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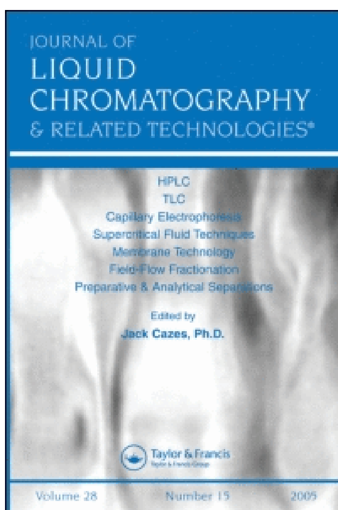

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COMPARISON OF ^{125}I-LABELED AND ^{14}C-LABELED PEPTIDES OF THE MAJOR OUTER MEMBRANE PROTEIN OF *Chlamydia Trachomatis* STRAIN L2/434 SEPARATED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The objective of this study was to determine if in-gel chloramine-T radioiodination adequately labels OM proteins to allow for accurate and precise structural comparison of these molecules. Therefore, intrinsically ^{14}C-amino acid labeled proteins and ^{125}I-labeled proteins were cleaved with two endopeptidic reagents and the peptide fragments separated by HPLC. A comparison of retention times of the fragments, as determined by differential radiation counting, thus indicated whether ^{125}I-labeling identified of all the peptide peaks seen in the ^{14}C-labeled proteins. Results demonstrated that radioiodination yields complete and accurate information about the primary structure of outer membrane proteins. In addition, it permits the use of extremely small amounts of protein allowing for method optimization and multiple separations to insure reproducibility.

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INTRODUCTION

In a previous study (2) we used the procedure of ^{125}I-labeled peptide mapping to investigate the primary structural relationships of the major outer membrane proteins (MOMPs) of five strains of *Chlamydia trachomatis* and one strain of *C. psittaci*. The results indicated there was a remarkable amount of homology among the MOMPs both within *C. trachomatis* strains and between the species.

These unexpected results suggested to some that the in-gel chloramine-T iodination procedure may be a poor way to label such proteins. If only a few select amino acids bind ^{125}I, many peptides may be unlabeled and therefore "invisible" in the two-dimensional peptide mapping system employed. This study addresses this question by comparing peptides generated by two different reagents to cleave to MOMP of *C. trachomatis* strain L2/434. The MOMP was either intrinsically radiolabeled using a mixture of ^{14}C-amino acids or ^{125}I-labeled using the chloramine-T in-gel procedure. Because of its great resolving power, high-performance liquid chromatography (HPLC) was used to separate the peptides (5) and gamma and liquid scintillation detectors used to identify radiolabeled peaks. Results demonstrated that ^{125}I-radiolabeling is an excellent method for labeling proteins, yielding a complete picture of the protein's primary structure.

MATERIALS AND METHODS

Organism

Chlamydia trachomatis strain L2/434(L2) were grown and elementary bodies (EBs) purified as described previously (2).

Gel Electrophoresis

The gel electrophoresis procedure of Laemmli (6) as modified by Caldwell and Judd (2) was used to separate the MOMP of ^{14}C-labeled and unlabeled EBs of the L2 strain. Briefly, EBs were solubilized in a 2% SDS solubilizing solution containing EDTA and

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2-mercaptoethanol. This lysate was then separated in a 12.5% acrylamide (30:0.8 acrylamide: bis-acrylamide) gel with a 5% acrylamide stacking gel. The sample was subjected to electrophoresis at 25 mA constant current for 3-3.5 h.
The gels were then stained using 0.25% Coomassie Brilliant Blue, 50% methanol, and 10% acetic acid. The MOMP bands were then excised, radioiodinated (unlabeled MOMP) or sham iodinated (^{14}C-MOMP), and subjected to enzyme cleavage.

