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The Primary Structure of Selected Peptides from Chymotryptic Digests of Nifungin

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THE PRIMARY STRUCTURE OF SELECTED PEPTIDES FROM CHYMOTRYPTIC DIGESTS OF NIFUNGIN

BY

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FROM CHYMOTRYPTIC DIGESTS OF NIFUNGIN

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser — 7-27-70 Date

Head, Chemistry Department — 7-27-70 Date

To

JAMES and EULA

They Knew Me First

and to

SHARON

Who Knows Me Best

THE PRIMARY STRUCTURE OF SELECTED PEPTIDES FROM CHYMOTRYPTIC DIGESTS OF NIFUNGIN

Abstract

BURTON DOYN CARDWELL

Under the supervision of Professor Oscar E. Olson

Nifungin, an antifungal peptide produced by Aspergillus giganteus, was studied and partially characterized.

The amino acid composition of the antifungal peptide was investigated, and eighty-seven percent of the peptide constituents were identified.

The presence of free -SH groups in the peptide was studied by spectrophotometric titration of the peptide with p-hydroxymercuribenzoate.

Performic acid oxidation was used to disrupt the disulfide bridges present in the molecule, and subsequent enzymatic digestion of the oxidized peptide was accomplished with alpha chymotrypsin. Chromatographic separation of the digestion mixture produced eleven chymotryptic peptides. Six of these peptides were selected for amino acid sequence analysis by the Edman degradation method.

The results of this study indicated that nifungin is a basic peptide containing a minimum of forty-nine amino acid residues. It contains no tryptophan, histidine or methionine. The peptide has no free -SH groups, but incorporates four disulfide bridges within its structure. The sequence studies on the six chymotryptic peptides showed that these peptides had unusual end-group residues in some instances, based on the specificity of the digesting enzyme, suggesting that the overall nifungin structure was complex.

ACKNOWLEDGEMENTS

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INTRODUCTION

In 1956, the Division of Antibiotics and Fermentation, Michigan Department of Public Health Laboratories, Lansing, Michigan, initiated a cancer screening program in cooperation with the National Institutes of Health. The purpose of the program was to isolate from soils those fungi which could produce antitumor compounds with "in vivo" activity against selected sarcoma, carcinoma and leukemia tumor strains transplanted in mice.

One of the earliest mold cultures found to produce an antibiotic having activity against these tumor strains was designated as Michigan Department of Public Health 18894 or (MDPH 18894). This mold was later found to be Aspergillus giganteus. The compound produced by A. giganteus was designated alpha sarcin and assigned the Cancer Chemotherapy National Service Center Number 46401 (CCNSC 46401).

During the isolation and subsequent purification of alpha sarcin it was determined that two of the four minor peptides present in the crude preparation of the drug possessed antifungal activity against MDPH 13462L mold. The MDPH 13462L mold culture was another soil organism isolated in this same screening program and later identified as Aspergillus restrictus.

The two antifungal peptides were readily separated from each other and from the alpha sarcin by ion exchange chromatography using Amberlite XE64 resin (Rohm and Haas) with sodium phosphate buffers and a convex

gradient elution pattern. The major antifungal peptide from this separation was then desalted and further purified by gel filtration using a column packed with Sephadex G-25 (fine). Using these methods of isolation and purification, a quantity of this major antifungal peptide was stockpiled for use in this investigation.

The antifungal peptide was initially designated as the alpha sarcin mold peak. However, after beginning the study of it, the Michigan Department of Public Health petitioned the United States Adopted Names Council (USAN) of the American Medical Association for a suggested name for the compound. The name offered by the council was NIFUNGIN. This name was accepted by the Michigan Department of Public Health and thus replaced the older designation.

The mode of action of nifungin is not completely understood. However, it is believed that the peptide inhibits the growth of Aspergillus restrictus rather than destroying the organism. Apparently it is a fungistat rather than a fungicide.

It appeared that characterization of nifungin might be helpful in establishing its mode of action. It was decided, therefore, that work should be undertaken in this direction. Efforts toward determination of its primary structure through studies on both trypsin and chymotrypsin digests were initiated. Mr. Marvin Withrow at South Dakota State University investigated the primary structure of the tryptic peptides from oxidized nifungin, and the author carried out the amino acid sequence studies reported here on the chymotryptic digests of oxidized nifungin.

HISTORICAL

The knowledge that proteins could be cleaved in acid or base to produce a mixture of amino acids was known prior to 1900. However, the bonding arrangement between amino acids in the intact protein remained unknown until 1902 when the concept of the peptide bond was introduced. The peptide theory introduced a fifty-three year search which led to the determination of the first complete amino acid sequence of a protein molecule in 1955. It is therefore with the peptide theory that we begin the historical development of the primary structure of proteins.

The peptide concept of protein structure was proposed independently by Emil Fischer and F. Holmeister in 1902 (1). The validity of the concept lay in the following experimental observations. (a) Alpha amino nitrogen and free carboxyl groups, except for beta and gamma carboxyl groups of aspartic and glutamic acid are liberated in equivalent amounts from proteins on hydrolysis. (b) Simple peptides occur commonly in nature. (c) The biuret reaction which characterizes structures with peptide bonds was positive for natural proteins but negative after complete hydrolysis of the protein. (d) Peptides isolated from partial hydrolysates of proteins were found to be identical to synthetic peptides of known constitution.

The structure of partial hydrolysis products was thoroughly examined during the early research into the primary structure of

proteins and peptides. Most of the research done during the first two decades of the twentieth century was for the purpose of gathering evidence for proof of the peptide theory. The methods in general were those of classical organic chemistry and generally crude by our present day standards. However, these investigations provided the essential background knowledge for today's research on amino acid sequence analysis. Synge (2) in an excellent review summarized the research on partial hydrolysis products from 1900 to 1943.

In 1902, Fischer and Bergell (3) reported the isolation as a napthalene sulfonyl derivative of a dipeptide from an acid hydrolysate of silk fibroin. Organic analysis of this dipeptide led them to describe it as glycylalanine. When they subsequently prepared the synthetic napthalene sulfonyl derivatives of L-glycyl-alanine and L-alanyl-glycine and compared them to the isolated peptide neither corresponded exactly to the natural product in its properties. These results suggested that the isolated material was actually a mixture of the two synthetic dipeptides. Fischer attempted to repeat the original isolation, but his efforts were unsuccessful because of the lack of dependable techniques. In a later investigation Fischer (4) converted the same natural dipeptide to a diketopiperazine by condensation of its esters. The L-glycyl-L-alanine anhydride formed in this manner was identical to the synthetic products.

Investigators such as Abderhalden, Schwarb and Bergmann carried out similar type research on still other partial hydrolysis products of silk fibroin (2).

The basic protamines and histones from fish sperm were also investigated during this early period. Kossel and Mathews (5) precipitated these compounds with phosphotungstic acid, or silver nitrate, and carried out primary structure studies on the trypsin hydrolysis products of the isolated compounds.

Abderhalden and Suzuki (6) investigated the group of proteins known as the keratins. Goose feathers were treated with 70 percent sulfuric acid for 5 days at room temperature. After the complete removal of the sulfuric acid with barium hydroxide, the aqueous solution was evaporated to dryness and extracted with boiling methyl alcohol. This extract gave products which were not only insoluble in water, but in some organic solvents as well. These products gave the characteristic biuret reaction, but did not give any other of the usual protein color reactions. Complete hydrolysis of the products with acid was unsuccessful. Abderhalden (7) later isolated a crystalline product from these same fractions. Complete acid hydrolysis of this crystalline product gave glycine, proline and a molecule of hydroxyproline. Abderhalden theorized that this crystalline product was an anhydride formed by two molecules of proline, one molecule of hydroxyproline and one molecule of glycine by the elimination of four molecules of water.

Osborne and Clappe (8) hydrolyzed the plant protein, gliadin, with sulfuric acid at room temperature. Crystallization of the hydrolysate with subsequent analysis indicated a dipeptide consisting of proline and phenylalanine.

Following these early investigations into the primary structure of proteins, the discovery was made that there existed in nature certain small di- and tripeptides. Their well defined crystalline structure and their low molecular weight made them excellent compounds for study (9). The knowledge gained in identifying the amino acids in the earlier more complex products was applied with great success to such dipeptides as carnosine and anserine and particularly to the tripeptide glutathione.

Glutathione was isolated by Hopkins in 1921 from a yeast source (10). Quastel et al., (11) initially described glutathione as a dipeptide consisting of glutamic acid and cysteine. Further, their description correctly indicated the dipeptide as the unusual gamma-glutamylcysteine. Later, Hunter and Eagles (12) suggested that glutathione contained a third component in its molecule. Hopkins (13) on this information undertook a more careful study of glutathione. Hopkins found that if he boiled the oxidized form of the pure compound in water for several hours a diketopiperazine was obtained. He characterized this diketopiperazine as anhydrodiglyclcystine. Similar treatment of the reduced glutathione gave the same compound. On this basis it appeared that a glycine residue was present in the peptide, and that it was directly linked to the cysteine. Glutathione was therefore actually a tripeptide consisting of glutamic acid, glycine and cysteine. Titration studies by Pirie and Pinhey (14) tentatively verified the glutamylcysteinylglycine structure of the compound.

In 1930 the COOH-terminal position of glycine was determined by three separate research groups using three different methods. Proof that glutamic acid was in the NH_2-terminal position was established by Gurin and Clark (15) when they converted oxidized glutathione to the N,N-dibenzenesulfonyl derivative, followed by butyl esterification and isolated the dibutyl ester of benzenesulfonyl-L-glutamic acid. In 1935, Harrington and Mead synthesized glutathione (16).

In spite of the success in determining the amino acid sequence of glutathione, Synge concluded his review of partial hydrolysis products with this statement (2): "It seems that the main obstacle to progress in the study of protein structure by methods of organic chemistry is the inadequacy of technique rather than theoretical difficulty. It is likely that the development of new methods of work in this field will lead us to a much clearer understanding of the proteins."

The period between 1940 and 1950 was marked by increased effort on the part of protein chemists to develop more systematic methods for establishing the amino acid sequence of proteins and peptides. In 1945 Fox (17) reviewed the methodology for determining the terminal amino acid sequence in both proteins and peptides. These methods plus the techniques of paper chromatography developed by Consden, Gordon and Martin (18) marked the introduction to present day studies of amino acid sequences.

Sanger (19) determined the first complete amino acid sequence of a protein in 1955. For this determination he chose the pancreatic hormone, insulin.

The study of insulin began with the determination of its molecular weight. Osmotic pressure measurements and ultracentrifugation data showed the minimum molecular weight to be 12,000. Repeating Sanger's experiments, Harfenist (20) obtained results which indicated that the molecular weight of the molecule was approximately 6000 rather than the 12,000 originally proposed by Sanger. Harfenist and Craig (21) verified the lower molecular weight using a method of partial substitution on the insulin molecule.

NH_2-terminal analysis of insulin by Sanger using 2,4-dinitro-1-fluorobenzene (DNFB) yielded one residue of Dinitrophenyl (DNP) glycine and one residue of DNP phenylalanine (19). These results suggested the presence of two peptide chains in the insulin molecule. COOH-terminal analysis of insulin showed one alanine and one asparagine residue (19). These findings substantiated the presence of two separate chains in the molecule.

Sanger was able to separate insulin into its two component chains by splitting the intact molecule with performic acid at the site of its disulfide bridges. Once split, the two resulting peptide fragments were readily fractionated from each other (19).

The oxidized insulin yielded an acidic fraction (A) which contained the NH_2-terminal glycyl residue and no basic amino acids, plus

the more basic fraction (B) which had an NH_2-terminal phenylalanyl residue and was insoluble at pH 6.5 (22). COOH-terminal analysis on both chains showed that the glycyl chain was terminated with asparagine and the phenylalanyl chain with alanine. The glycyl chain contained twenty amino acids, while the phenylalanyl chain contained thirty amino acid residues. Further, four cysteic acid residues were found in the glycyl chain, and two in the phenylalanyl chain.

Sanger and Tuppy (23) reported the amino acid sequence of the phenylalanyl chain in 1951. The phenylalanyl chain was subjected to partial hydrolysis in acid and alkali and the resulting peptide mixture was separated by paper chromatography using the methods of Consden, et al. (18). NH_2-terminal groups were identified and several amino acid sequences were determined. The entire sequence of the phenylalanine chain could not be determined because of the difficulty which occurred in the fractionation of some of the non-polar peptides.

Sanger and Tuppy (23) in an effort to avert these difficulties next digested the phenylalanyl chain with the proteolytic enzymes, pepsin, chymotrypsin and trypsin, fractionated the resulting peptides by paper chromatography, and from the terminal analysis data obtained, were able to determine the complete structure of the phenylalanyl chain.

The presence of more hydrophobic amino acid residues in the glycyl chain made its structure more difficult to determine. Sanger et al., reported its sequence in two papers (24,25). The first paper

discussed the peptides obtained by partial hydrolysis with strong acid, while the second was devoted to the larger peptides obtained by enzymatic digestion with pepsin and chymotrypsin. Separation of the peptides was accomplished by ionophoresis on silica gel and they were identified further by paper chromatography using 2,4 dinitro-1-fluorobenzene. The sequences obtained after acid hydrolysis plus those obtained from the peptides after enzymatic action were enough to deduce the complete sequence of glycyl chain.

