decreased about ten-fold for serum thymic factor. A likely explanation is that at lower pH the protonated ε-amino group of serum thymic factor reacts poorly with fluorescamine.

Serum thymic factor was injected with several other peptides to produce the chromatogram shown in Fig. 1. Peptides were readily discernable with UV detection in the upper profile and by reaction with fluorescamine buffered to pH 9 in the lower profile. Both serum thymic factor and ranatensin (pEVPQWAVGHFM) have their N-terminus blocked by pyroglutamic acid, but only the former produces a response with fluorescamine. Presumably fluorescamine reacts with the lysine amino group at the third residue.

A similar peptide separation is shown in Fig. 2. The melanotropin (MSH)-releasing inhibitor factor peptide (PLG) is blocked by an N-terminal proline and xenopsin (pEGKRPWIL) by the pyroglutamyl group. Neither group reacts with fluorescamine, but xenopsin is observed in the fluorescamine profile by virtue of its lysine residue. The internal lysine group in xenopsin has a free primary amino group,

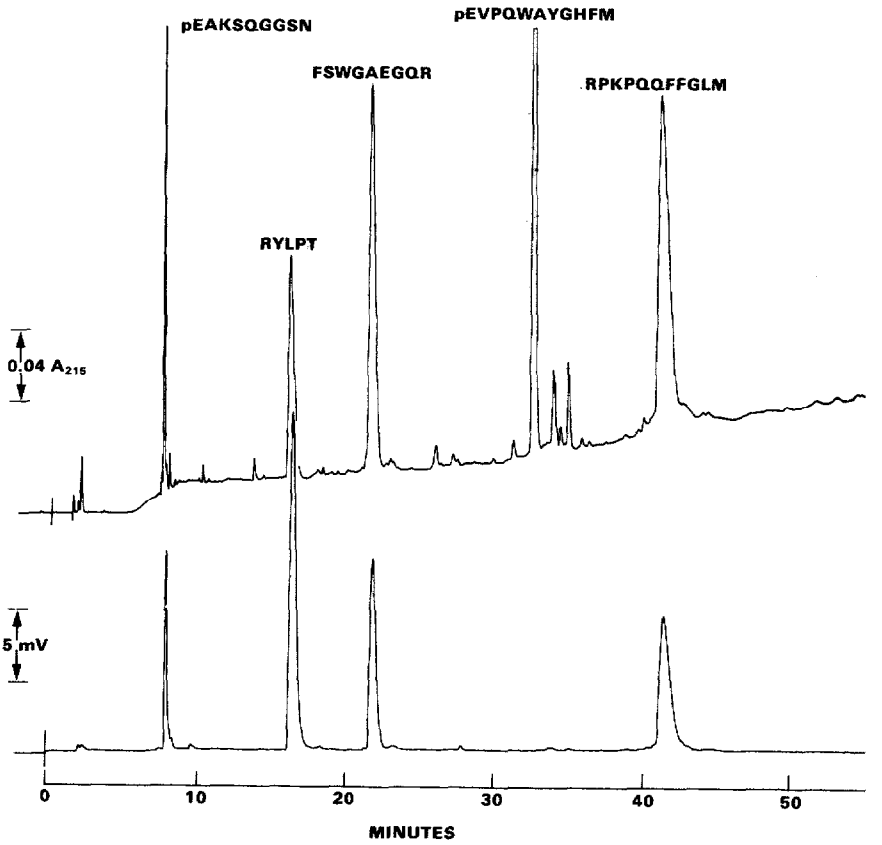

Fig. 1. Combined absorbance and fluorescamine reaction–detection of chromatographically separated peptides. Only ranatensin (pEVPQWAVGHFM) is not detected with the fluorescamine reaction shown in the lower tracing. Note that the amino terminus is blocked by pyroglutamic acid (pE).