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Intrinsic Radiolabeling

The L2 strain was intrinsically radiolabeled using 50 µCi of uniformly ^{14}C-labeled amino acids (ICN, Irvine, CA) as described in great detail elsewhere (2). The use of 17 ^{14}C-amino acids ensured that each peptide of MOMP would have the ^{14}C label.

Iodination

Unlabeled MOMP of the L2 strain was excised from a sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel of a whole EB lysate. The MOMP band was then radioiodinated with ^{125}I (as NaI, 50 µCi/µl, ICN) using the in-gel chloramine-T (Sigma, St. Louis, MO) procedure of Elder (3) and Swanson (8). Intrinsically ^{14}C-labeled MOMP bands were sham iodinated using the same procedure except that 30 µM cold NaI was substituted for Na^{125}I.

Enzyme Cleavage

Two enzymes were used to cleave the L2 MOMP bands: α-chymotrypsin (α-chymo), which has a broad specificity (1) and *Staphylococcus aureus* strain V8 protease (V8 protease), which cleaves at the carboxyl side of glutamate under the conditions used (4).

α-Chymotrypsin Cleavage

The dehydrated radiolabeled MOMP bands were rehydrated in 250 µl of 0.05 M NH_4HCO_3, pH 8.5. Twenty-five microliters of α-chymotrypsin (Calbiochem-Behring, LaJolla, CA), 1 mg/ml in 0.01 NHCl, was then added. Digestion was allowed to procede for 4 h at 37°C. The supernatant, which contained peptides liberated from the gel, was aspirated, dried in a Savant Speed-Vac (Savant, Hicksville, NY) and the peptide residue washed eight times with 250 µl of distilled water. The washed peptides were rehydrated to 2 x 10^5

cpm/200 µl (^{125}I) or 1 x 10^4 cpm/200 µl (^{14}C) in distilled water containing phenylalanine (1 mg/ml), tryptophane (1 mg/ml) and tyrosine (0.4 mg/ml) as markers.

V8 Protease Digestion

The dehydrated radiolabeled MOMP bands were rehydrated in 250 µl of 0.05 M NH_4HCO_3, pH 7.8. Twenty-five microliters of V8 Protease (Miles Laboratories, Elkhart, IN), 1 mg/ml in distilled water, was added. After 4 h digestion at 37°C, the peptide containing supernatant was aspirated and prepared in the same manner as the α-chymo peptides.

HPLC Separation of Peptides

All HPLC separations were performed on a Waters System (Waters Associates, Milford, MA) equipped with dual M600A pumps. A Waters µ Bondapak C_{18} reverse phase column (3.9 mm ID x 30 cm) was used to separate peptides.

Two hundred microliters of each MOMP peptide preparation was injected by the WISP 710 injector at time 0. The dual pump system, pump A delivering filtered, distilled water - 0.05%-TFA - and pump B delivering acetonitrile - 0.05%-TFA (AcN-TFA) (reagent grade, Sigma), then delivered the gradients used to separate the peptides. The α-chymo peptides were separated using a 1 ml/min solvent flow of a 0 to 10% ACN-TFA linear gradient (06 program) in the first 10 min. From 10-55 min a 10-100% ACN-TFA gradient (09 program) was generated followed by a 5 min 100% ACN-TFA wash. The V8 protease peptides were separated on an 0-10% ACN-TFA linear gradient in 8 min followed by an 8% to 100% ACN-TFA gradient (09 program) in 35 min with a 9 min 100% ACN-TFA wash. Due to limited amounts of V8 ^{14}C-labeled peptides, improved gradients were not generated using ^{14}C-labeled MOMP petides. However, ^{125}I-labeled peptides generated by V8 protease were plentiful (one of the reasons that radioiodination is such a useful method of protein labeling). Therefore, an improved gradient separation was developed using the ^{125}I-labeled peptides. The bulk of V8 protease peptides, which elute late in the ACN-TFA gradient, were first separated on a 0-25% ACN-TFA linear gradient over the first 2 min

