A Diagonal Electrophoretic Method for Selective Purification of Methionine Peptides

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1. A method is described that selectively purifies methionine peptides from enzymic digests of a protein. The peptides, after paper electrophoresis, are treated on paper with iodoacetamide at acid pH. This specifically converts methionine residues into their sulphonium salts. When the paper is submitted to electrophoresis at right angles to the original direction, the carbamoylmethylmethionine peptides emerge from an undifferentiated diagonal. 2. Heating at neutral pH converts carbamoylmethylmethionine into homoserine and thereby specifically cleaves the peptides. 3. The effect of the modifications on amino acid composition and sequence analyses of the peptides was studied. 4. When the method was applied to a tryptic digest of *S*-aminoethyl-chymotrypsinogen A, two peptides were selectively purified that had the expected amino acid sequence.

Separation and purification of peptides has become the rate-limiting step in the study of primary structure of proteins. Although conventional techniques of ion-exchange chromatography, paper chromatography, paper electrophoresis and molecular sieving have great resolving power, they are stretched to their limits when applied to enzymic digests of proteins of molecular weight much greater than 30 000. There is therefore a need for methods that will alleviate this problem.

Another problem arises from the considerable interest that has recently been generated in evolutionary homology of sequence in similar proteins. An approximation to the evolutionary closeness of the proteins can be obtained by comparing sequences in some unique part of their peptide chains, such as the *N*-terminus, the active centre or around disulphide bridges. A technique that uniquely purifies these selected areas of the sequence would greatly assist this sort of study.

Such a selective purification technique was developed by Brown & Hartley (1963, 1966) for their study of cystine bridges in chymotrypsinogen A, and has since been applied to many other proteins containing disulphide bridges (Hartley, Brown, Kauffman & Smillie, 1965). It seemed possible that this technique of 'diagonal electrophoresis' could be applied to amino acid residues other than cystine. The essential features of such a method must be: that a specific chemical modification of the residue be carried out on a paper strip; that the modification should change the net charge of the peptide; that the modified peptide should be stable in subsequent sequence studies;

and, finally, that the residue modified should be relatively rare in the protein, since otherwise too many peptides would emerge from the diagonal.

Methionine is present in low amount in most proteins and offers opportunities for chemical modification. Indeed, Mikeš & Holeysovský (1957) have already shown that a 'chromatographic diagonal' can be used to demonstrate the presence of methionine in amino acid mixtures by exposing paper strips to performic acid vapour. We have preferred to utilize the reaction between methionine and iodoacetamide (Scheme 1). Lawson, Gross, Foltz & Witkop (1961) and Stark & Stein (1964) have shown that this reaction is very specific at acid pH to yield the sulphonium salt CM-methionine,* which has an extra positive charge. The

Scheme 1. Reaction between methionine and iodoacetamide.

* Abbreviations: CM-Met or CM-methionine, *S*-carbamoylmethylmethionine; AE-Cys, *S*-aminoethylcysteine; Hsr, homoserine; DNS, 1-dimethylaminonaphthalene-5-sulphonyl.

present paper describes a diagonal electrophoretic technique based on this reaction, which selectively purifies methionine peptides from a mixture. As a test of the method we have investigated the 'methionine diagonal' of a tryptic digest of aminoethyl-chymotrypsinogen.

MATERIALS AND METHODS

Chemicals. Bovine chymotrypsinogen A and trypsin were crystalline salt-free products from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Peptides were from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A., and other chemicals were of the highest purity available from commercial sources and used without further purification.

High-voltage paper electrophoresis. For peptides and amino acids the apparatus of Michl (1951) was used as described by Brown & Hartley (1966) with the following buffers: pH 6.5: pyridine-acetic acid-water (25:1:225, by vol.); pH 3.5: pyridine-acetic acid-water (1:10:90, by vol.); pH 2.0: formic acid-acetic acid-water (1:4:45, by vol.). For DNS-amino acids a flat-plate apparatus was used with 0.1 M-pyridine-acetate buffer, pH 4.38 (Gray & Hartley, 1963a).