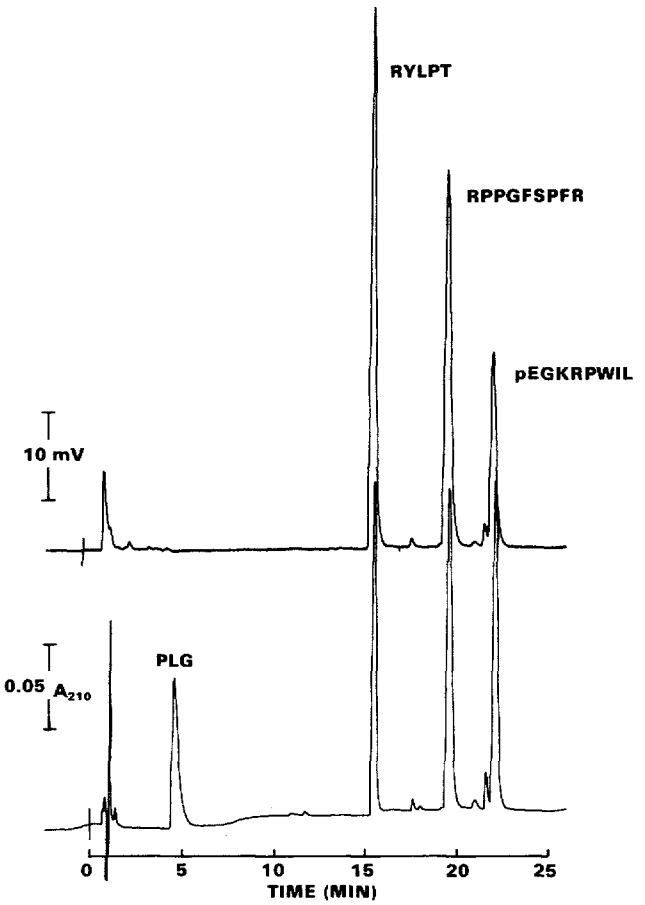

Fig. 2. Dual detection of peptides with UV and fluorescamine reaction. Peptides were separated on a 2-mm I.D. column. Only MSH-releasing inhibitor factor (PLG) with proline at the amino terminus is not observed in the upper fluorescence profile.

which apparently does react with fluorescamine when the pH is 9.0. The MSH releasing inhibitor factor peptide goes undetected because it lacks a primary amino group. In the absence of fluorescamine, no fluorescent peaks were observed in either chromatogram, because the excitation and emission wavelengths are too distant for those used for native fluorescence which requires excitation at 280 nm or below with emission collected around 350 nm or less[15].

The response of the fluorescamine reaction to internal lysine residues can be substantially reduced by lowering the reaction pH. At pH 10.4 both serum thymic factor and xenopsin can be readily detected with the fluorescamine reaction, as seen in Fig. 3. Both blocked peptides have an internal lysine residue which reacts with fluorescamine, as seen in the previous chromatograms. Reducing the reaction pH to 8.4 greatly reduces the reactivity of these two peptides with fluorescamine, as shown in Fig. 4. Lowering of the pH reduced the F/A ratio about five-fold for xenopsin and nearly ten-fold for serum thymic factor.