The sequence of both chains (A and B) of insulin was now known. However, two problems remained: (1) allocation of the amide groups to particular dicarboxylic amino acid residues, and (2) the assignment of the position of the cystine molecules in the original insulin compound.

Sanger, et al., (26) determined the location of the amide groups by comparing ionophoretically, those peptides from enzymatic digests having intact amide groups with those of acid hydrolysates in which the amide group had been split off. The relative rates of migration gave an indication as to the presence of charged groups on the peptides and also suggested the number of carboxyl groups masked as amides.

Ryle, et al., (27) determined the position of the disulfide bridges in insulin. They subjected the intact insulin to partial hydrolysis and fractionated the resulting cystine containing peptides from one another. These peptides were then oxidized with performic acid and the cysteic acid peptides obtained were identified. Through

comparison of the amino acid sequences in these peptides with those of the tryptic and chymotryptic peptides of the A and B chain the disulfide bridge locations were determined.

Once the disulfide bonds were located, it became a matter of fitting all the pieces of the compound together to complete the sequence.

The general methods used by Sanger to determine the primary structure of insulin established the pattern for all subsequent amino acid sequence determinations. These methods included (a) determination of the amino acid composition of the peptide or protein, (b) NH_2-terminal and COOH-terminal group determination of the peptide, (c) oxidation of the disulfide bonds in the protein structure, (d) enzymatic degradation of the oxidized protein, (e) separation and purification of the peptide mixture, (f) determination of the amino acid composition and sequence of each peptide fragment, (g) determination of location of the disulfide bridges, (h) fitting together the complete sequence from the sequence of overlapping peptides obtained from the digestion of the protein with two or more enzymes.

The second major achievement in the establishment of amino acid sequences for proteins was the work of Hirs, et al. on ribonuclease (28). This single-chain enzyme, composed of 124 amino acids and containing four intrachain disulfide bridges was approached in a manner similar to that employed for insulin. Hirs (29) disrupted the bridges with performic acid, and Moore and Stein (28) digested the oxidized ribonuclease with trypsin and chymotrypsin in separate experiments. The

resulting peptide mixtures were separated on Dowex 50 x 2 ion exchange resin using volatile pyridine acetate buffers of varying concentrations and ionic strengths.

Moore and Stein et al., (30) determined the amino acid sequences of the small tryptic and chymotryptic peptides of ribonuclease. The DNFB method and the newer Edman degradation (31) which involved the formation of cyclic phenylhydantoins was used to determine the amino terminal sequences (31). The sequence of COOH-terminal amino acids was determined with carboxylpeptidase A and B. The overlapping sequences of the various peptides were then fitted together to complete the protein structure.

The use of Dowex 50 x 2 ion exchange resin for the separation of low molecular weight peptides and the automatic amino acid analyzer developed by Spackman, Moore and Stein (32) for the quantitative determination of the amino acid composition of the peptides were the principal advances in technique employed to establish the primary structure of this larger protein.

The Dowex 50 x 2 is a strongly cationic, sulfonated polystyrene ion exchange resin crosslinked with 2% divinylbenzene. The percentage of divinylbenzene used in the resin network determines the porosity of the resin, and thus the size of the peptides which are resolved best by the resin.

Dowex 50 x 8 gives excellent resolution of di- and tripeptide mixtures, however for peptides containing 4-7 residues the Dowex

50 x 4 resin becomes more practical. The Dowex 50 x 2 resin is preferred for peptides of 8-10 amino acid residues. The lower percentage of divinylbenzene incorporated into the resin allows the high molecular weight peptides to enter the resin matrix where exchange can occur.

Since the clarification of the complete structures of insulin and ribonuclease, the structure of numerous other proteins and peptides have been established. Eck and Dayhoff (33) have compiled an atlas of most of the published and some unpublished partial and complete amino acid sequences.

MATERIALS

Equipment - Amino acid analyses were made using a Beckman Model 120 B Amino Acid Analyzer as described by Spackman (34). Absorbance measurements were carried out with a Cary Model 14 Recording Spectrophotometer having matching quartz sample and reference cuvettes. A Sargent Model S 30240 Recording pH Stat with combination pH electrodes, automatic temperature control and stirring unit, was used for determining the rate of enzyme reaction. The Model V-10 automatic fraction collector, with a 200 fraction turntable capacity was used for the collection of eluates. Peptide samples were lyophilized using a Buchler cold finger condenser having five lyophilizing flasks. Reduced pressure was maintained with a Welch Duoseal Vacuum pump.

Chemical Reagents - All inorganic and organic chemicals used in the preparation of solutions for this investigation were reagent grade unless otherwise specified. Manufacturer and specification of reagents will be described in the proper sections under Methods. Buffer solutions used in this investigation will also be described in the proper sections under Methods.

METHODS

Isolation and Purification of Nifungin - The antifungal peptide, nifungin, used in this investigation was isolated and purified by Olson and Goerner using the methods described by them in 1965 (35).

The moisture content of the peptide, determined by drying to a constant weight at 105°, was 0.0%. Its ash content, 1.49%, was determined by heating 40.2 mg of the peptide at 600° for two hours in a small platinum crucible and weighing the residue.

The ultraviolet absorption spectrum of nifungin showed a single absorption maximum at 2780 Å and a minimum at 2540 Å. It had been found that the quantity of the peptide in a solution could be determined from the ultraviolet spectrum by using the method of Camiener et al. (36). Using this method, the spectrum of a solution containing the peptide was run on the Cary Model 14 Spectrophotometer and a tangent was drawn common to the curve at the minimum near 2500 Å and the inflection near 3050 Å. The height of the curve above this line at 2800 Å was used as a measure of the peptide content of the solution. The purified nifungin used in this study gave 113 absorption-height units per mg. This value had been found by Olson and Goerner (35) to be representative of pure nifungin.

Amino Acid Analysis - Amino acid analyses on acid hydrolysates were made as described by Spackman et al. (34) with the use of the Spinco Model 120 B Amino Acid Analyzer.

The amino acid content of the nifungin was determined on acid hydrolysates prepared as follows: A 1.00 ml aliquot of a solution containing 1.2 mg of nifungin per ml was added to each of four 20 ml heavy walled Pyrex ampoules, frozen and lyophilized, then 0.9 ml of constant boiling HCl was added to each ampoule, and after freezing its contents in a dry ice-alcohol bath and evacuating the ampoule it was sealed at the neck with an oxygen flame. The ampoules were then placed in a 120° gravity convection oven and one tube was withdrawn at each 20,40,70 and 116 hours. After removing and cooling, each tube was placed in a dry ice-alcohol bath for about 10 minutes, the seal was broken, and the tube lyophilized. After adding 5.00 ml of diluting buffer, pH 2.2 (34), 2.00 ml was used for amino acid analysis on both the short and the long columns of the analyzer. The analyzer was standardized using Beckman amino acid calibration mixture Type 1 just before and just after the analysis of the hydrolysates and average "C" values were used in making calculations.

No tryptophan was noted on analysis of the acid hydrolysates. However, because of the lability of this amino acid during acid hydrolysis, the method of Bencze and Schmidt (37) was used in an effort to detect tryptophan in the intact peptide. Barium hydroxide hydrolysates (38) were also examined on the amino acid analyzer.

Oxidation of Nifungin with Performic Acid - Nifungin was oxidized with performic acid according to the method of Hirs (29).

Performic acid was prepared by mixing formic acid, 97-100% (Matheson, Coleman Bell: Norwood, Ohio) with 30% hydrogen peroxide, (Mallinckrodt Chemical Works, St. Louis, Missouri) in the ratio of 19:1. The solution was carefully agitated and allowed to stand open at room temperature for two hours.

A 1.00 g portion of nifungin was dissolved in 25 ml of formic acid (97-100%) and transferred to a 250 ml volumetric flask which served as the reaction vessel. To prevent the freezing of reactants during the oxidation period, 5.0 ml anhydrous methanol was also added to the reaction vessel.

After two hours, the solution of performic acid was covered and placed in the cold room at 4°. At the same time, the reaction vessel containing nifungin was immersed in an alcohol bath maintained at -10° by a cold finger containing dry ice. After 30 minutes, the performic acid solution was removed from the cold room and 93.5 ml was transferred to the reaction vessel. This volume represented a portion equivalent to twelve times the amount of acid necessary to effect complete oxidation of four disulfide bonds. The reaction mixture was agitated in the reaction vessel and the oxidation was allowed to proceed for 2.5 hours at -10°. After this period, the reaction mixture was transferred quantitatively into 200 ml of distilled water at 4° with several rinsings. The solution was mixed thoroughly, and then divided equally between four round-bottomed lyophilizing flasks, shell frozen and freeze dried for 24 hours. The drying procedure was

then repeated after redissolving the dried oxidized peptide in 40 ml of cold distilled water. After the final drying, the oxidized peptide was transferred to a tared screw cap tube and its weight determined at 934 mg.

Amino acid analysis of the peptide after oxidation indicated the presence of 8 moles of cysteic acid and the complete absence of half-cystine residues in the molecule. This showed that the oxidation of the disulfide bonds was essentially complete. Except for the oxidation of half-cystine, the amino acid composition of the peptide was not changed by the oxidation.

Digestion of Nifungin with Alpha Chymotrypsin - Alpha chymotrypsin (3.4.4.5., Worthington, LOT No. CDl 6093) was used to hydrolyze the oxidized nifungin into shorter peptides for sequence studies. The alpha chymotrypsin was assayed for activity by the method of Hummel (39). Its activity was found to be 11 units/mg. This is essentially the activity expected of crystalline alpha chymotrypsin (39).

The peptide (934 mg) was dissolved in 93 ml of distilled deionized water. The temperature of the solution was adjusted to 37° and maintained there using the automatic temperature controller on the Sargent Recording pH Stat. When the temperature stabilized at 37°, the pH of the solution was adjusted to 8.0 using 0.1 N NaOH. At zero time 19 ml of a 1% solution of chymotrypsin in 0.001 N HCl was added via pipette, giving a 50:1 substrate - enzyme ratio.

The rate of enzyme reaction was measured as the amount of 0.1 N

NaOH required to maintain the pH of the solution at 8.0 (Fig. 1). The Sargent Recording pH Stat was used for automatic pH and temperature control. The reaction was carried out for 24 hours and additional 10 ml of enzyme solution was added after 4 hours. The reaction was terminated at the end of the digestion period by adjusting the pH to 2.0 with 1 N HCl to inactivate the enzyme. The pH of the mixture was then raised to 7.0 with 1 N NaOH and the hydrolysate was transferred quantitatively to a round-bottomed lyophilizing flask. The hydrolysate was shell frozen and freeze dried for 24 hours. The dried peptide was transferred to a sterile screw cap tube and held under refrigeration.

Chromatographic Separation of Peptides - The chymotryptic peptides were separated by ion exchange chromatography using Amberlite XE64 resin (Rohm and Haas). A 2.2 cm x 160 cm column was packed to a resin bed height of 150 cm with the Amberlite resin. The resin was then equilibrated with 0.1 M ammonium acetate buffer at pH 5.92. The dried peptide digest was dissolved in 20 ml of this same buffer and applied to the top of the column. The column was eluted with a convex gradient system consisting of 0.1 M ammonium acetate buffer pH 5.92 in the mixing vessel (2,200 ml) and 0.9 M ammonium acetate pH 6.0 in the reservoir. Because of the column height, a metering pump was placed between the mixer and the top of the column to insure a flow rate of 80 ml per hour.