Alkylation by iodoacetamide on paper strips. The paper was either sprayed or wetted carefully with drops of 0.1 M-iodoacetamide in pyridine-acetate buffer, pH 3.5, and incubated at room temperature on a glass rack in a desiccator containing a tray of water for the appropriate period. It was hung in a hood to dry and then rinsed with acetone to remove excess of iodoacetamide.

Preparation of CM-methionine and CM-methionine peptides. About 1–2 μ moles of methionine or peptide were treated with a tenfold molar excess of iodoacetamide in 0.2 ml. of 0.2 M-pyridine-acetate buffer, pH 3.5, at 37° for 5 hr. The mixture was applied as a band to Whatman 3MM paper and separated by electrophoresis at pH 6.5. The alkylated compounds migrated towards the cathode and were identified by dipping a marker strip in cadmium-ninhydrin reagent (Heilmann, Barollier & Watske, 1957). They were eluted with pyridine-acetate buffer, pH 6.5, and stored at –5°.

Amino acid analysis. Quantitative analyses were made in an automatic amino acid analyser by the procedure of Spackman (1963). Peptides were hydrolysed in 5.7 N-HCl for 18–20 hr. at 105° in evacuated tubes.

Peptides containing CM-methionine were first heated for 2 hr. at 100° to convert the CM-methionine into homoserine, as described below. The hydrolysates of these homoserine peptides were evaporated to dryness, dissolved in 50 μ l. of aq. 2 N-NH₃, and incubated 1 hr. at 37° to convert homoserine lactone into homoserine, and then evaporated to dryness. The first buffer used in the analyser was pH 3.10 rather than pH 3.28 and homoserine emerged as a discrete peak between serine and glutamic acid. The area constant used for homoserine was the average of that for serine and threonine. A calibration curve for destruction of homoserine during hydrolysis was made by hydrolysing an authentic sample for various times. Destruction was 17% in 20 hr. and this correction factor is applied in the following results.

Tryptic digest of S-aminoethyl-chymotrypsinogen A

(Hartley & Kauffman, 1966). Chymotrypsinogen A (250 mg.) was reduced with mercaptoethanol in 8 M-urea, treated with ethylenimine and dialysed. The trypsin (1.5 mg.) used for the digestion was pretreated with diphenylcarbamoyl chloride to inhibit chymotrypsin contamination. Although a considerable precipitate remained after digestion overnight at pH 8.0, the 'fingerprints' of the soluble peptides indicated that this was almost entirely undigested protein. No indication was found in these 'fingerprints' of any characteristic chymotryptic cleavages.

'Methionine diagonal' of tryptic peptides from S-aminoethyl-chymotrypsinogen. The soluble peptides from the tryptic digest were run as a band on Whatman 3MM paper in high-voltage electrophoresis at pH 6.5. A 1 cm. strip was cut from this paper parallel to the direction of electrophoresis and suspended horizontally between two stainless-steel clips. Fluorescent markers of 5 μ l. of 0.1 M-DNS-arginine (Brown & Hartley, 1966) were applied at 2 cm. intervals and dye markers (Sanger, Brownlee & Barrell, 1965) at 5 cm. intervals. These markers are very useful to calibrate electrophoretic mobility of peptides and to correct for any migration of the peptides along the wet paper during the alkylation reaction.

The dry strip was sprayed evenly in a hood with 0.1 M-iodoacetamide in pyridine-acetate buffer, pH 3.5, until thoroughly wet but not running. It was then arranged on a glass rack in a desiccator containing a tray of buffer, care being taken to avoid contact between different parts of the paper. After 14–16 hr. at room temperature the paper was hung in a hood to dry and rinsed several times with acetone.

The strip was then stitched across the middle of a full sheet of Whatman 3MM paper and submitted to electrophoresis at pH 6.5 at right angles to the original direction. The fluorescent and dye markers were marked out on the dry paper and the peptides revealed by dipping in cadmium-ninhydrin reagent.

RESULTS

Diagonal electrophoresis of methionine and methionine peptides

The electrophoretic mobility of CM-methionine or peptides containing it is considerably different from that of the non-alkylated compounds (Table 1). The increased cationic mobility is obviously due to the extra positive charge on the sulphonium group. With each compound the results were the same regardless of whether alkylation was carried out in solution or on paper strips.