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followed by a 25%-100 ACN-TFA gradient using the 09 gradient profile over the next 18 min with a 4 min 100% ACN-TFA wash. The peptides eluting between 10 min and 24 min were pooled, dried in a Speed-Vac, and rehydrated in 80% H_2O-TFA; 20% ACN-TFA. These peptides were then reseparated using 80% H_2O-TFA; 20% ACN-TFA for 42 min followed by a 100% ACN-TFA wash for 6 min. In all separations, an LKB 7000 Ultrorac (LKB Instruments, Sweden) was started at injection. Two tenth minute fractions were collected during the entire separation. These were then counted in a Beckman Biogamma Counter (Beckman Instruments, Palo Alto, CA) to detect ^{125}I emission or in a Beckman LS 8000 Scintillation Counter after the addition of 1.5 ml of Beckman Aquesol Scintillation Cocktail. Counts per minute were then plotted vs retention time (RT) as derived from the fraction number. The elution of amino acid standards was monitored at 254 nm. No peptides could be detected in an sample by UV absorption.

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RESULTS

Figure 1 shows an SDS-PAGE separation of whole EBs of 5 strains of *C. trachomatis* and one strain of *C. psittaci* (MN). The MOMPs are readily identified because they bind the greatest amount of Coomassie Brilliant Blue. Unlabeled and ^{14}C-labeled L2 MOMP bands were cleaved with α-chymotrypsin or V8 protease, and the peptides separated by HPLC. Figure 2 shows a composite chromatogram of HPLC separation of ^{14}C-labeled and ^{125}I-labeled peptides generated by α-chymo cleavage. Out of the 58 observable ^{14}C peaks, all but 5 are clearly represented by ^{125}I peaks indicating that ^{125}iodination is an excellent method of labeling proteins for these analyses. Figure 3 shows a composite chromatogram of HPLC separation of ^{14}C-labeled and ^{125}I-labeled peptides generated by V8 protease cleavage. This enzyme is considerably more specific than α-chymo as seen by the greatly diminished number of peptide peaks seen in those chromatograms. Clearly, each peptide visualized by ^{14}C-emission is represented by a ^{125}I-emitting peak, again, demonstrating that ^{125}I-labeling yields an accurate picture of these proteins' primary structure.

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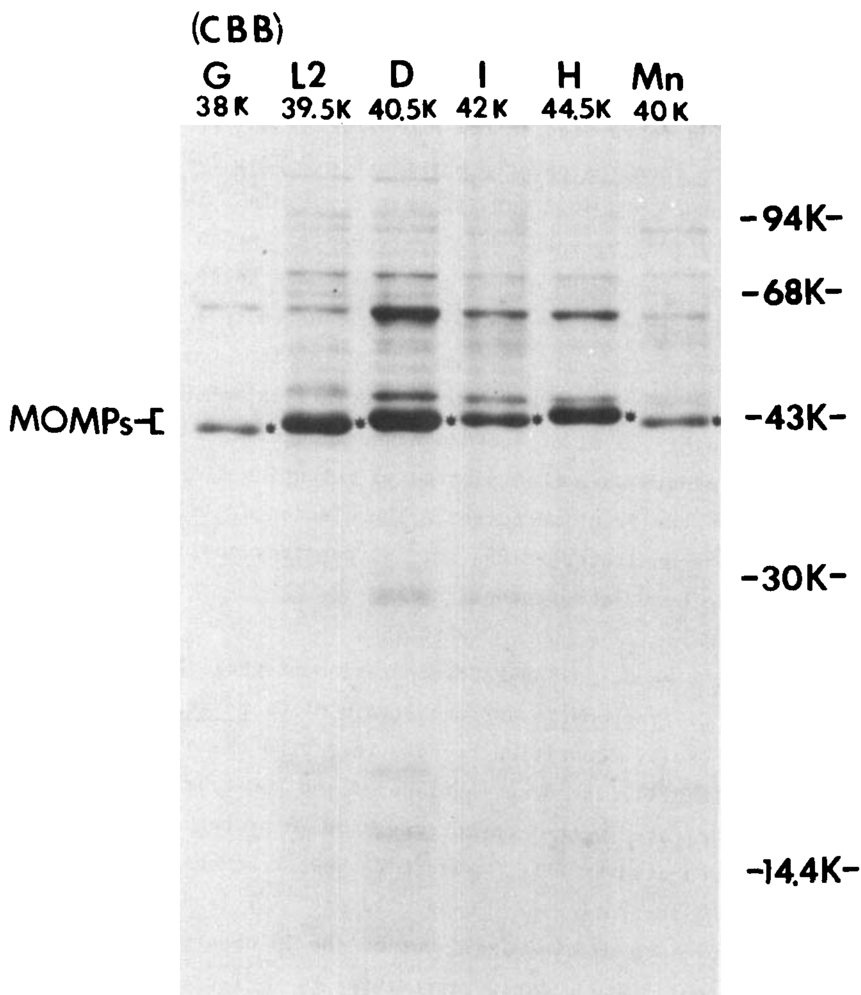