A total of 440 eluates containing 10 ml per tube was collected on the Gilson fraction collector. The optical densities of the eluates

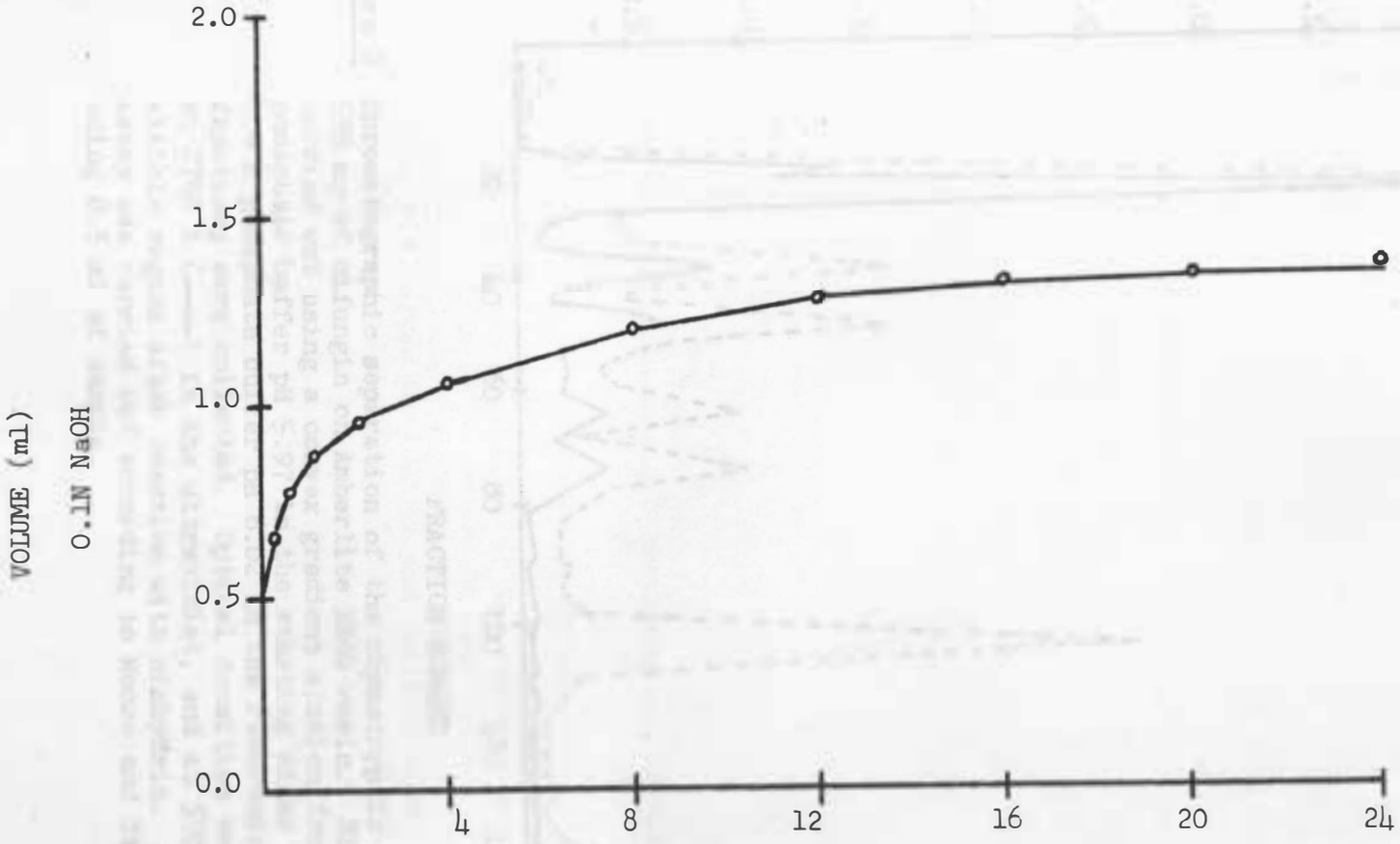

Figure 1 Rate of Reaction for the Chymotryptic Digestion of Oxidized Nifungin

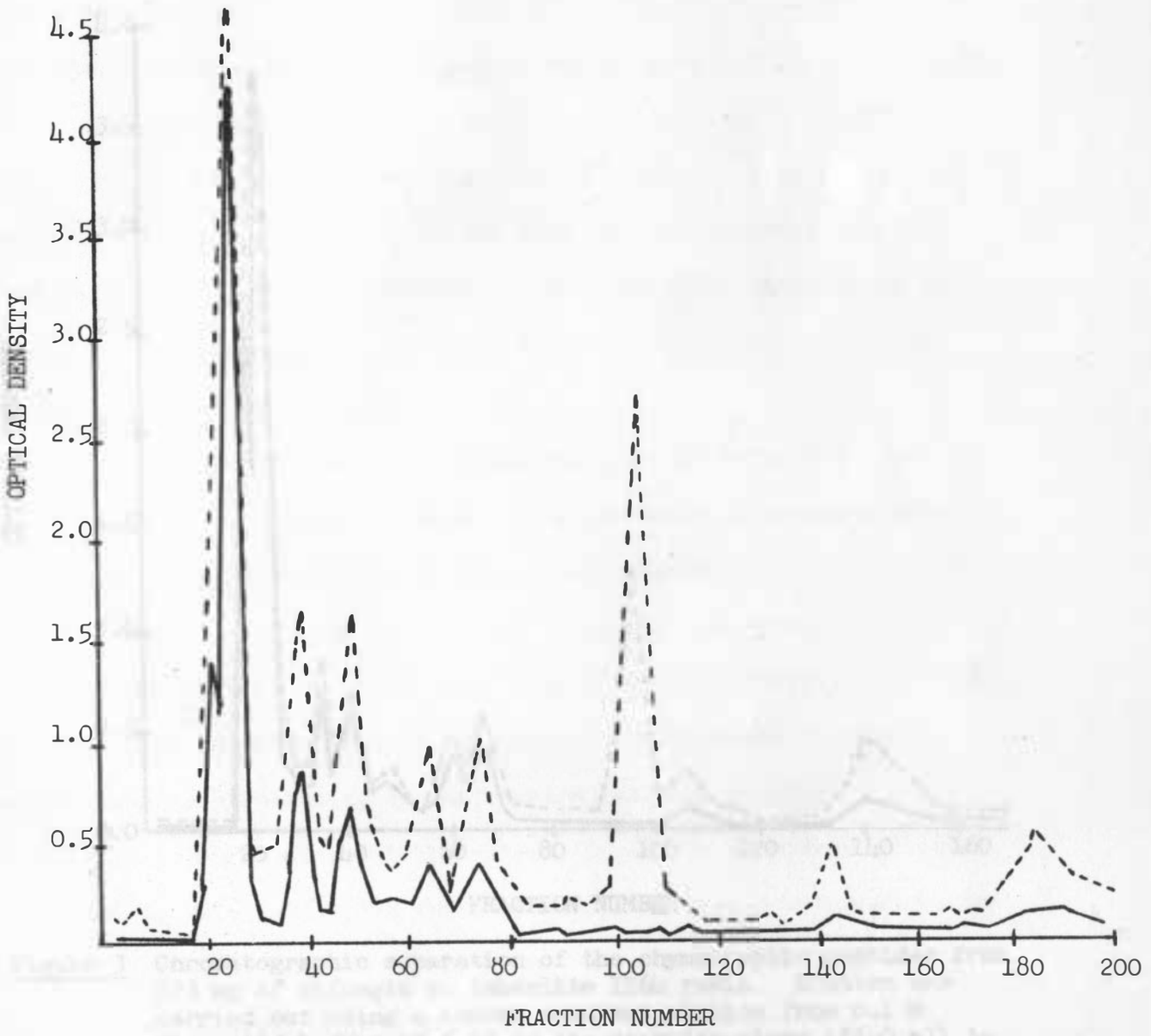

Figure 2 Chromatographic separation of the chymotryptic peptides from 595 mg of nifungin on Amberlite XE64 resin. Elution was carried out using a convex gradient elution from 0.1 M phosphate buffer pH 5.97 in the starting mixer (2140 ml) to 0.9 M phosphate buffer pH 6.02 in the reservoir. Twenty ml fractions were collected. Optical densities were determined at 2760 Å (——) in the ultraviolet, and at 5700 Å (---) in visible region after reaction with ninhydrin. The ninhydrin assay was carried out according to Moore and Stein (40) using 0.5 ml of sample.

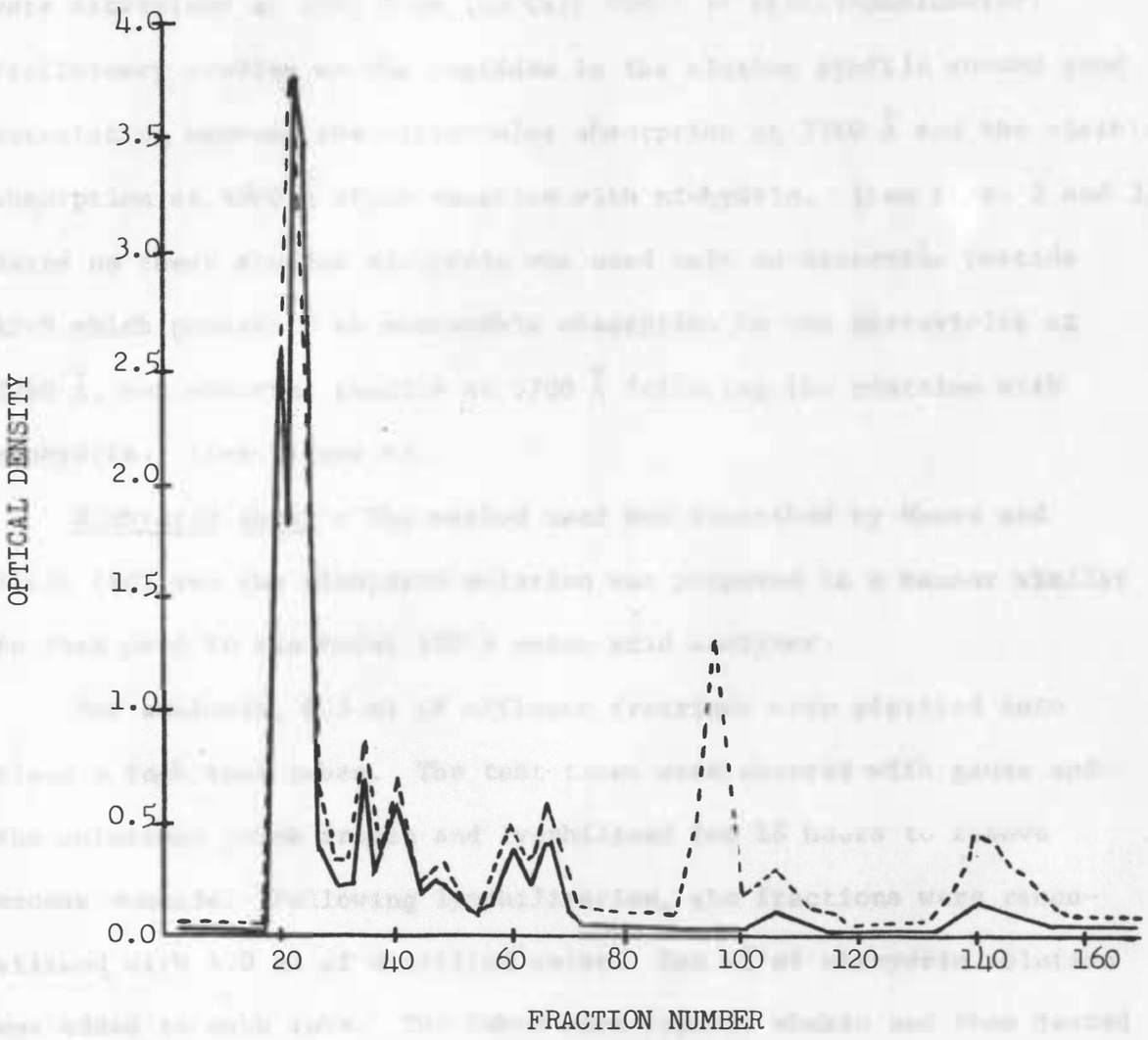

Figure 3 Chromatographic separation of the chymotryptic peptides from 523 mg of nifungin on Amberlite XE64 resin. Elution was carried out using a convex gradient elution from 0.1 M phosphate buffer pH 5.97 in the starting mixer (2140 ml) to 0.9 M phosphate buffer pH 6.02 in the reservoir. Twenty ml fractions were collected. Optical densities were determined at 2760 Å (——) in the ultraviolet, and at 5700 Å (---) in visible region after reaction with ninhydrin. The ninhydrin assay was carried out according to Moore and Stein (40) using 0.5 ml of sample.

were determined at 2760 Å on the Cary Model 14 spectrophotometer. Preliminary studies on the peptides in the elution profile showed good correlation between the ultraviolet absorption at 2760 Å and the visible absorption at 5700 Å after reaction with ninhydrin. (See Figs. 2 and 3). Based on these studies ninhydrin was used only to determine peptide AF-9 which possessed no measurable absorption in the ultraviolet at 2760 Å, but absorbed readily at 5700 Å following the reaction with ninhydrin. (See Figure 4).

Ninhydrin Assay - The method used was described by Moore and Stein (40) and the ninhydrin solution was prepared in a manner similar to that used in the Model 120 B amino acid analyzer.

For analysis, 0.5 ml of effluent fractions were pipetted into clean 3 inch test tubes. The test tubes were covered with gauze and the solutions quick frozen and lyophilized for 16 hours to remove excess ammonia. Following lyophilization, the fractions were reconstitued with 5.0 ml of distilled water. One ml of ninhydrin solution was added to each tube. The tubes were capped, shaken and then heated in a boiling water bath for 15 minutes. After development of the characteristic purple color the contents of the tubes were cooled, and diluted with 5 ml of ethanol-water (1:1) and shaken vigorously, the absorbance of each was determined at 5700 Å on the spectrophotometer.