The specificity of the alkylation by iodoacetamide at pH 3.5 towards the methionine residue was demonstrated by 'diagonal electrophoresis'. A mixture of all the amino acids found in proteins together with glycylmethionine (2.5 μ moles each) was separated by electrophoresis at pH 2 and the paper strip, when dry, was sprayed with iodoacetamide. After 5 hr. incubation, the excess of reagent was washed away with acetone and the strip was stitched to a full sheet of Whatman 3MM paper. Electrophoresis at pH 2 at right angles to

the original direction gave the pattern shown in Fig. 1. Only two major spots lie off the diagonal, and their electrophoretic mobilities identify them as CM-methionine and glycyl-CM-methionine. The trace component above glycyl-CM-methionine was found to be CM-methionylglycine derived from a methionylglycine impurity in the dipeptide sample. Fig. 1 clearly shows that the method is specific for methionine or methionine peptides.

Rate of alkylation of methionine peptides on paper

The time required for this reaction was investigated by using methionine and methionine dipeptides as model compounds. Samples of each peptide (50mμmoles) were applied to rectangles (2cm. x 3cm.) of Whatman 3MM paper. Iodoacetamide (0.1M) (0.1ml.) in pyridine-acetate buffer, pH 3.5, was added to the papers, which were then incubated for various times in a damp desiccator. After drying and removing excess of reagent with acetone, the rectangles were stitched across the middle of a full sheet of Whatman no. 1 paper and submitted to electrophoresis at pH 6.5. The residual unchanged methionine peptides remained in the neutral band and were separately eluted, hydrolysed and analysed for amino acid content. Controls of 50mμmoles of untreated peptides were included in the electrophoresis to correct for elution losses (methionylalanine, 86% recovery; phenylalanylmethionine, 74% recovery). The results (Fig. 2) show that in all cases reaction was complete in 8hr. incubation at room temperature.

Conversion of CM-methionine into homoserine

The stability of CM-methionine peptides is obviously an important consideration for subsequent sequence work. We found that they were indefinitely stable at -5° at neutral pH and could be run on electrophoresis at pH 2, pH 3.5 or pH 6.5

without loss. During acid hydrolysis, however, CM-methionine decomposed to products that appeared as multiple peaks in the amino acid analyser.

Lawson *et al.* (1961) showed that CM-methionine in a peptide may undergo a reaction that cleaves a peptide bond to give C-terminal homoserine. This

Fig. 1. Diagonal electrophoresis (pH2/pH2) of a mixture of amino acids and methionine peptides. The mixtures were applied originally at the spot marked ⊕ in the horizontal strip. After electrophoresis at pH2 and treatment with iodoacetamide, the strip was stitched to a full sheet of paper and submitted to electrophoresis at pH2 in the vertical direction. Met-SO₂, methionine sulphone.

Table 1. Electrophoretic mobility of CM-methionine peptides

Mobilities are expressed relative to the distance from the centre of the neutral band at pH6.5 or the origin at pH2 to the leading edge of the lysine marker spot.

Peptide	Mobility relative to lysine	
	pH 6.5	pH 2
Met	0	0.52
CM-Met	0.91	0.85
Gly-Met	0	0.64
Gly-CM-Met	0.85	0.99
Met-Ala	0	0.61
CM-Met-Ala	0.63	0.98

Fig. 2. Rate of alkylation of methionine peptides on a paper strip. Experimental conditions are given in the text. ▲—▲, Methionine; ●—●, glycylmethionine; ○—○, phenylalanylmethionine; △—△, methionylalanine.

reaction, although slow at room temperature, was greatly accelerated by higher temperatures. We therefore sought to use this conversion as an aid to the quantitative analysis of CM-methionine. The CM-peptides were incubated under various conditions, and the extent of conversion into homoserine was estimated qualitatively by paper electrophoresis at pH 2 or quantitatively by amino acid analysis, with or without acid hydrolysis. Free CM-methionine or *C*-terminal CM-methionine in a peptide was quantitatively converted into homoserine after 2 hr. at 60° in 0.05 M-sodium hydrogen carbonate, but CM-methionylalanine and CM-methionylphenylalanine were only slightly split under these conditions. Although we tried the effect of many acids and bases, we found that incubation at 100° in water for 2 hr. in sealed tubes

gave the more nearly quantitative and specific cleavage of these CM-peptides. Fig. 3 shows the pattern of products of this heating after electrophoresis at pH 2, and Table 2 shows the extent of cleavage after 2 hr. at various temperatures.