Figure 1. A Coomassie Brilliant Blue (CBB) stained SDS-PAGE gel (15%) of whole EB lysates of C. trachomatis strains G/UW-57, L2/434, D/UW-3, I/UW-12, H/UW-4, and a C. psittaci meningopneumonitis (MN) Cal-10 strain. These organisms were used in a previous study to investigate the structural relations of their major outer membrane proteins (MOMPs, designated by asterisks). The L2 strain was used in this study. The MOMP band of unlabeled and ^{14}C-labeled EBs was excised from the gel and cleaved with endopeptidases. Molecular weight markers (Bio Rad, Richmond, CA) were phosphorylase B-94,000 d (94 k), bovine serum albumine (68 k), ovalbrimin (43 k), carbonic anhydrase (30 k), and lysozyme (144 k).

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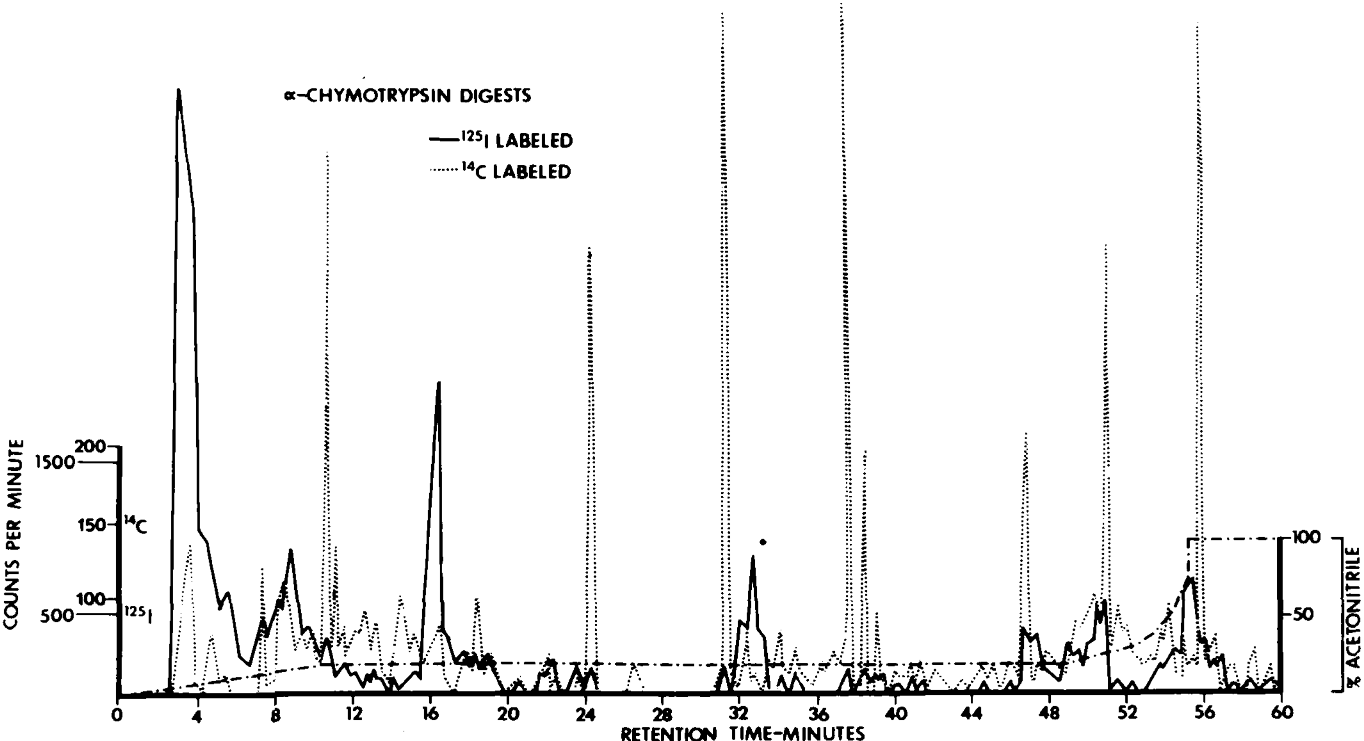