The Edman Degradation - The Edman degradation of the chymotryptic

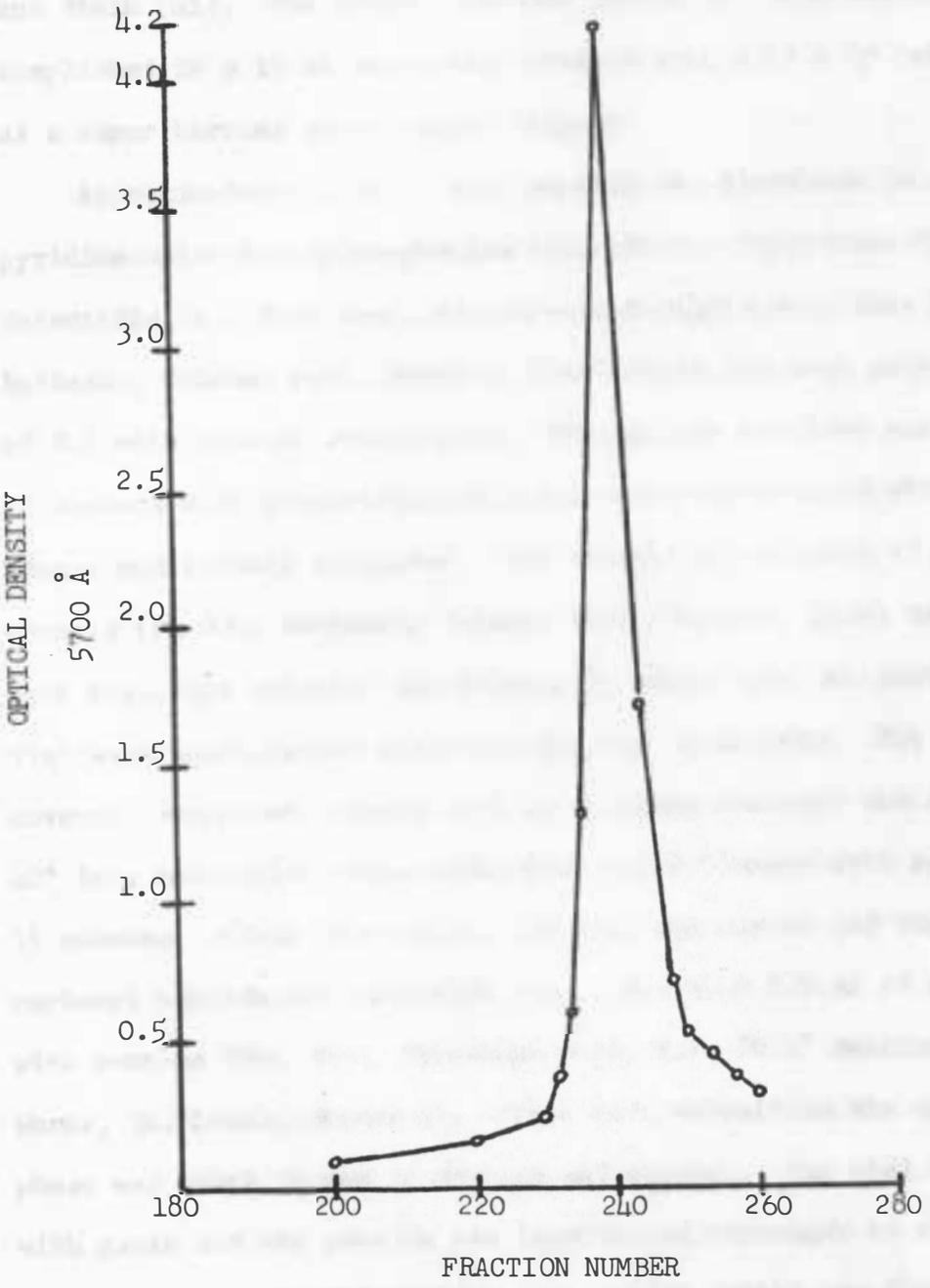

Figure 4 Location of Peptide AF-9 Using the Ninhydrin Method

peptides from nifungin was carried out as described by Daphiede, Moore and Stein (41). The entire reaction series for each peptide was accomplished in a 15 ml serum vial covered with a 1" x 1" teflon square as a vapor barrier and a rubber stopper.

Approximately 10 uM of each peptide was dissolved in 1.0 ml of pyridine-water-N-ethylmorpholine (150:100:29) (Pyridine: P368 Fischer Scientific Co., Fair Lawn, New Jersey: N-ethylmorpholine: P7832, Matheson, Coleman Bell, Norwood, Ohio) which had been adjusted to pH 8.5 with glacial acetic acid. The peptide solution was bathed for 30 seconds with prepurified nitrogen, the vial covered with the teflon square and loosely stoppered. One hundred microliters of phenylisothiocyanate (Px 800, Matheson, Coleman Bell, Norwood, Ohio) was added to each vial, the solution was thoroughly mixed, and the contents of the vial were again bathed with nitrogen for 30 seconds. The vial was covered, stoppered, capped with an aluminum overseal and incubated at 40° in a controlled temperature bath for 2.5 hours with agitation every 15 minutes. After incubation, the vial was opened and the phenylthiocarbamyl peptide was extracted five times with 2.0 ml of water saturated with benzene (No. 3856, Thiophene Free, B.P. 80.1° Mallinckrodt Chemical Works, St. Louis, Missouri). After each extraction the upper solvent phase was quick frozen in dry ice and alcohol. The vial was covered with gauze and the peptide was lyophilized overnight to remove the excess benzene. After lyophilization the peptide sample was dissolved in 0.1 M

of trifluoroacetic acid (No. 7454, B.P. 71-73° Matheson, Coleman Bell, Norwood, Ohio) and bathed again with nitrogen. The vial was sealed again and allowed to remain at room temperature for one hour. The vial was then opened and the excess trifluoroacetic acid was removed under a stream of nitrogen. The time required for this was 2-3 minutes. One ml of 0.4 M acetic acid was added to the vial, and the resulting solution extracted three times with 2.0 ml of benzene saturated with 0.4 M acetic acid to remove the phenylthiohydantoin. After extraction a suitable aliquot was removed from the vial for acid hydrolysis and amino acid analysis. The remainder of the peptide was lyophilized in preparation for the next degradation.

For the amino acid analysis, an aliquot (0.1 - 0.5 ml) of the peptide after each Edman degradation step was placed in a 3 inch test tube. The sample was quick frozen and lyophilized for 16 hours to remove excess solvent. The dried peptide in the tube was dissolved in 2.0 ml of 6N HCl and cooled. The tube was sealed under vacuum and the sample was hydrolyzed for 24 hours at 110° in a gravity convection oven. At the end of the hydrolysis period, the tube was opened, and the sample was transferred quantitatively to a 25 ml round-bottomed flask. Excess hydrochloric acid was removed on the rotary evaporator, and the peptide sample was dissolved in 2.1 ml of pH 2.2 diluting buffer. The peptide sample (1.0 ml/column) was then run on the amino acid analyzer.

The complete amino acid composition of each purified peptide had

been determined previously by amino acid analysis. For each successive Edman degradation of an individual peptide the NH_2-terminal amino acid was removed as the cyclic phenylthiohydantoin in the discarded solvent phase. The original amino acid chromatogram for each peptide was compared quantitatively with the chromatogram obtained after each stage of Edman degradation. The amino acid "subtracted" or greatly reduced on successive chromatograms was assumed to be the NH_2-terminal amino acid for that stage of the degradation. For (n) number of amino acids, the subtractive method was carried out (n-1) times.

-SH Group Determination - The free -SH groups in unoxidized nifungin were determined by spectrophotometric titration of the peptide at pH 7.0 and 4.6 using p-hydroxymercuribenzoate (42).

For analysis, 3.0 ml of 6.4×10^{-5} N p-hydroxymercuribenzoate in 0.05 M phosphate buffer pH 7.0, previously standardized with 5.7×10^{-3} N reduced glutathione, was pipetted into the 1.0 cm quartz sample cuvette in the Beckman DU spectrophotometer. An equal volume of 0.05 M phosphate buffer pH 7.0 alone was placed in the matching reference cuvette, and the absorption of the p-hydroxymercuribenzoate was determined at 250 mu. To titrate, identical increments of the .0057 M peptide solution were added to both the cuvette containing the known quantity of buffered p-hydroxymercuribenzoate and to the reference cuvette containing buffer. After mixing, the change in absorption with the addition of peptide was measured at 250 mu and recorded. With the addition of peptide increments, resulting absorption readings were corrected for dilution. The end point of the titration is normally reached when the slope of the absorption curve becomes zero. The titration was repeated at pH 4.6 to determine the presence of total -SH groups.

RESULTS

Amino Acid Composition of Purified Nifungin - The amino acid composition of nifungin after acid hydrolysis is given in Table I. For each hydrolysis period the number of micromoles of each amino acid residue present per mg of nifungin was calculated. Based on time study results either a maximum value per residue or an average extrapolated value for the number of micromoles per weight of sample was determined (Footnote c, Table I).

The amino acid residues accounted for slightly over 85% of the peptide, with ash and moisture content contributing an additional 1.5% to the composition. Approximately 13% was undetermined. Unpublished studies ruled out the presence of phosphate ion, reducing sugars or tryptophan. The appearance of an unknown peak in each of the amino acid chromatograms was continually noted. The unknown peak was located in the chromatograms from the 15 cm chromatographic column in the region immediately preceding the lysine residue. The identity of the material responsible for this peak was not determined for this investigation.

Table I

Amino acid composition of nifungin after acid hydrolysis

Amino acid residue	micromoles of amino acid residue per mg nifungin hydrolysates					Amino acid residues per mole of nifungin (minimum)		Mg amino acid residue per mg nifungin
	20 hr	40 hr	70 hr	120 hr	Average or extrapolated value [c]	Calculated [d]	Nearest whole number	
Lysine (suppressed)[a]	1.6860	1.6942	1.7220	1.7170	1.7048	11.76	12	---
Lysine (normal)[b]	1.7908	1.8453	1.7608	1.8043	1.8003	12.24	12	0.23070
Histidine	0.000	0.000	0.000	0.000	0.000	0.00	0	0.00000
Arginine	0.1465	0.1464	0.1398	0.1389	0.1484*	1.01	1	0.02318
Aspartic acid	0.7430	0.7394	0.7335	0.7417	0.7394	5.03	5	0.08510
Threonine	0.2643	0.2371	0.1954	0.1160	0.2984*	2.03	2	0.03017
Serine	0.2282	0.1709	0.1107	0.0344	0.3000*	2.04	2	0.02612
Glutamic acid	0.2974	0.2893	0.2890	0.2919	0.2919	1.98	2	0.03769
Proline	0.1660	0.1685	0.1714	0.1718	0.1694	1.15	1	0.01645
Glycine	0.5917	0.5914	0.5960	0.6006	0.5948	4.04	4	0.03394
Alanine	0.5876	0.5966	0.5960	0.6011	0.5953	4.05	4	0.04231
Half cystine	1.1324	0.9287	0.4932	0.5133	1.1763*	8.00	8	0.12013
Valine	0.1489	0.1500	0.1500	0.1473	0.1491	1.02	1	0.01478

Methionine	0.0000	0.0000	0.0000	0.0000	0.0000	0.00	0	0.00000
Isoleucine	0.2959	0.2953	0.3002	0.2890	0.2951	2.00	2	0.03339
Leucine	0.0000	0.0000	0.0000	0.0000	0.0000	0.00	0	0.00000
Tyrosine	0.8142	0.7801	0.7457	0.5696	0.8824	6.00	6	0.14398
Phenyl-alanine	0.1411	0.1388	0.1367	0.1385	0.1388	0.94	1	0.02043
							51	0.85837
Tryptophan	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		
Ammonia	0.6782	0.7466	1.1467	1.2096	0.5756			
				Corrected - -	0.5405	3.67	4	

Footnotes to Table

a Data from suppressed 570 nm

b Data from normal 570 obtained by dilution of sample

c Except for those values marked by an asterisk, the data in this column are averages. Those values marked by an asterisk were obtained by extrapolation to zero time.

d Since the molecular weight of the antifungal peptide was not known, this column was calculated by assuming from inspection of the data that the peptide contained the number of residues indicated below, dividing the micromoles of the amino acid per milligram of nifungin by this number and taking the average of the values obtained.

Amino Acid	Assumed uM/mg	Factor
Lysine	12	0.1500
Aspartic Acid	5	0.1479
Glutamic Acid	2	0.1460
Glycine	4	0.1487
Alanine	4	0.1488
Valine	1	0.1491
Isoleucine	2	0.1476
Phenylalanine	1	0.1388
	Average - -	0.1471

Separation and Purification of the Chymotryptic Peptides - The results of the chromatographic separation of the chymotryptic peptides from oxidized nifungin are shown in Figure 5.

The acidic peptides AF-1 through AF-6 were eluted in rapid succession after the column was started. Peptide AF-1 was a yellowish colored peak which appeared in those eluates corresponding to the void volume of the ion exchange column. All of the other peptides were colorless. Peptides AF-1 through AF-6 all required further purification by ion exchange chromatography.

The peptides AF-7 through AF-11 appeared to be resolved well enough that further purification was unnecessary. The tubes containing each of these peptides were therefore pooled. Each pool included those eluates on both sides of the peak whose optical density at 2760 Å was equal to or greater than the optical density representing one-half the height of the peak tube. The pooling was done in this manner to obtain the most homogenous sampling possible for each of the peptides. The volume of each pool was determined, and the solution was filtered through a 28 mm millipore filter pad, using a small Buchner funnel. Each peptide pool was then transferred quantitatively to a previously tared 30 ml sterile screw-cap tube. The tubes were immediately covered with two layers of gauze and the solutions shell frozen in an alcohol-dry ice bath, and lyophilized for forty-eight hours. After lyophilization, the dried peptides were immediately weighed, and placed under refrigeration.