This specific cleavage reaction is clearly very convenient for our purpose. The CM-methionine peptides can be easily purified by the diagonal technique. Heating in water at 100° converts the relatively unstable CM-methionine into the more stable homoserine, and splits the peptide neatly into two fragments that can be more easily subjected to sequence analysis. A simple electrophoresis at pH 2 of the heated CM-methionine peptide immediately establishes whether methionine was *N*-terminal, *C*-terminal or internal, since homoserine plus a peptide, one peptide or two peptides respectively will result.

Fig. 3. Cleavage of methionine peptides after heating. The tracing shows the cadmium-ninhydrin spots after electrophoresis at pH 2. R is an amino acid mixture: (left to right) cysteic acid, methionine sulphone, tyrosine, phenylalanine, methionine + proline, leucine, valine, arginine, lysine. MA is methionylalanine. CMA, CMA, 70°, and CMA, 100°, are CM-methionylalanine untreated or heated for 2 hr. in water at 70° or 100°. MP is methionylphenylalanine. CMP, CMP, 70°, and CMP, 100°, are CM-methionylphenylalanine untreated or heated for 2 hr. at 70° or 100°. T is an amino acid mixture: (left to right) aspartic acid, glutamic acid, threonine, isoleucine + serine, alanine, glycine, histidine. A marker spot of authentic homoserine, Hsr, is also included.

CM-methionine and homoserine in the 'dansyl'-Edman procedure

Since the 'dansyl'-Edman method of Gray & Hartley (1963b) is a useful technique for sequence analysis, it is important to know the behaviour of CM-methionine and homoserine in this procedure. 'Dansylation' of CM-methionine, CM-methionylphenylalanine and CM-methionylalanine (Gray & Hartley, 1963a) followed by acid hydrolysis gave no fluorescent spot corresponding to DNS-CM-methionine, but a series of minor fluorescent bands were found that probably represent breakdown products of this compound. Treatment of the CM-methionine or CM-peptides at 100° before 'dansylation' did, however, yield DNS-homoserine, which was found to run in the same place as DNS-threonine on electrophoresis at pH 4.38. These two DNS-amino acids were easily resolved by stitching the ambiguous area to another sheet of paper and re-running in high-voltage electrophoresis at pH 3.5 (100 v/cm. for 1 hr.) as shown in Fig. 4.

Table 2. Cleavage of CM-methionine peptides at various temperatures

The peptides were incubated in water in sealed glass tubes for 2 hr. The percentage cleavage was calculated from the yield of alanine or phenylalanine in the amino acid analyser.

Peptide	Temperature	Cleavage (%)
CM-Met-Ala	70°	19
	80	27
	90	57
	100	100
CM-Met-Phe	70	15
	100	100

Fig. 4. Identification of DNS-homoserine by electrophoresis at pH 3.5. The ambiguous region from the usual pH 4.38 electrophoresis can be stitched as shown and subjected to electrophoresis at pH 3.5 in a solvent-cooled tank. DNS-OH, 1-dimethylaminonaphthalene-5-sulphonic acid.