Figure 2. HPLC elution profiles of ^{125}I-labeled and ^{14}C-labeled peptides of C.trachomatis L2 MOMP generated by -chymotrypsin digestion. Peptides were separated on a reverse phase C_{18} column using H_2O-TFA and Acn-TFA in the mobile phase. Two-tenths of a minute fractions were collected and counted in a Beckman Biogamma Counter (^{125}I) of in a Beckman LS 8000 Counter after the addition of Aquesol (^{14}C). This chromatogram is a composite of two separations. Note that all ^{14}C peaks are represented by an ^{125}I peak with but five exceptions located a 26 min, 37-37 min, and 42-43 min. Internal standards, TYR, PHE, TRP, eluted at 8.53, 9.11, 11.5 respectively and varied less than 0.05 min/run.

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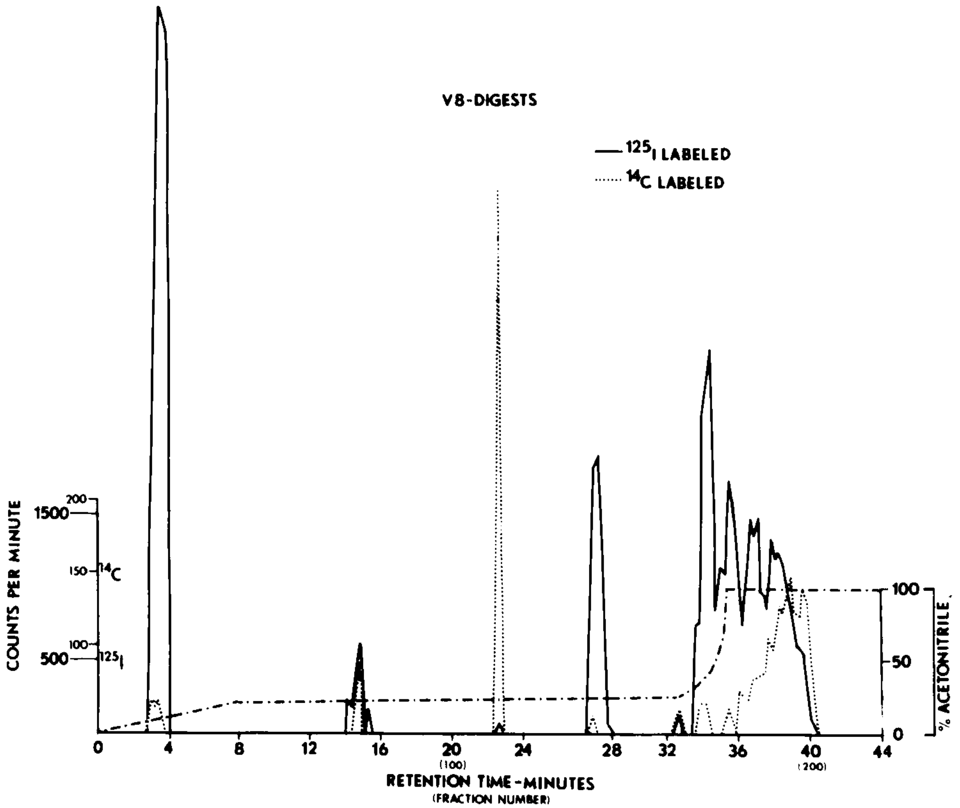