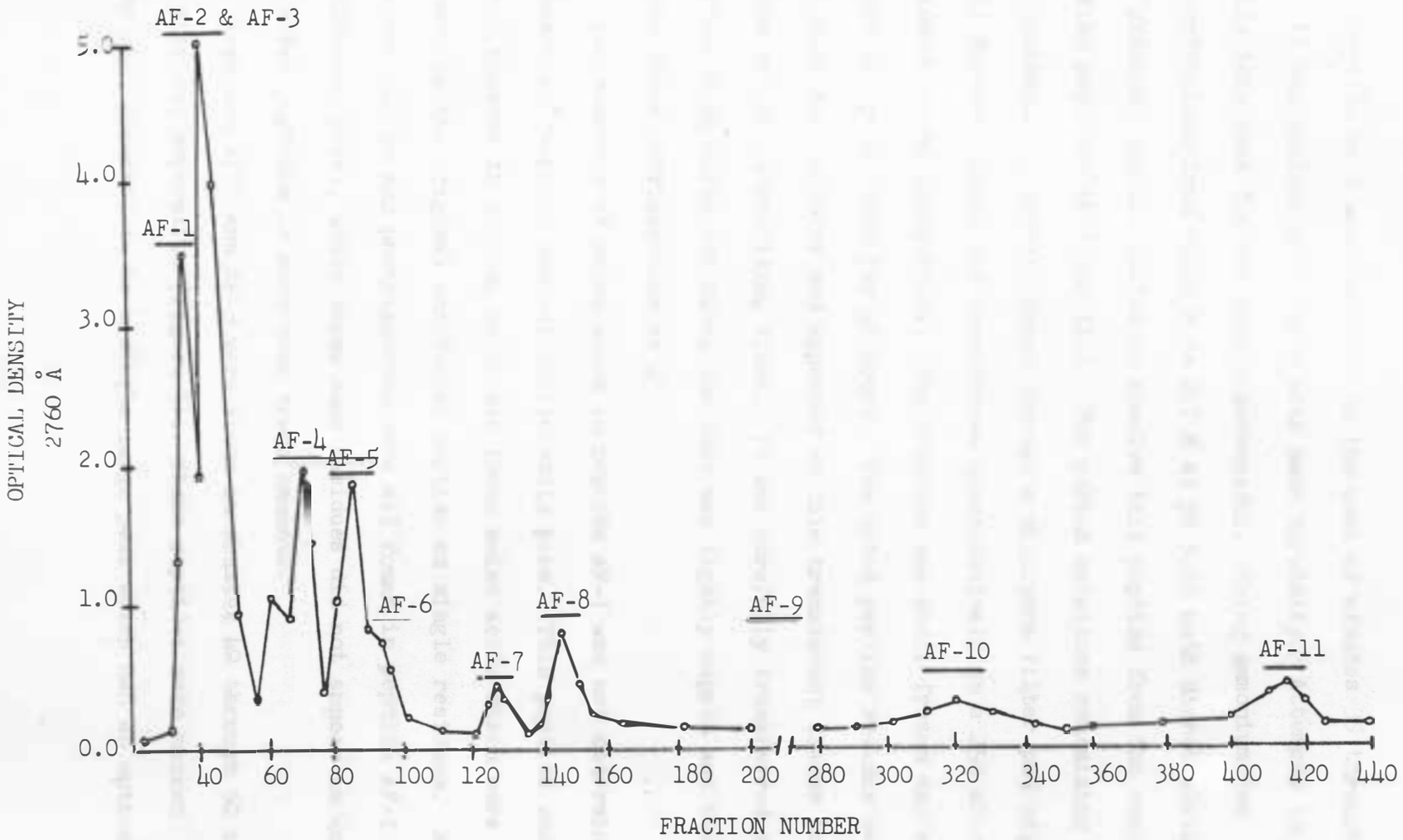

Figure 5 The Chromatographic Separation of the Chymotryptic Peptides from nifungin on Amberlite XE64 Ion Exchange Resin

Peptide AF-1 was contained in the pool of eluates 35 through 43. It was yellowish in color with some turbidity. Attempts to purify this peak further were unsuccessful. Using ammonium ion concentrations from 0.001 M to 0.1 M at pH 5.95 both direct elution and gradient elution failed to resolve this peptide from the contaminating peptides AF-2 and AF-3. The pooled solutions containing it and the contaminants were filtered through a millipore filter pad using a small Buchner funnel and transferred quantitatively to a 250 ml round-bottomed lyophilizing flask. The solution was shell frozen and subsequently lyophilized for 48 hours. The dried peptide residue was yellowish tan in color and appeared as thin translucent flakes in the bottom of the lyophilizing flask. It was carefully transferred to a sterile 30 ml screw cap tube; the tube was tightly capped and then stored under refrigeration at 4°.

The sequence of amino acids in peptide AF-1 was not determined because the compound was not sufficiently pure. This peptide was useful however in helping to locate those amino acids which were present in the original antifungal peptide as single residues. Arginine, proline, valine and phenylalanine were all found in peptide AF-1 at a significant level, while these same residues did not appear in any of the other peptides in more than trace amounts.

Peptides AF-2 and AF-3 were found in eluates 40 through 50 in the original separation (Figure 5). These peptides were masked under what appeared to be a single large peak which had an optical

density of 5.0 at 2760 Å. The acidity of this peptide fraction, based on its position in the elution pattern, suggested that the strongly acidic sulfonic acid resin, Dowex 50 W x 2 (Dow Chemical Co.) could be used to separate any peptides present. Preliminary studies on the Dowex 50 W x 2 using ammonium acetate buffers at pH 3.8 were successful in achieving this separation.

The pool (120 ml) which contained the combined peptides AF-2 and AF-3 was quantitatively transferred to a 500 ml round-bottomed flask and evaporated to dryness on the Buchler rotary evaporator. The dried residue was reconstituted in 20.0 ml of 0.02 M ammonium acetate buffer at pH 3.8. This sample was immediately applied to the top of a 2.0 x 60 cm Dowex 50 W x 2 ion exchange column which had been equilibrated with 0.02 M ammonium acetate buffer pH 3.8. The solution was allowed to percolate into the resin matrix. The resin surface was then rinsed twice with 1.0 ml of the equilibrating buffer. The column was eluted with a gradient system consisting of 0.02 M ammonium acetate buffer pH 3.8 in the mixer (1000 ml) and 0.3 M ammonium acetate buffer pH 3.8 in the reservoir. Ten ml eluates were collected using the Gilson automatic fraction collector at a flow rate of 60 ml per hour. A total of 60 fractions was collected. The optical density of each fraction was determined at 2760 Å and plotted as a function of the eluates collected (Figure 6).

Peptide AF-4 was originally believed to be contaminated with another peptide fragment. Because of this, attempted purification

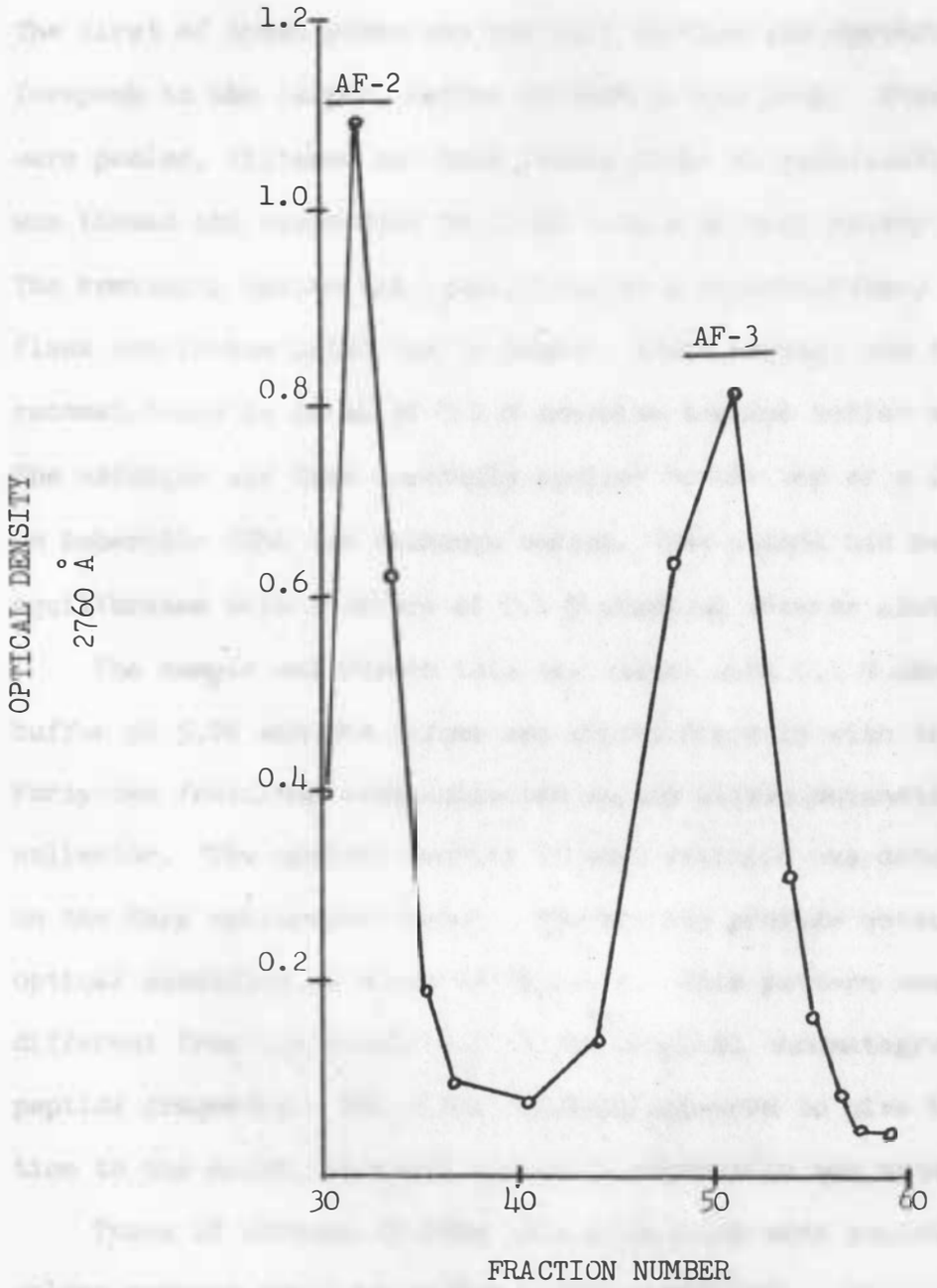

Figure 6 Chromatographic Separation of Peptide AF-2 and AF-3 on Dowex 50W x 2 Ion Exchange Resin

of the peptides was carried out as follows.

Eluates 54 through 75 (Figure 5) appeared to include two peaks. The first of these peaks was not well defined and appeared as a small forepeak to the larger, better defined second peak. These 21 eluates were pooled, filtered and held frozen prior to purification. The pool was thawed and evaporated to 25 ml with a Buchler rotary evaporator. The remaining volume was transferred to a round-bottomed lyophilizing flask and freeze dried for 24 hours. After drying, the residue was reconstituted in 10 ml of 0.1 M ammonium acetate buffer at pH 5.92. The solution was then carefully applied to the top of a 2.0 cm x 60 cm Amberlite XE64 ion exchange column. The column had been previously equilibrated with 2 liters of 0.1 M ammonium acetate eluting buffer.

The sample was rinsed into the column with 0.1 M ammonium acetate buffer pH 5.92 and the column was eluted directly with the same buffer. Forty-two fractions were collected on the Gilson automatic fraction collector. The optical density of each fraction was determined at 2760 Å on the Cary spectrophotometer. The elution profile obtained from the optical densities is shown in Figure 7. This pattern was not essentially different from the separation in the original chromatogram of the peptide fragments. The direct elution appeared to give better definition to the peaks, however, a further separation was attempted.

Tubes 18 through 28 from this separation were pooled and the volume reduced to 10 ml on the rotary evaporator. The solution was quantitatively transferred to the top of another 2.0 cm x 60 cm