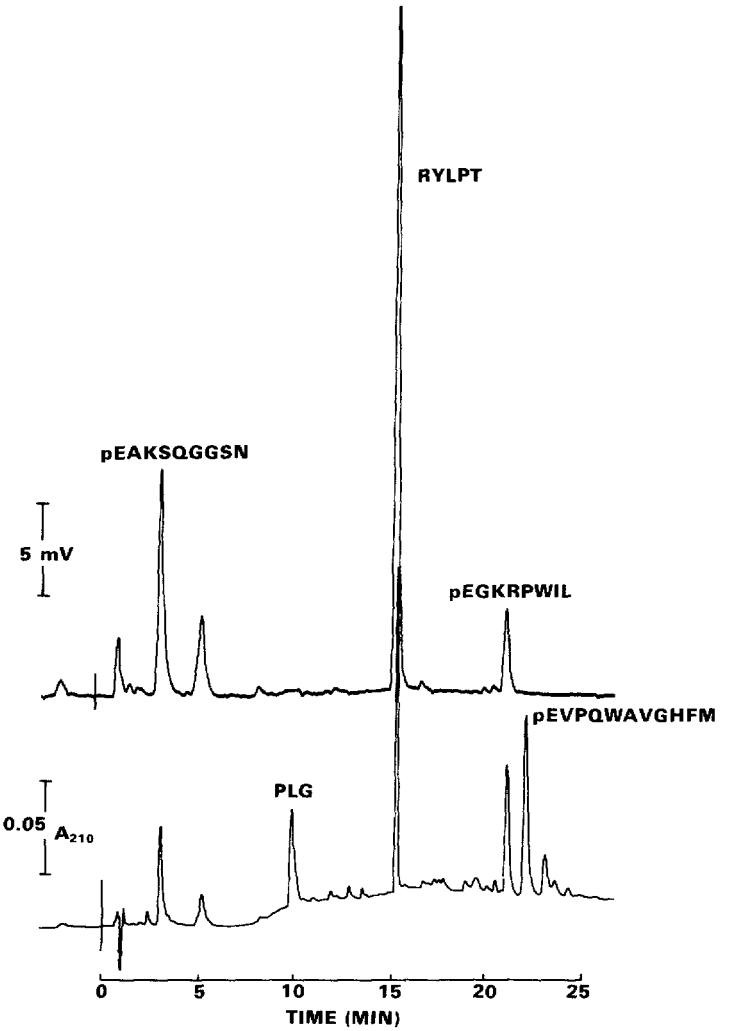

Fig. 3. Peptide reactivity with the fluorescamine reaction at pH 10.4. A 2-mm I.D. column was used, as in Fig. 2. Neither ranatensin nor MSH-releasing inhibitor factor are detected with the fluorescamine reaction. Note that xenopsin (pEGKRPWIL) and serum thymic factor (pEAKSQGGSN) have pyroglutamic acid at their amino terminus, as does ranatensin, but that the former peptides also have an internal lysine group (K).

Combined absorbance and fluorescamine reaction detection can be used for the selective identification of peptides blocked at the N-terminus when the pH is lowered to 8.4. This observation was substantiated by Nakai *et al.*[12], who reported that the ε-amino group contributed insignificantly to the fluorescence yield of peptides reacting with fluorescamine at pH 8.5. Peptides that are blocked by pyroglutamic acid at the N-terminus can be treated with pyroglutamate peptidase to remove this group from the peptide for sequencing. Proline does not block isothiocyanate coupling, and such peptides can be directly sequenced. Unfortunately, the fluores-

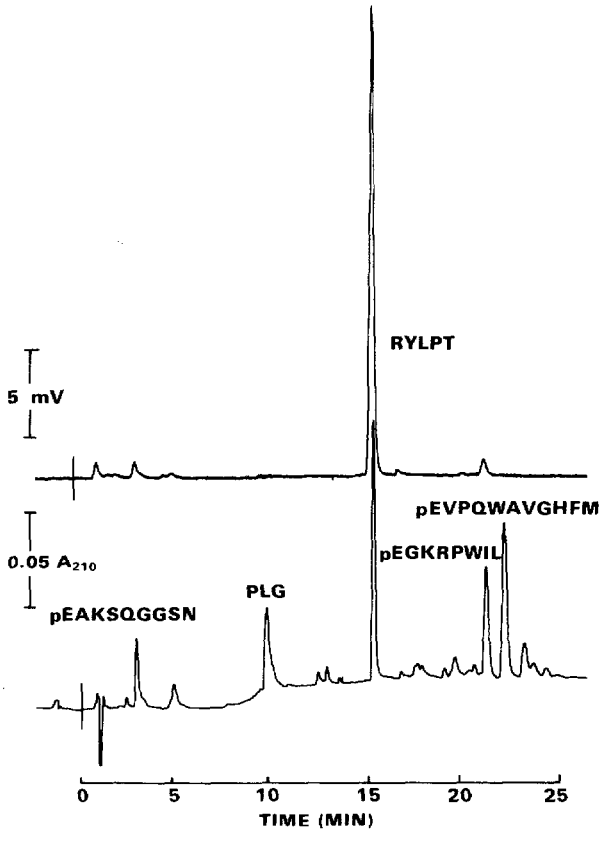

Fig. 4. Peptide reactivity with the fluorescamine reaction at pH 8.4. Other conditions are as in Fig. 3.

camine technique does not distinguish between these two N-terminal residues. On-line proline detection requires a different method, described below.

Proline can be detected by its reaction with OPA or fluorescamine after oxidative decarboxylation by reaction with an active chlorine agent. Hypochlorite is used in conjunction with OPA[16] and chlorosuccinimide with fluorescamine[17]. Because the former is a two-step reaction, whereas the latter is a three-step reaction, the OPA method was investigated for detecting prolyl groups in peptides. Unlike fluorescamine, OPA does not readily produce a strong fluorophore with the α-amino group in peptides. OPA does, however, form a strongly fluorescent product with lysine residues[3].

In the two-step OPA method hypochlorite is added to the column effluent followed by the OPA reagent. Like the fluorescamine method, two reagent pumps are required, and the UV detector preceeds the post-column reaction system. To accelerate the opening of the proline ring by hypochlorite, the first reaction coil is heated to 40°C or higher. The liberated amino group then reacts with the OPA reagent which is introduced by a separate pump.