Table 3. 'Dansyl'-Edman analysis of CM-methionine and homoserine peptides

Peptide	DNS-amino acid identified in the Edman degradation				
	First residue	Second residue	Third residue	Fourth residue	Fifth residue
Dipeptides:					
Gly-CM-Met	Gly	None	—	—	—
Gly-Hsr	Gly	Hsr	—	—	—
Phe-CM-Met	Phe	None	—	—	—
Phe-Hsr	Phe	Hsr	—	—	—
CM-Met-Ala	None	Ala	—	—	—
CM-Met-Ala (heated)	Hsr + Ala	—	—	—	—
CM-Met-Phe	None	Phe	—	—	—
CM-Met-Phe (heated)	Hsr + Phe	—	—	—	—
Chymotrypsinogen peptides:					
CM1 (Asp-Ala-CM-Met-Ile-AE-Cys)	Asp	Ala	None	Ile	AE-Cys*
CM1 (heated)	Asp + Ile	Ala + AE-Cys*	Hsr	—	—
CM2 (CM-Met-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-AE-Cys)	None	Gly	Asp	—	—
CM2 (heated)	Hsr + Gly	Asp	—	—	—

* Bis-DNS-AE-Cys runs in approximately the same position as bis-DNS-lysine on electrophoresis at pH 4.38.

Several peptides containing CM-methionine or homoserine were tested by the 'dansyl'-Edman procedure (Table 3). As observed above, no stable DNS-derivative is found when CM-methionine is *N*-terminal, but DNS-homoserine can be clearly identified. The Edman degradation nevertheless removes *N*-terminal CM-methionine at the appropriate stage and allows the sequential analysis to proceed.

Methionine and CM-methionine peptides from chymotrypsinogen

The practical validity of the above methods was tested by applying them to an enzymic digest of bovine chymotrypsinogen A, which contains two methionine residues in known sequence (Hartley, 1964). Fig. 5 shows the pH 6.5 'methionine diagonal' of a tryptic digest of *S*-aminoethyl-chymotrypsinogen A. Two peptides, CM1 and CM2, lie off this diagonal and both gave a red colour with cadmium-ninhydrin reagent. They must arise from methionine peptides that were originally neutral at pH 6.5.

Isolation of the CM-methionine peptides. The neutral band from half of the original pH 6.5 electrophoresis of the tryptic digest was cut out and treated with iodoacetamide at pH 3.5 as described above, except that the reagent solution was applied with a dropper to the strip folded into a pad in a Petri dish. After rinsing the dry paper with acetone, a band of fluorescent marker was applied along the strip, which was then stitched to Whatman 3MM paper for electrophoresis at pH 6.5. The positions of the two basic bands CM1

Fig. 5. Methionine diagonal (pH 6.5/pH 6.5) of a tryptic digest of aminoethyl-chymotrypsinogen. The electrophoretic mobility of the peptides relative to lysine is indicated.

and CM2 were identified with cadmium-ninhydrin reagent on a guide strip, and the remainder of the bands eluted with pyridine-acetate buffer, pH 6.5.

Isolation of the unmodified methionine peptides. To compare directly the CM-methionine peptides with their unmodified precursors, the latter were

purified directly from the remaining half of the neutral band of the original preparative pH 6.5 electrophoresis. This strip was stitched to a full sheet of Whatman 3MM paper and submitted to electrophoresis at pH 2. Two ninhydrin-positive bands were found: M1 and M2 (Fig. 6a). A strip from this electrophoresis was treated with iodoacetamide in the usual way and submitted to electrophoresis at right angles at pH 6.5 (Fig. 6b). In this procedure unmodified peptides remain in the neutral band, whereas CM-methionine peptides become cationic. Band M1 clearly represents the pure methionine precursor of peptide CM1, whereas band M2 contains the precursor of peptide CM2 plus another peptide that was found to be Pauly-positive. [Amino acid analysis showed this to be Tyr-Thr-Asn-Ala-Asn-Thr-Pro-Asp-Arg, derived from residues 146-154 of chymotrypsinogen A (Hartley, 1964).] The latter was removed from peptide M2 by further electrophoresis at pH 3.5 (Fig. 6c) and both peptides M1 and M2 were eluted.