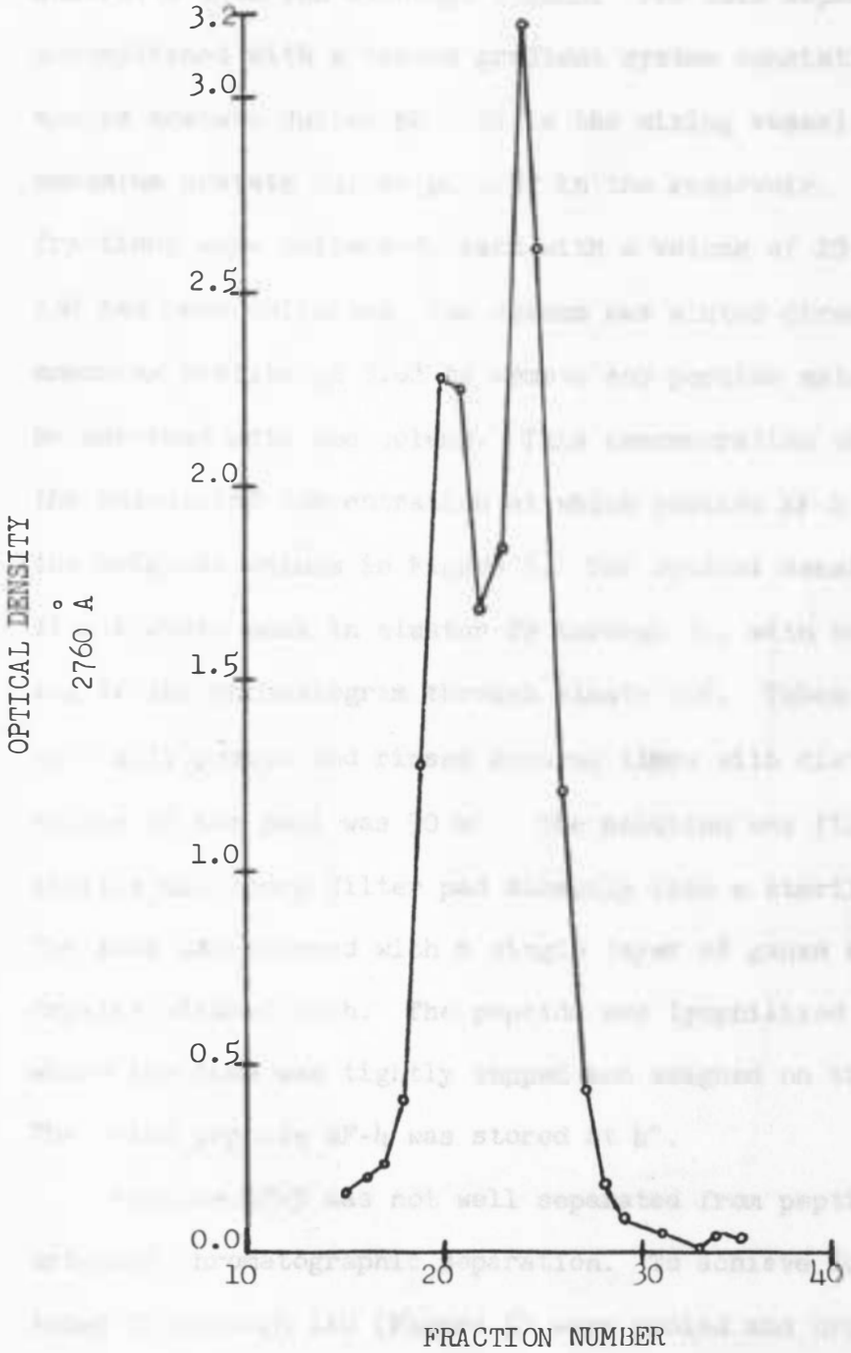

Figure 7 Initial Separation of Peptide AF-4 on Amberlite XE64 Ion Exchange Resin

Amberlite XE64 ion exchange column. For this separation, elution was accomplished with a convex gradient system consisting of 0.01 M ammonium acetate buffer pH 5.82 in the mixing vessel (1,000 ml) and 0.1 M ammonium acetate buffer pH 5.82 in the reservoir. A total of 168 fractions were collected, each with a volume of 10 ml. After fraction 130 had been collected, the column was eluted directly with 0.2 M ammonium acetate pH 5.82 to remove any peptide material which might be adsorbed onto the column. This concentration was selected based on the calculated concentration at which peptide AF-4 had been eluted from the original column in Figure 5. The optical density appeared as a single sharp peak in eluates 29 through 34, with no other peaks appearing in the chromatogram through eluate 168. Tubes 29 through 34 were carefully pooled and rinsed several times with distilled water the final volume of the pool was 50 ml. The solution was filtered through a sterile millipore filter pad directly into a sterile tared screw cap tube. The tube was covered with a single layer of gauze and quick frozen in a dry-ice alcohol bath. The peptide was lyophilized for 48 hours after which the tube was tightly capped and weighed on the analytical balance. The dried peptide AF-4 was stored at 4°.

Peptide AF-5 was not well separated from peptide AF-6 in the original chromatographic separation. To achieve further purification, tubes 76 through 110 (Figure 5) were pooled and dried under vacuum on the Buchler rotary evaporator. The dried residue was redissolved in 20 ml of deionized water and transferred quantitatively to a 250 ml

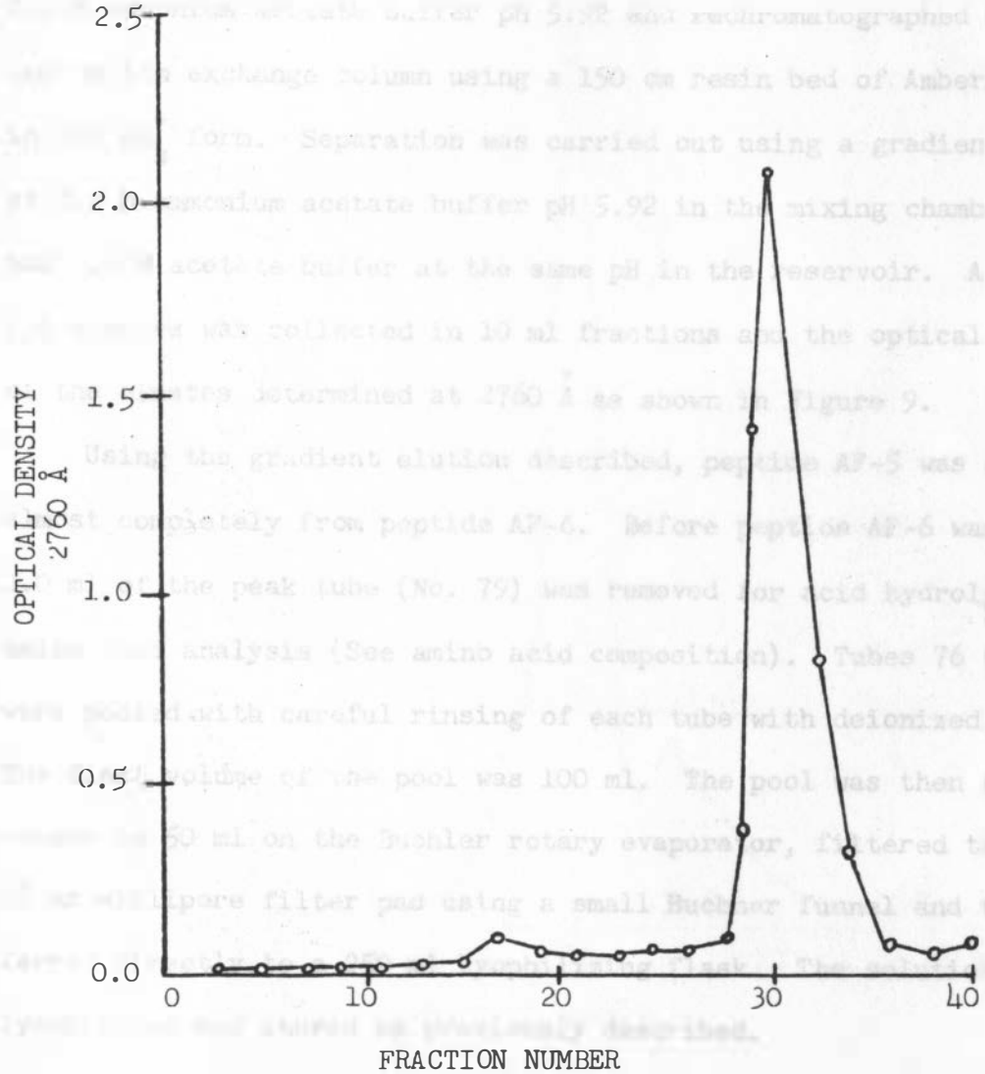

Figure 8 Final Chromatographic Separation of Peptide AF-4 on Amberlite XE64 Ion Exchange Resin

lyophilizing flask. The solution was shell frozen in the flask and lyophilized for 24 hours. After drying, the flasks were stoppered and held under refrigeration.

The dried AF-5 and AF-6 peptides were reconstituted in 10 ml of 0.2 M ammonium acetate buffer pH 5.92 and rechromatographed on a 2.2 x 160 cm ion exchange column using a 150 cm resin bed of Amberlite XE64 in the NH_4^+ form. Separation was carried out using a gradient elution of 0.2 M ammonium acetate buffer pH 5.92 in the mixing chamber (2.200 ml) and 0.6 M acetate buffer at the same pH in the reservoir. A total of 136 eluates was collected in 10 ml fractions and the optical densities of the eluates determined at 2760 Å as shown in Figure 9.

Using the gradient elution described, peptide AF-5 was separated almost completely from peptide AF-6. Before peptide AF-6 was pooled, 1.0 ml of the peak tube (No. 79) was removed for acid hydrolysis and amino acid analysis (See amino acid composition). Tubes 76 through 84 were pooled with careful rinsing of each tube with deionized water. The final volume of the pool was 100 ml. The pool was then reduced in volume to 50 ml on the Buchler rotary evaporator, filtered through a 28 mm millipore filter pad using a small Buchner funnel and transferred directly to a 250 ml lyophilizing flask. The solution was lyophilized and stored as previously described.

Peptide AF-6 was prepared by pooling eluates 86 through 102, (Figure 9) filtering through a millipore filter pad and lyophilizing for 20 hours. The dried residue was dissolved in 10 ml of 0.2 M

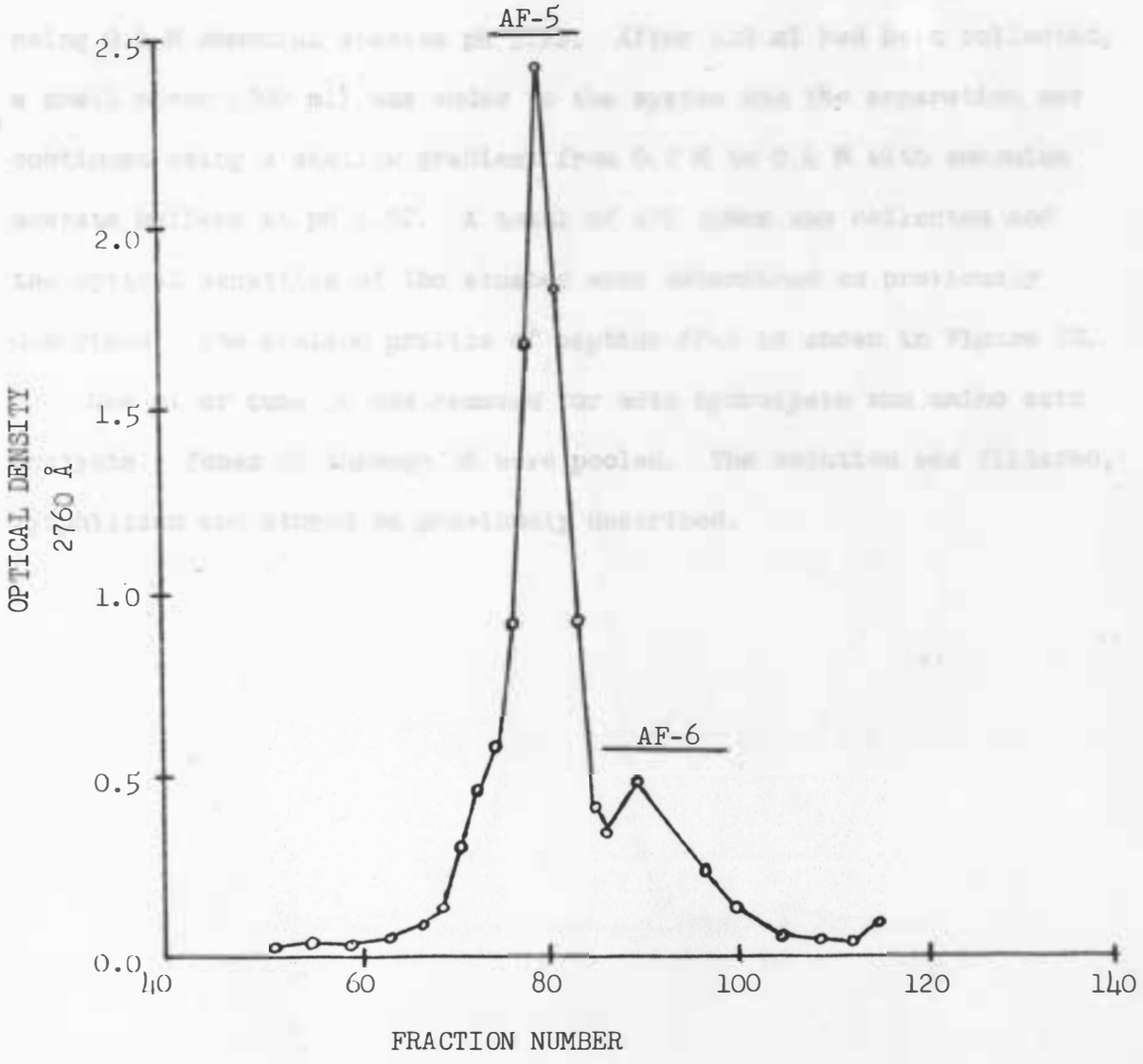

Figure 9 Chromatographic Separation of Peptides AF-5 and AF-6 on Amberlite XE64 Ion Exchange Resin

ammonium acetate buffer pH 5.92 and chromatographed on a 2.0 x 60 cm column of Amberlite XE64 in the NH_4^+ form. The purification of the peptide AF-6 was carried out with an initial direct elution of the column using 0.2 M ammonium acetate pH 5.92. After 110 ml had been collected, a small mixer (200 ml) was added to the system and the separation was continued using a shallow gradient from 0.2 M to 0.4 M with ammonium acetate buffers at pH 5.92. A total of 105 tubes was collected and the optical densities of the eluates were determined as previously described. The elution profile of peptide AF-6 is shown in Figure 10.

One ml of tube 36 was removed for acid hydrolysis and amino acid analysis. Tubes 30 through 36 were pooled. The solution was filtered, lyophilized and stored as previously described.