The peptide chromatograms shown in Fig. 5 were obtained with UV detection and the dual-reagent OPA method. All peptides observed at 215 nm were also present

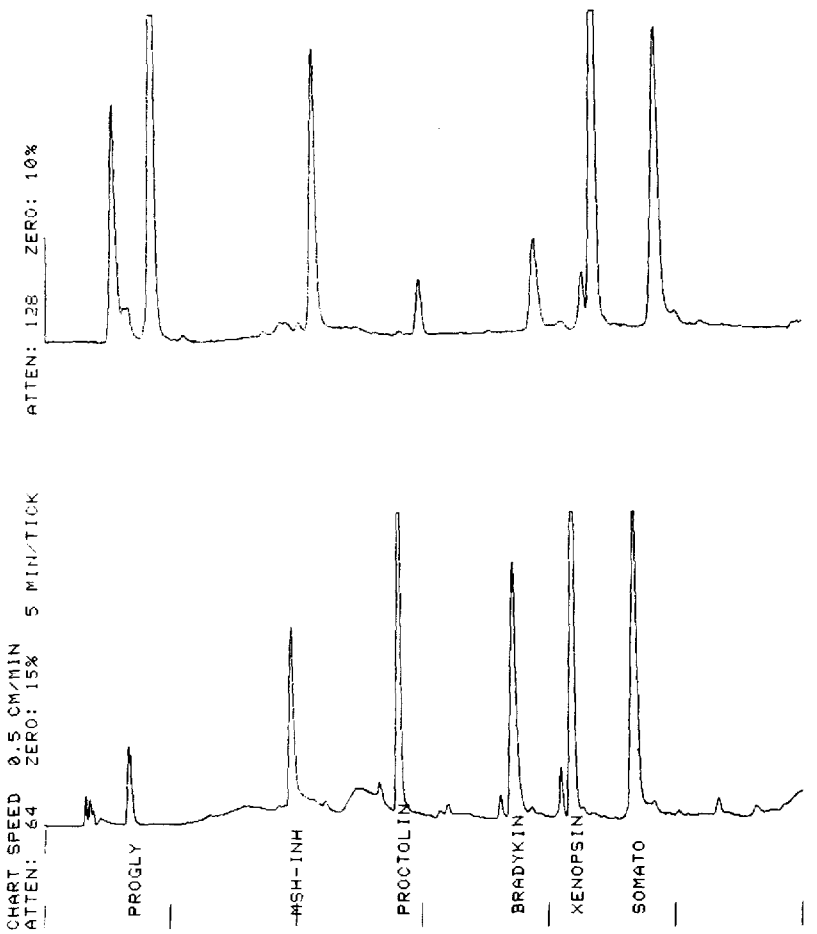

Fig. 5. Peptide detection with the hypochlorite–OPA post-column reaction system. All peptides are observed in both the lower tracing (absorbance at 215 nm, 0.128 absorbance units full scale) and in the upper (fluorescence) profile, 256 mV full scale. Proctolin (RYLPT) and bradykinin (RPPGFSPFR) are the smaller peaks in the fluorescence profile. Xenopsin and somatostatin (AGCKNFWKTFTSC) peaks are much larger; both have lysine (K) residues.

in the OPA reaction chromatogram in the upper profile. The offset between peak retention times in the upper and lower chromatograms represents a residence time of *ca.* 50 sec for the peak in the post-column reaction system. All four peptides containing proline but no lysine are apparent in the fluorescence profile; however, only prolylglycine produced strong fluorescence. Xenopsin contains both proline and lysine and exhibits a very prominent, fluorescence peak as does somatostatin, which contains lysine but no proline. The strong fluorescence peak at the beginning of the chromatogram corresponds to the minor UV peaks that appeared in the void volume and are probably free amino acids that are not retained.