Amino acid composition of the peptides. The methionine peptides M1 and M2 were hydrolysed

directly, but peptide CM1 was heated at 100° for 2hr. before hydrolysis, to convert the CM-methionine into homoserine. Peptide CM2 was heated at 60° for 2hr. in 0.05M-sodium hydrogen carbonate and the low yield of homoserine probably represents incomplete conversion by this inferior procedure. The amino acid ratios found were: peptide M1: Asp (1.1), Ala (1.0), Met (0.9), Ile (0.9), AE-Cys (0.7); peptide M2: Asp (1.0), Ser (1.0), Pro (1.0), Gly (2.7), Val (0.9), Met (0.8), Leu (1.0), AE-Cys (0.6); peptide CM1: Asp (1.0), Ala (1.1), Hsr (1.0), Ile (0.9), AE-Cys (0.7); peptide CM2: Asp (1.0), Ser (0.9), Pro (0.8), Gly (3.1), Val (0.9), Hsr (0.9), Leu (1.0), AE-Cys (0.7). It is clear that peptide CM1 is derived from peptide M1, which has the sequence Asp-Ala-Met-Ile-AE-Cys (residues 178-182), and peptide CM2 from peptide M2, which has the sequence Met-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-AE-Cys, which is the active-centre serine sequence of chymotrypsin (residues 192-201).

Specific cleavage of the CM-methionine peptides. Aqueous solutions of peptides CM1 or CM2 were heated in sealed tubes at 100° for 2hr. When cool, a drop of 2N-ammonia was added to each and the solutions were incubated at room temperature for 15min. to convert homoserine lactone into homoserine. The products of peptide CM1 separated into two ninhydrin-positive bands on electrophoresis at pH 6.5. The acidic band had the composition (Asp₁, Ala₁, Hsr₁) and the basic band was (Ile₁, AE-Cys₁). The heated peptide CM2 gave free homoserine plus a peptide on electrophoresis at pH 2, showing that CM-methionine was *N*-terminal. Hence both peptides were specifically cleaved by the heat treatment.

Sequence of the CM-methionine peptides. Table 3 includes the results of the 'dansyl'-Edman procedure (Gray & Hartley, 1963b) applied to the various methionine peptides from chymotrypsinogen. As with the dipeptides that we initially studied, we found that *N*-terminal CM-methionine gave no DNS derivative, but that the sequential Edman degradation nevertheless continued beyond this residue. The position of the CM-methionine could be established unequivocally by previous heat treatment of the peptide to convert the sulphonium salt into homoserine. The expected end groups were found at each step and the DNS-homoserine could be identified unequivocally.

DISCUSSION

The principle of diagonal electrophoresis appears to be a useful approach to the problem of selective purification of biological materials. It is clearly not necessarily restricted to work with peptides, since any substance that can be separated on paper, and then specifically modified on paper to change

Fig. 6. Isolation of the unmodified methionine peptides from the neutral band of the tryptic digest of *S*-aminoethyl-chymotrypsinogen. (a) Marker strips from a pH 2 electrophoresis of the neutral band. (b) A pH 2/pH 6.5 methionine 'diagonal' of a marker strip from (a). After treatment with iodoacetamide the strip is run at pH 6.5 at right angles. The CM-methionine peptide arising from peptide M1 is CM1 (Fig. 5). Peptide M2 gives the CM-methionine peptide CM2, and leaves the contaminant in the neutral band. (c) Peptide M2 can be freed from this contaminant by electrophoresis at pH 3.5.

its separation properties, is amenable to purification. Separation could be by paper electrophoresis or chromatography, modification could be chemical or enzymic, and the principle would still apply.

This 'methionine diagonal' for peptides may prove most useful in conjunction with the cyanogen bromide technique of Gross & Witkop (1962) for specific cleavage of peptide chains at methionine residues. It would allow one to select specifically from an enzymic digest peptides that 'overlap' the fragments obtained by cyanogen bromide treatment and thus arrange these larger pieces in the correct sequence. A further felicity is that the CM-methionine peptides can themselves be specifically cleaved to yield fragments that will correspond to the *N*- and *C*-termini of the appropriate cyanogen bromide derivatives.

A battery of such selective purification techniques could offer a new approach to the study of the primary structure of proteins. The degree of homology of sequence between two polypeptide chains could be approximated by determining the sequence around each cystine residue or each methionine residue, for example. Such an approach has already revealed structural homologies around the disulphide bridges of chymotrypsinogen A, chymotrypsinogen B, trypsinogen and elastase (Hartley *et al.* 1965), and further development of

diagonal techniques will extend the range for such studies.

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