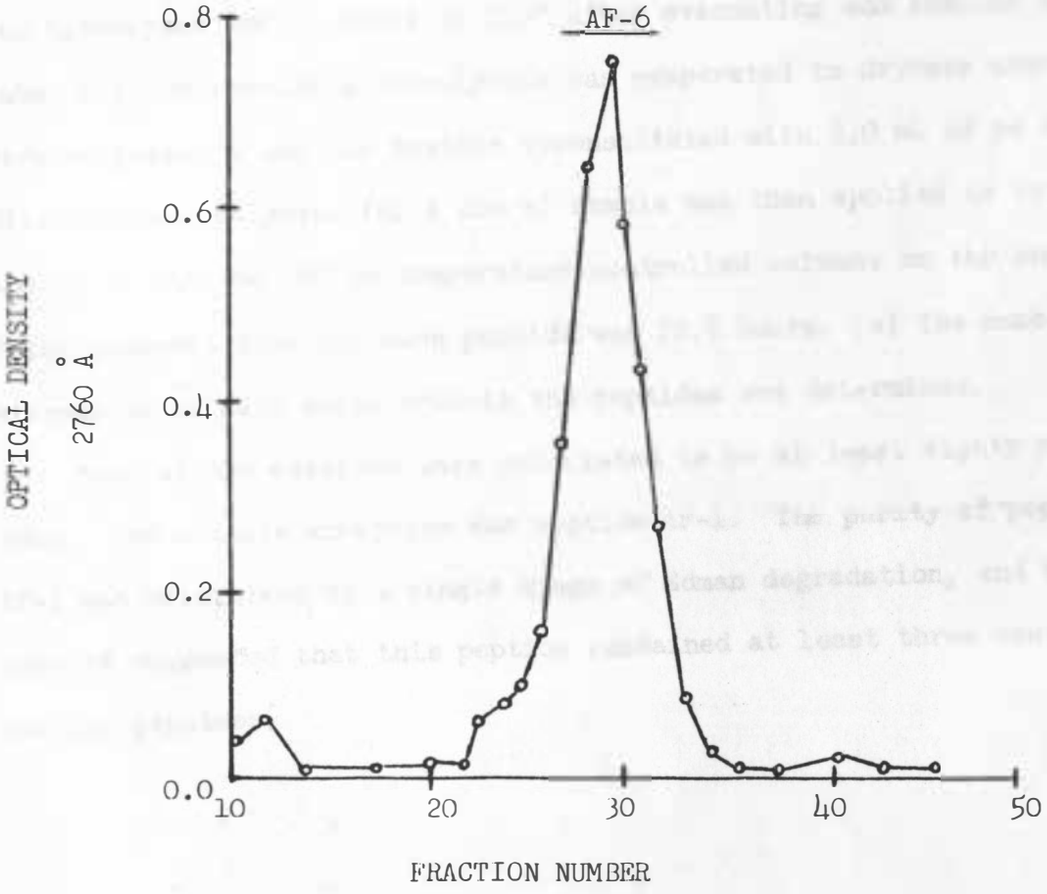

Figure 10 Final Purification of Peptide AF-6 on Amberlite XE64 Ion Exchange Resin

Amino Acid Composition of the Chymotryptic Peptides - The amino acid composition of the chymotryptic peptides from nifungin is given in Table II . The acid hydrolysis and analysis of each peptide was accomplished as follows: (a) Ten percent (1.0 ml) from each of the peak tubes was lyophilized overnight in a clean tube to remove excess ammonia. (b) The dried peptide was then dissolved in 2.0 ml of 6 N HCl and hydrolyzed for 24 hours at 110° after evacuating and sealing the tube. (c) The resulting hydrolysate was evaporated to dryness under reduced pressure and the residue reconstituted with 2.0 ml of pH 2.2 diluting for analysis. (d) A one ml sample was then applied to both the 15 cm and the 150 cm temperature controlled columns on the analyzer. Total analysis time for each peptide was 22.5 hours. (e) The number of micromoles of each amino acid in the peptides was determined.

Most of the peptides were calculated to be at least eighty percent pure. The notable exception was peptide AF-1. The purity of peptide AF-1 was determined by a single stage of Edman degradation, and the results suggested that this peptide contained at least three contaminating peptides.

Table II

Amino acid composition of peptides obtained by chymotryptic hydrolysis of oxidized nifungin

The composition of each peptide is given in terms of micromoles of the constituent amino acids in the aliquot analyzed. Values for the major constituents are underlined. Values for residues present in less than 0.01 of a micromole are omitted. Molar ratios are shown in parentheses and are calculated without any correction for destruction of amino acids during hydrolysis. The peptides are numbered as in the test.

Amino acid residue	Peptide AF1	Peptide AF2	Peptide AF3	Peptide AF4	Peptide AF5	Peptide AF6	Peptide AF7	Peptide AF8	Peptide AF9	Peptide AF10	Peptide AF11
Lysine	0.44	0.38	0.19(1)	0.02	0.25(1)	0.15(1)	0.23(2)	0.45(2)	0.32(1)	0.15	0.53(4)
Histidine	--	--	--	--	--	--	--	--	--	--	--
Arginine	0.08	--	--	--	--	--	--	--	--	--	--
Cysteic Acid	0.55	0.18	0.35(2)	0.01	0.26(1)	0.15(1)	0.26(2)	0.51(2)	0.01	0.05	0.13(1)
Aspartic Acid	0.29	0.14	--	--	0.04	0.12(1)	0.01	0.02	0.01	0.09	0.23(2)
Threonine	0.50	--	--	0.57(1)	0.45(2)	0.05	--	--	--	--	--
Serine	0.12	--	--	0.01	0.04	--	--	--	0.04	--	--
Glutamic Acid	0.14	--	--	--	0.02	--	0.01	--	0.28(1)	--	--
Proline	0.10	--	--	--	--	--	--	--	--	--	--
Glycine	0.19	--	--	0.01	0.13	0.12(1)	0.12(1)	0.23(1)	0.02	--	--
Alanine	0.17	0.15	0.13(1)	0.15(1)	0.50(2)	0.03	0.01	--	0.27(1)	--	--

Table II continued on page 47

Table II continued

Amino acid residue	Peptide AF1	Peptide AF2	Peptide AF3	Peptide AF4	Peptide AF5	Peptide AF6	Peptide AF7	Peptide AF8	Peptide AF9	Peptide AF10	Peptide AF11
Half Cystine	--	--	--	--	--	--	--	--	--	--	--
Valine	0.05	--	--	--	--	--	--	--	--	--	--
Isoleucine	0.07	--	0.15(1)	0.05	0.05	--	--	--	--	0.07	0.12(1)
Leucine	--	--	--	--	--	--	--	--	--	--	--
Tyrosine	0.12	0.19	--	0.57(1)	0.02	--	0.09(1)	--	--	0.03	--
Phenylalanine	0.13	--	--	--	--	--	--	--	--	--	--

The Edman Degradation - The usual manner of presenting amino acid sequence data is to express the results in terms of the molar ratio of amino acids remaining after successive degradations. In this investigation however, the data will be expressed as the number of micromoles of amino acids present after each stage of degradation. This method of presentation was necessitated by the lack of uniformity in sampling after each degradation. The lack of uniformity was not intentional, and a detailed review of sampling technique will be given in the discussion section.

The amino acid sequence of those peptides selected for analysis in this investigation are presented in the following order: (a) The amino acid composition, without regard to order, is given first. (b) Directly below the composition is the number of residues of each acid present as shown in Table II. (c) The Edman degradation is then listed according to the number of significant stages in the sequence analysis. The results of each stage are expressed in micromolar quantities. The amino acid "subtracted" from the peptide after each sequence stage is identified by underlining its value. (d) The amino acid sequence is then given for the peptide. Any pertinent information concerning the peptide follows the amino acid sequence.

From the eleven chymotryptic peptides originally isolated, six were selected for amino acid sequence studies using the Edman degradation. These included peptides AF-3, AF-4, AF-6, AF-7, AF-8 and AF-9.

Peptide AF-3

Amino Acid Composition	Lys	$CySO_3H$	Ala	Ile
Number of Residues	2.0	2.0	1.0	1.0
Edman Degradation				
Stage one	.35	.63	.01	.12
Stage two	.27	.53	.00	.00
Stage three	.15	.30	.00	.00
Stage four	a	a	a	a
Stage five	.17	.62	.00	.00
Amino Acid Sequence				

Ala-Ile ($CySO_3H$, $CySO_3H$, Lys, Lys)

a. Data not available. Recorder malfunction.

The stepwise removal of the alanine and isoleucine residues from AF-3 was evident from the data for the Edman degradation. Interpretation of the data beyond the second stage of the degradation was difficult, therefore only two residues were assigned positions in the peptide.

Peptide AF-4

Amino Acid Composition	Thr	Ala	Tyr
Number of Residues	1.0	1.0	1.0
Edman Degradation			
Stage one	.19	.02	.20
Stage two	.05	.00	.43
Amino Acid Sequence			
		Ala-Thr-Tyr	

The position of alanine and threonine in peptide AF-4 is clearly shown by the data. The specificity of alpha chymotrypsin for the carboxyl side of the aromatic amino acids suggested tyrosine as the COOH-terminal residue, and the data presented verify this.

Peptide AF-6

Amino Acid Composition	Lys	Cys	Asp	Gly
Number of Residues	1.0	1.0	1.0	1.0
Edman Degradation				
Stage one	.09	.08	.01	.06
Stage two	.10	.11	.02	.04
Stage three	.02	.05	.00	.02
Amino Acid Sequence				

Asp-Gly-Lys-$CySO_3H$

Peptide AF-6 was the trailing peptide initially separated from AF-5. The sequence of amino acids in this peptide was readily defined by the Edman degradation. The COOH-terminal position of cysteic acid is unusual based on the specificity of alpha chymotrypsin.

Peptide AF-7

Amino Acid Composition	Lys	$CySO_3H$	Gly	Tyr
Number of Residues	1.0	2.0	1.0	1.0
Edman Degradation				
Stage one	.21	.46	.09	.20
Stage two	.09	.59	.09	.24
Amino Acid Sequence				

Gly-Lys ($CySO_3H$, $CySO_3H$, Tyr)

The position of glycine as the NH_2-terminal residue in peptide AF-7, and the position of the lysine residue as the penultimate residue is well documented by the data presented. The cysteic acid residues in the peptide have not been assigned positions in the sequence. Assuming that tyrosine was the COOH-terminal in the peptide, the cysteic acid residues would follow after lysine and before the tyrosine terminus, as shown.

Peptide AF-8

Amino Acid Composition	Lys	$CySO_3H$	Gly
Number of Residues	2.0	2.0	1.0
Edman Degradation			
Stage one	.12	.26	.11
Stage two	.23	.61	.11
Stage three	.05	.51	.05
Amino Acid Sequence			

Lys-Gly-$CySO_3H$-$CySO_3H$

Following the first stage of Edman degradation, only cysteic acid remained in a 2:1 ratio with glycine. This indicated that lysine was the NH_2-terminal residue in peptide AF-8. After the second stage degradation, both lysine and cysteic acid showed increases because of sampling methods, but the glycine level did not increase proportionately. On this basis glycine was assigned the second position in the amino acid sequence. The third stage of analysis indicated that another lysine was removed from the peptide. The final two positions in the peptide were therefore assigned to the remaining cysteic acid residues.

Peptide AF-9

Amino Acid Composition	Lys	Glu	Ala
Number of Residues	1.0	1.0	1.0
Edman Degradation			
Stage one	.02	.52	.51
Stage two	.00	.55	.05
Amino Acid Sequence			

Lys-Ala-Glu

This peptide was the only one found to possess no absorption in the ultraviolet range at 2760 Å. Its presence was determined by the ninhydrin method with its absorbance measured at 5700 Å.

-SH Group Determination - The results of the determination of the reactive -SH groups in the unoxidized nifungin are shown in Table III.

Table III

Titration of 3 ml of 6.4×10^{-5}M p-Hydroxymercuribenzoate with nifungin

pH	5.7×10^{-3}M nifungin, ml	O.D. @ 250 nm	O.D. corrected for dilution
	0.000	0.293	0.293
7.0	0.025	0.290	0.292
	0.125	0.289	0.301
	0.000	0.178	0.178
4.6	0.100	0.178	0.184
	0.100 after 50 minutes	0.179	0.185

At pH 7.0 there was no tendency in the absorption to change with time following each addition of the peptide solution to the cuvettes. Similarly, at pH 4.6 only a 0.001 change in optical density occurred after the fifty minute reaction period. Since the molarity of the antifungal peptide was equal to that of the reduced glutathione (5.7×10^{-3}M), the 0.100 ml used at pH 4.6 should have completed the titration. On the basis of this analysis, it was concluded that nifungin possessed no reactive -SH groups, and the eight half-cystine residues found in the peptide were bound through four disulfide bonds.

DISCUSSION

The disruption of disulfide bonds by oxidation with performic acid has been a standard technique in protein chemistry since its introduction by Sanger (23). The value of the method is that cysteic acid and the oxidation products of cystine contain sulfur in a stable oxidation stage. In this stable state these amino acids can be determined with precision in acid hydrolyzates of proteins and peptides. The major shortcomings of the method were not a problem in this investigation because nifungin contained no tryptophan residues. The reagent is capable of transforming tryptophan residues into a number of derivatives in which the indole ring has been ruptured (29).