Replacing hypochlorite with water but continuing to add OPA with the second reagent pump makes the post-column reaction selective for only those peptides con-

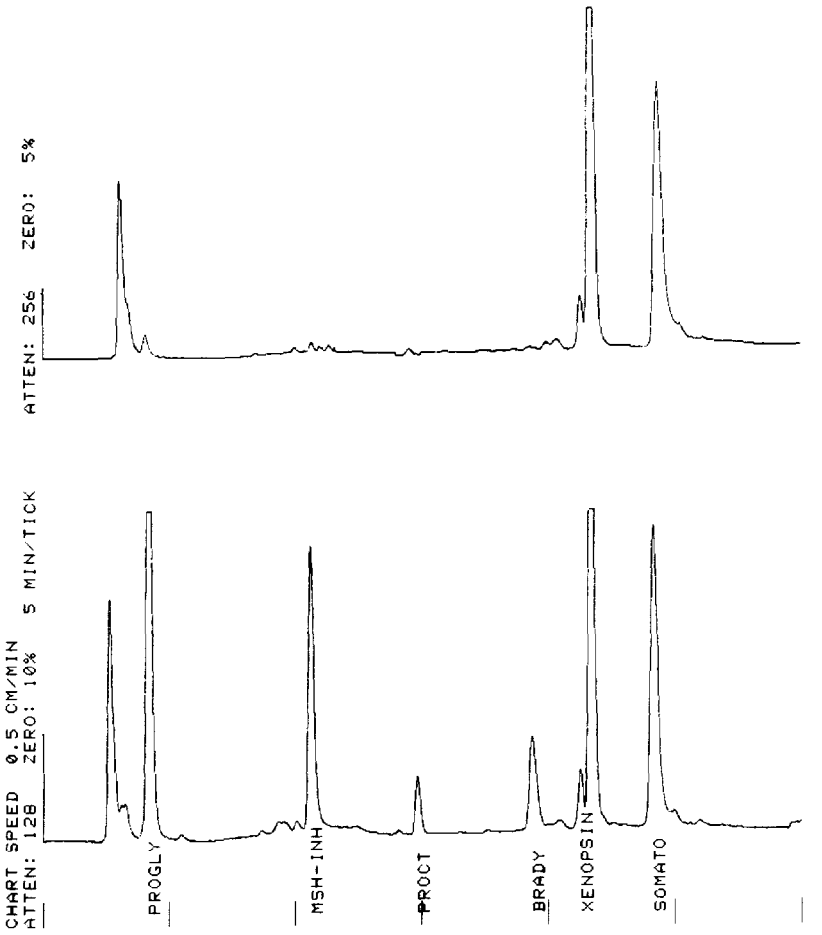

Fig. 6. Comparison of peptide detection with an OPA post-column reaction and a combined hypochlorite–OPA reaction. The lower, OPA/hypochlorite chromatogram is the same as shown in Fig. 5. Only xenopsin and somatostatin are evident in the upper (OPA) chromatogram, where a full scale response is 256 mV or twice that in the lower profile. Note that only xenopsin and somatostatin have lysine.

taining lysine[3]. Peptide detection with this reaction system is directly compared to the dual reagent system with hypochlorite in Fig. 6. The four peptides containing only proline are not observed when hypochlorite is omitted from the reaction system. Both systems respond to peptides with either two lysine residues (somatostatin) or with both a lysine and proline residue (xenopsin). Hypochlorite did, however, reduce the xenopsin peak by 54% and the somatostatin peak by 55%. This effect has also been observed in the detection of amino acids with OPA when hypochlorite is added to detect proline. The hypochlorite lowers the response of the primary amino acids (18), probably by converting them into chloramines which no longer react with OPA.

Eliminating the lysine response would make the reaction system specific for proline residues. It has been reported[18] and confirmed in this laboratory that an increase in the temperature of the hypochlorite reaction system nearly eliminates the