Figure 3. HPLC elution profiles of ^{125}I-labeled and ^{14}C-labeled peptides of C. trachomatis L2 MOMP generated by Staphylococcus aureus V8 protease digestion. Peptides were separated on a reverse phase C_{18} column using H_2O TFA and ACN-TFA in the mobile phase. Two-tenths minute fractions were collected and counted in a Beckman Biogamma Counter (^{125}I) or a Beckman LS 8000 Counter (^{14}C) after the addition of Aquesol. This chromatogram is a composite of two separations. Note that all ^{14}C peaks are represented by ^{125}I peaks. Internal standrads, TYR, PHE, TRP, eluted at 7.25, 8.37, and 10.92 respectively and varied less than 0.05 min/run.

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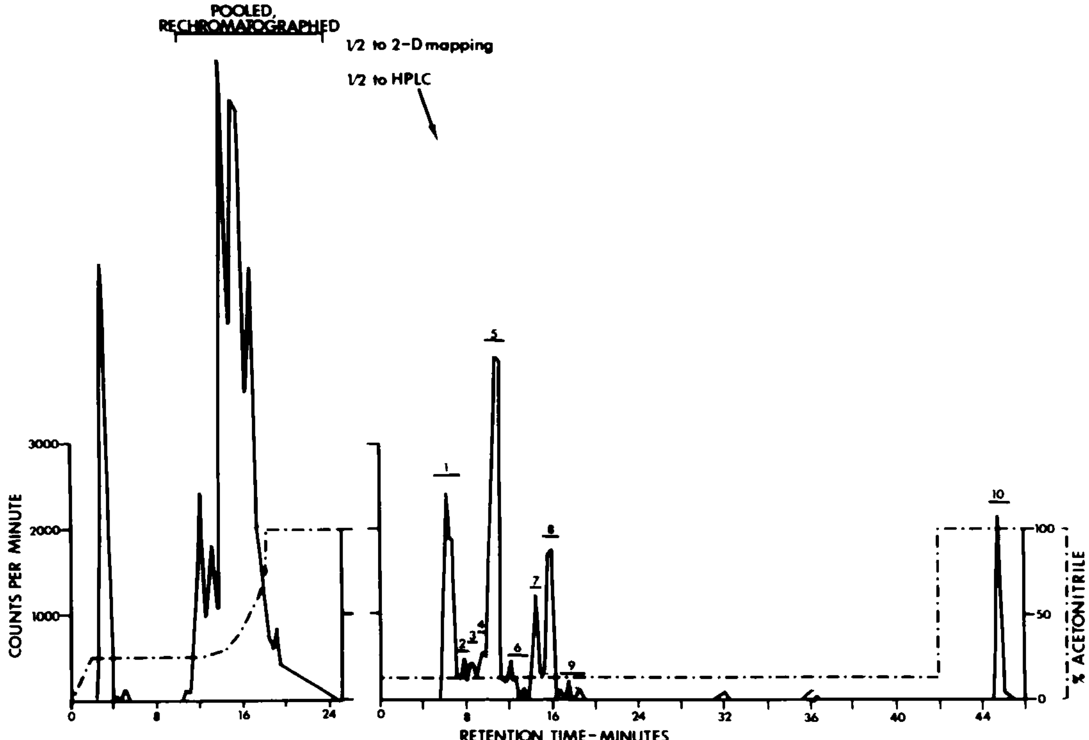