Performic acid is a powerful oxidizing agent. In addition to the sulfur containing amino acids, it will oxidize the phenolic group of tyrosine and the hydroxyl group of threonine and serine. Hirs noted that performic acid involves the oxidation of cysteine to cysteic acid (29). The conversion of the disulfide bond in cystine to cysteic acid proceeds at a somewhat slower rate. The modification of tyrosine proceeds at a rate slower than either of the other conversions.

The presence of halide ions in the peptide reaction mixture interferes with the oxidation procedure. The reagent is capable of converting halides to the corresponding halogen. These halogens subsequently attack tyrosine with the formation of a mixture of halotyrosine derivatives.

The Dowex 50 x 2 and the companion Dowex 50 W x 2 cation exchange resins have been used most frequently for the separation of peptide mixtures. These resins however, have some disadvantages. Most notable among these is their strong adsorption capacity for peptides which contain the cyclic amino acid residues, i.e. tyrosine and phenylalanine. The use of alpha chymotrypsin in this investigation made the recovery of the peptides with these amino acids critical to the sequence determination. For this reason the Dowex resins were not employed for the separation of the chymotryptic peptides.

The resin chosen for this investigation was the weakly acidic carboxylic exchange resin, Amberlite XE64 (also known as IRC50). This resin has limited use for peptide separation, and has been employed primarily for the fractionation of proteins (43). It has also proven useful in the separation of either large peptides of highly basic peptides not readily fractionated on ion exchange resins similar to Dowex 50 x 2.

The choice of Amberlite XE64 for the separation of peptides in this investigation was based on the following considerations. The Amberlite XE64 resin had been successfully used in the MDPH laboratory for the isolation and purification of the antifungal peptide. Conditions for the effective use of the resin had been studied, and suitable companion buffer systems had been investigated. The large number of basic and aromatic amino acid residues in the antifungal peptide necessitated the use of an ion exchange resin whose primary exchange groups possessed a

relatively high pKa. The carboxylic acid groups of the XE64 resin are half-ionized at pH 6.0, and complete dissociation occurs at pH level greater than 7.0. The use of this resin is therefore practical with peptides whose net charge is essentially positive in the pH 5.7 range. (c) Preliminary studies using Dowex 50 x 2 for separation of the peptides from nifungin confirmed the strong adsorption of some nifungin peptides to this resin. The presence of ninhydrin positive materials on the Dowex 50 x 2 resin following elution with phosphate buffers at strengths up to 0.9 M made its use appear impractical for this investigation.

Alpha chymotrypsin catalyzes the hydrolysis of the peptide bonds between the COOH group of tyrosine, phenylalanine or tryptophan and the NH_2 group of the adjacent amino acid (44). It also catalyzes the hydrolysis of esters, amides and other acyl derivatives. Slower hydrolysis has also been found to occur at the carboxyl group of other amino acids including lysine, serine, threonine and cysteic acid. The rate at which this enzyme attacks susceptable sites on the substrate is influenced by the amino acid residue adjacent to the susceptable bond and the conditions used to carry out the hydrolysis.

In this investigation the specificity of alpha chymotrypsin was found to be more broad than anticipated. Four of the six peptides examined in the study possessed no tyrosine or phenylalanine residue in their structure.

Preliminary experiments separating the chymotryptic peptides from

nifungin on Amberlite XE64 were followed by measurement of both the ultraviolet and visible absorption of the eluates at 2760 Å and 5700 Å. The absorption measurements at 5700 Å were made after the eluates had been reacted with ninhydrin. Results from these experiments showed good correlation between the two series. (Figure 2 and 3). Based on the observed correlation, the chymotryptic peptides isolated for this study were identified solely for their absorption at 2760 Å. The one exception to this was peptide AF-9 which had no visible absorption at 2760 Å and required the ninhydrin assay for identification.

The reason for the correlation between the two absorption measurements was not known. Normally, for peptides and proteins, absorption in the 2760 Å region is selectively based on the presence of tyrosine, phenylalanine and tryptophan in the structure. Conversely, absorption at 5700 Å based on the ninhydrin reaction is general in nature where the characteristic Ruhemann's Purple is produced when the amino nitrogen in the peptide reacts with two moles of ninhydrin reagent.

Possible explanations for the observed correlation are: (a) contamination of peptides not containing the aromatic amino acids by traces of tyrosine or phenylalanine; (b) measurable absorption in 2760 Å region based on peptide concentration, where the actual absorption maximum is at a lower wave length, i.e. 2500 Å or 2100 Å; or (c) absorption in the 2760 Å region influenced by the peptide bond.

The successive steps involved in the subtractive method of Edman degradation have been described in the method section. The purpose

here is to discuss the scope and limitations of the method as applied to the results obtained in this investigation.

The two conditions essential for the coupling of the peptide with phenylisothiocyanate are an alkaline pH and a solvent system in which both the peptide and the reagent are appeciably soluble. In the former, the alkaline pH is required to insure that the amino group which attacks the thiocarbonyl groups of phenylisothiocyanate is unprotonated. In the latter case a number of different solvents have been used (45). The combination of pyridine, water and N-ethyl morpholine in the ratio (150:100:29) used in this investigation appears to be the solvent system of choice in the current literature.

The complete coupling reaction can be achieved by the use of a 50 fold excess of phenylisothiocyanate at 37° for 2 hours (45). This same reaction for the peptides of nifungin was carried out using only a 10 fold excess of the phenylisothiocyanate. The effect of this decrease in the amount of reagent is unknown, however, it is thought that the reaction leading to the formation of the phenylisothiocarbamyl peptide PTC was not complete. Statistically, this would limit the quantity of PTC available for cyclization later in the reaction. Further, continuous sampling of unreacted peptide would lead to confusing results.

The importance of excluding oxygen during the coupling reaction has been stressed by Edman (31, 46). Atmospheric oxygen is capable of replacing the sulfur in the thiocarbonyl group of the PTC. According

to Konigsberg (45) the oxygen of the phenylcarbamyl group will not attack the carbonyl carbon of the amino terminal acids at any appreciable rate under the conditions used for cyclization. If the phenylcarbamyl peptide is formed, but does not cyclize, it will interfere with the interpretation of the results from the subsequent stages. Since the phenylisothiocyanate has already bound the free amino group in the peptide, it cannot be degraded further. The uncyclized phenylthiocarbamyl will be carried along in the peptide fraction. Upon acid hydrolysis, this fraction will yield some portion of all the amino acids present in the original peptide. This will occur no matter how far the degradation has been carried out. If some phenylcarbamyl peptide is formed during each coupling reaction the accumulation of these products during several stages will contribute to the non-integral loss of the NH_2-terminal amino acid that is being removed.

After the phenylthiocarbamyl peptide was formed, the reaction mixture was extracted 3-5 times with 2.0 ml of benzene. These extractions removed excess phenylisothiocyanate, phenylurea and residual solvent. The presence of benzene at this point is believed to further aid the removal of the last traces of water. The benzene-water azeotropic mixture formed evaporates at a lower temperature than water alone.

The requirement for maintaining anhydrous conditions during cyclization has been stressed in the literature (46). These conditions are necessary in order to avoid cleavage of the acid sensitive bonds

in the remainder of the peptide.

The cyclization step for the nifungin peptides was carried out under mild conditions using 100 microliters of anhydrous trifluoroacetic acid at 25° for one hour. Although mild, these conditions have been found to be effective in obtaining complete cyclization of all potentially degradable material (45).

Following cyclization, the trifluoroacetic acid was evaporated in a nitrogen stream and the residue was dissolved in 0.4 M acetic acid. The acetic acid solution containing the phenylthiohydantoin was extracted 3 times with 2 ml of benzene saturated with 0.4 M acetic acid. This extraction removes the phenylthiocarbamyl amino acids and the phenylthiohydantoins of most acidic and neutral amino acids except for the derivatives of arginine, histidine, aspartic acid, serine, threonine and cysteic acid. While these derivatives can be extracted into ethyl acetate, care must be taken with this solvent. Ethyl acetate will extract peptide materials as well as amino acids; this is particularly true for peptides containing non-polar amino acid residues.

The use of the subtractive method for the determination of amino acid sequence as used in this investigation was only partially successful. Side reactions which led to the accumulation of products either not capable of reacting with phenylthiohydantoin or undergoing cyclization made the data for some of the peptides difficult to interpret. These difficulties can be overcome however, by purification of the remaining peptide in the aqueous solution before removing an aliquot

for hydrolysis and amino acid analysis. Konigsberg and Hill (47) purified the remaining peptides in human hemoglobin from their side reaction products using the following method. After cyclization and extraction of the water solution with benzene, the aqueous phase was adsorbed onto a 0.3 x 6 cm column of Dowex 50 x 2 in the hydrogen form. The column was first washed with 4.0 ml of water, and then eluted with a small quantity of pyridine acetate buffer to remove the peptide. The pyridine buffer was 1 M in pyridine at pH 5.6. An aliquot of the purified peptide was then taken for amino acid analysis. The material recovered from the water wash was ninhydrin negative, but after acid hydrolysis, all the amino acids present in the parent peptide were found. This indicated that the materials eluted in the water wash was a peptide derivative containing no free amino groups. This procedure cannot be applied to acidic peptides containing cysteic acid as these peptides are not adsorbed nor can it be used with peptides that have many basic or aromatic residues. While the method does not permit the quantitative determination of the subtracted amino acid it does eliminate a major source of contamination.

In this investigation the purification and sampling method described was not employed. The limitations of the method involving those peptides containing cysteic acid and aromatic amino acid residues were too closely related to the characteristics of the nifungin peptides. An aliquot of the aqueous phase thought to correspond to 10 percent of the remaining peptide after each cyclization was withdrawn for hydrolysis and amino

acid analysis. However, as the results showed, this sampling technique proved to lack uniformity and gave analyses which were difficult to interpret through a series of degradations. The lack of uniformity in sampling is believed to have been caused by several factors. Larger peptides which undergo the Edman degradation tend to retain their solubility in aqueous solution. Many small peptides similar in size to the nifungin peptides become insoluble as they are degraded and accurate sampling of these peptides is therefore difficult (45). The presence of uncyclized phenylthiocarbamyl peptide in the aqueous phase can lead to the removal of more side products and unreacted materials than peptide (45).

The spectrophotometric titration of the -SH groups in nifungin with p-hydroxymercuribenzoate indicated that no reactive -SH groups were present in the molecule. The method has the advantage that the ultraviolet absorption of the mercury-sulfur bond formed between the peptide and reagent can be measured directly in the region of 250 nm. Benesch et al. (48) have indicated that the p-hydroxymercuribenzoate is the only compound of its type which shows an adequate increase in absorption in the useful spectral region as the result of mercaptide formation.

The titration is carried out at two pH levels. The reaction at pH 7.0 is used to allow the readily exposed -SH groups in the protein structure to react with the mercury compound. At the lower pH level (4.6.), the titration is a timed reaction in which any groups which

may be masked in the protein matrix are given an opportunity to undergo reaction with the mercury compound.

The size of the nifungin peptide was such that all of the -SH groups should have been readily accessible to the p-mercuribenzoate reagent almost instantaneously, at either level. If there were any reactive -SH groups present, an end point should have been reached at pH 4.6. after the fifty minute time period allotted. Depending upon the size of the protein being titrated, the end point for reactive -SH groups present will be reached from several minutes to four or five hours.

The complete amino acid sequence of the antifungal peptide nifungin, cannot be determined from the results of a single enzyme digestion as carried out in the current investigation. However, the results from this study can be used in two ways, (1) The sequence of amino acids obtained in this study can be compared with the sequence of amino acids obtained from the digestion of the same antifungal peptide with a different proteolytic enzyme. A comparison of this type involving all of the purified peptides from each digestion could theoretically yield the complete primary structure of the nifungin molecule. (2) The results of this study can be used to partially characterize the compound under investigation.

In the current study, the results were used to make the following observations about the antifungal peptide.

(a) The antifungal peptide is basic in nature and has 49 amino

acid residues in its structure.

(b) The peptide is composed of 1.5% ash and moisture, and 85% amino acid residues. The remaining 13% was not identified in this investigation.

(c) The peptide does not possess any free -SH groups, but has four disulfide bonds in its molecular structure.

(d) There are no tryptophan, histidine or methionine residues in the peptide.

(e) Four chymotryptic peptides examined in this study had COOH-terminal residues which did not correspond to the expected chymotrypsin specificity. While random fragmentation was anticipated, the level here appeared excessive. Based on this, and preliminary studies involving the nifungin terminal residues, the antifungal peptide was thought to have a rather complex structure.

SUMMARY

In this study the antifungal peptide, nifungin, was investigated and partial characterization was achieved.

The amino acid composition of the peptide was determined. Analyses, following acid hydrolysis, accounted for 85% of the peptide structure as amino acid residues. An additional 1.5% was found as ash and moisture. The remaining portion of the structure was not identified.

The peptide was oxidized with performic acid to disrupt the disulfide bonds, and subsequently digested with alpha chymotrypsin. The peptide mixture was separated on Amberlite XE64 resin with ammonium acetate buffers. Eleven chymotryptic peptides were isolated by this method. Six peptides were selected for amino acid sequence analysis and partial characterization by the Edman degradation, using the subtractive method. The chymotryptic peptides chosen for analysis possessed unusual end-group residues in some instances based on enzyme specificity.

Spectrophotometric titration of nifungin with p-hydroxymercuri-benzoate showed that the antifungal peptide possessed no free -SH groups suggesting that the half-cystine residues in the molecule were all involved in disulfide linkages.

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