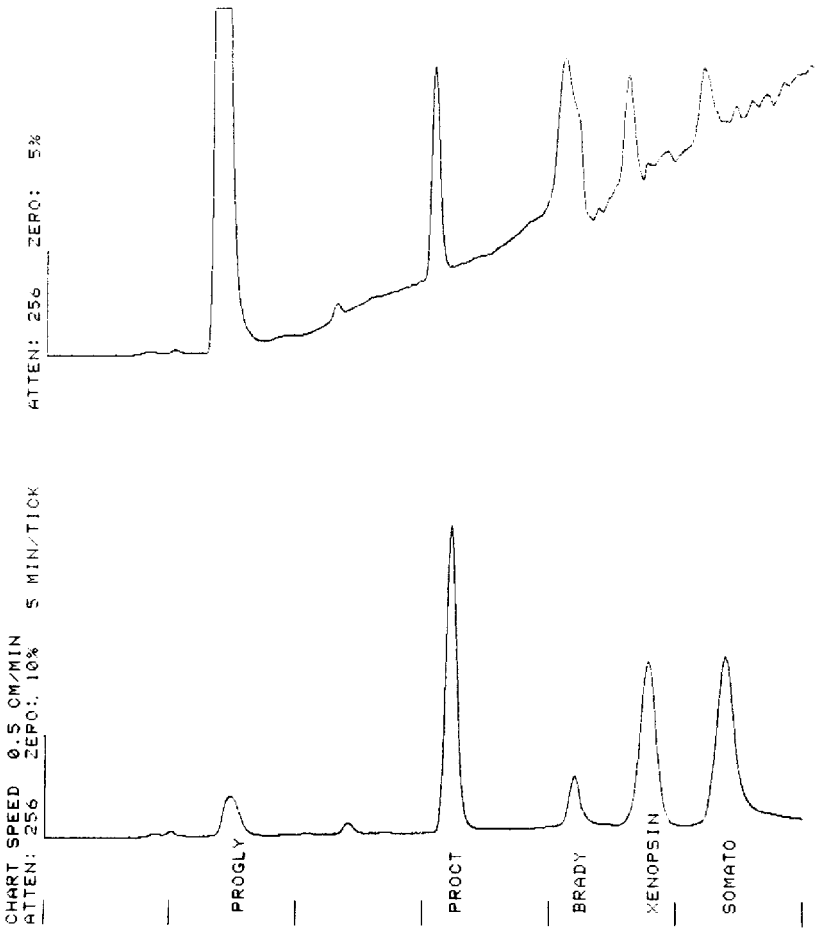

Fig. 7. Effect of temperature on proline and lysine reactivity in the hypochlorite–OPA, post-column reaction system. The lower chromatogram was obtained at a reaction temperature of 40°C and the upper at 60°C. Full scale was 512 mV in both traces. All peptides peaks are present in both chromatograms, although the alignment of xenopsin and somatostatin in both has been distorted by the rising baseline.

response to primary amino acids without greatly reducing the proline peak. Increasing the reaction temperature in the hypochlorite reaction coil had little effect on the lysine response and slightly enhanced the proline response. Because a longer reaction time was needed, a larger diameter coil was used that increased the reaction time with hypochlorite from *ca.* 30 to 120 sec. The effect of increased reaction temperature on prolyl- and lysylpeptide detection is seen in Fig. 7. The lower chromatogram was obtained at a reaction temperature of 40°C, and the upper at 60°C. The higher temperature greatly enhanced detection of the dipeptide, prolylglycine, and slightly increased the response to bradykinin, which has three proline residues. The proctolin peak with a single proline residue was slightly depressed at the higher temperature. The somatostatin peak with just lysine was reduced two-fold at 60°C compared to 40°C. At higher temperatures the response to somatostatin is reduced even more, but

the baseline rise becomes becomes even more extreme. This baseline increase is not evident when methanol replaces acetonitrile as the mobile phase. It is very likely that acetonitrile is decomposing under these conditions and liberating ammonia. Although it would be attractive to eliminate the lysine response and make the method specific for proline, this is not practical because of the increased band broadening associated with the longer hypochlorite reaction time and because of the intractable baseline problem. Thus, the lysine response cannot be readily eliminated, at least not with the most popular mobile phase for peptide separations.

CONCLUSIONS

The fluorescamine post-column reaction has been shown to detect peptides with a blocked amino terminus. The response to lysine residues was suppressed by lowering the pH of the reaction to 8.4. Peptides that have a free amino terminus are detectable in both the UV and fluorescamine-reaction chromatograms. Peptides lacking a free amino terminus are only apparent in the UV chromatogram. Common blocking groups at the amino terminus are acetyl, pyroglutamyl and prolyl derivatives. The first two groups interfere with peptide sequencing, and coupling yield may be a problem with the third. The presence of proline is detectable with the combined hypochlorite–OPA reaction. The experiment must be performed twice, *i.e.* two sample injections are required. The first reaction chromatogram is obtained in the presence of hypochlorite and the second in its absence. Fluorescence peaks present only in the first experiment contain proline but no lysine residues. Peaks present in both chromatograms certainly contain lysine and may also have a proline residue.

Thus, only one experiment is necessary to confirm that a particular peptide is not blocked and is ready for sequencing. If the peptide is blocked at the amino terminus, then two more experiments are required to substantiate that proline is the blocking group. The presence of proline residues in the peptide can be positively determined if the peptide does not contain both lysine and proline residues.

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