Figure 4. HPLC elution profiles of ^{125}I-labeled peptides of C. trachomatis MOMP generated by V8 protease digestion. Peptides were separated on a reverse phase C_{18} column using H_2O-TFA and ACN-TFA in mobile phase. This figure is included to demonstrate how poorly resolved peptide peaks can be rechromatographed to enhance separation. Those peaks uner the "Pooled, Rechromatographed" bar were reseparated using a different elution gradient. Note the marked improvement in peak separation. The ^{14}C-labeled material was not of adequate activity to perform this experiment, stressing the usefullness of radioiodination in generating elution profiles which adequately separate peptidic fragments.

Figure 4 is a chromatogram demonstrating that the separation of V8 protease generated peptides of ^{125}I-labeled MOMP can be greatly improved by modifying the elution gradient. Unfortunately, a severe paucity of ^{14}C-peptides generated by V8 protease prevented us from using this gradient to compare ^{14}C and ^{125}I-labeled peptides. In this figure a rapid separation of ^{125}I-labeled V8 protease peptides was performed. The poorly separated peptides were pooled and rechromatographed resulting in greatly improved separation.

DISCUSSION

The objective of this study was to determine if in-gel chloramine-T radioiodination adequately labels OM proteins to allow for accurate and precise structural comparison of these molecules. Therefore, intrinsically ^{14}C-amino acid labeled proteins and ^{125}I-labeled proteins were cleaved with two endopeptidic reagents and the peptide fragments separated by HPLC. A comparison of retention times of the fragments, as determined by differential radiation counting, thus indicated whether ^{125}I-labeling identified of all the peptide peaks seen in the ^{14}C-labeled proteins.

The results presented here indicate that radioiodination is an excellent method for labeling proteins in structural analysis studies. Only a few peptides failed to have ^{125}I activity when a very large number of peptides were generated by α-chymo digestion. The large number of peptides generated by α-chymo is somewhat surprising, especially when compared with two-dimensional peptide maps (2) which show far fewer radioemitting peptides. As demonstrated in a previous paper (5), the large number of peptides probably represents both multiple iodination states of the peptides and the increased sensitivity of gamma counting <u>vs</u>. autoradiography.

It would appear that many amino acid residues accept the ^{125}I and not just tyrosine. There is evidence that the other aromatic amino acids and histidine will bind ^{125}I as well (7). Since α-chymo preferentially cleaves at the carboxyl side of the aro-

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matic amino acids (1), each peptide should acquire the ^{125}I label. Those few peptides which were not labeled may represent α-chymo cleavage at leucine residues which occurs at very low levels compared with the aromatic amino acid cleavage. The complete labeling of V8 protease peptides with ^{125}I is expected since this enzyme cleaves at only glutamate, thereby generating larger fragments which almost surely contain at least one ^{125}I-labeled residue.

Perhaps the utility of ^{125}I-labeling is best seen in Figure 4. Because of the relatively weak emission of ^{14}C and the low efficiency of V8 protease cleavage of L2 MOMP in acrylamide gels, it was difficult to generate enough peptidic fragments to optimize the HPLC separations. However, the much greater emission of ^{125}I-labeled fragments allowed for the multiple sample separations needed to improve the HPLC peptide separation.

Radioiodination appears to be an excellent method of labeling proteins for structural studies. The labeling procedure is rapid, reliable, and allows for studies using extremely small amounts of protein. The addition of a new in-line gamma counter available from Beckman Instruments, Inc., Palo Alto, CA, has greatly improved precision and has dropped the detection limits. An injection of as little as 6,000 cpm of radioiodinated peptides can be detected (personal observations) and direct integration of elution allows for extremely reproducible chromatograms for HPLC peptide mapping. Future studies will present data acquired using the new in-line gamma detector and its application to peptide analyses.

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