THE REACTION OF CYANOGEN BROMIDE WITH S-METHYLCYSTEINE: FRAGMENTATION OF THE PEPTIDE 14-29 OF BOVINE PANCREATIC RIBONUCLEASE A

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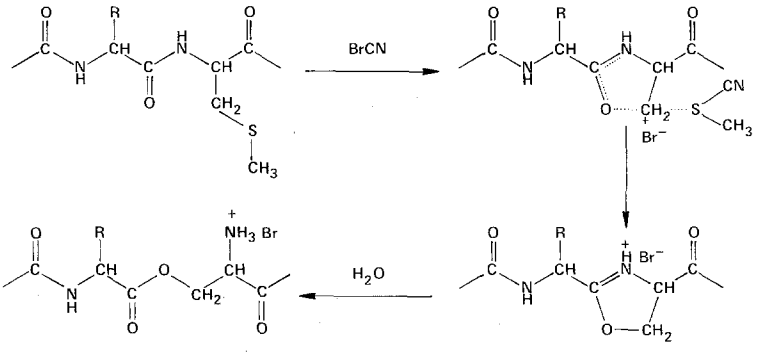
SUMMARY: The S-methylated peptide 14-29 of bovine pancreatic ribonuclease A is cleaved by cyanogen bromide with the formation of the expected fragments in 88% yield based on the conversion of S-methylcysteine.

INTRODUCTION: The high level of selectivity and the mild conditions of the cyanogen bromide reaction (1) are applicable to S-methylcysteine. While cyanogen bromide (BrCN) has been widely applied to the structural elucidation of peptides and proteins with methionine, the utility of the reaction in the case of peptides and proteins with reduced disulfide bridges and/or cysteine subsequent to alkylation remained to be demonstrated despite initial reports that appeared on the feasibility of the reaction (2;3;1).

Studies on simple di- and tripeptides indicated that the reaction proceeds by two different mechanisms - A or B (3) - depending on the conditions employed. Mechanism A involves the transformation of the S-methylcysteine peptide to an O-serine peptide as shown in equation 1. Mechanism B entails β-elimination of the intermediate cyanosulfonium compound to give a dehydroalanine residue.

The relative amounts of product obtained by each pathway depend to some extent on adjacent amino acid residues, but at 37°C in 0.1 N hydrochloric acid the ratio was typically 5:1 in favor of serine formation. By lowering the temperature to 0°C, the elimination reaction was almost completely suppressed. The reaction required, however, six days to advance to the extent of 75%. On the other hand, raising the temperature to 100°C



(1)

and conducting the reaction in 0.001 N hydrochloric acid, resulted in the complete consumption of S-methylcysteine at a ratio of 4:1 in favor of mechanism B in the space of one hour.

Ordinarily it is better to suppress mechanism B since the subsequent cleavage of the dehydroalanine residue results in the production of a COOH-terminal amide and pyruvyl peptide, neither of which is particularly suited for sequence studies.

Preliminary experiments on larger polypeptide fragments indicated that, although they generally reacted more slowly, the tendency to proceed by mechanism B was less pronounced at a given temperature. Accordingly, temperature conditions of 15-20°C were selected for the studies to be described below.

METHODS AND RESULTS: Bovine pancreatic ribonuclease A (Wilson Laboratories, Chicago, Illinois) was first reacted with a twelvefold excess of BrCN in 60% formic acid overnight at room temperature to cleave the methionyl peptide bonds. After lyophilization, the product was dissolved in 0.2 N acetic acid and chromatographed on a Sephadex G 25 Fine column (6 x 120 cm) with the same solvent as eluent. The peptide 1-13 was well separated from the remainder of the molecule still held intact by disulfide bonds. This core 14-124 upon reaction with mercaptoethanol and its disulfide (4) at pH 8.5 in phosphate buffer exposed to air followed by gel filtration on the above column yields the mixed disulfide of mercaptoethanol and the peptide 14-29 as shown in Figure 1. Reduction with dithioerythritol (5) and methylation with methyl p-nitrobenzene sulfonate (6) in pH 8.5 phosphate buffer under nitrogen gave the desired S-methylated peptide the amino acid analysis of which is shown in Table I.

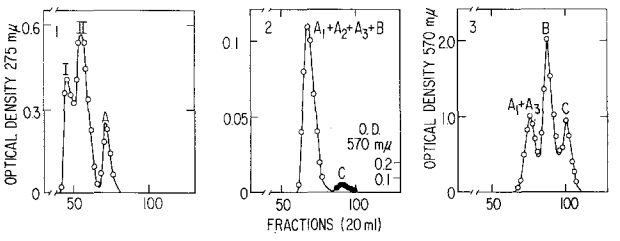


Figure 1

ɔvine Pancreatic Ribonuclease A: BrCN-Fragment 14-29 Reduced and S-Methylated

$$H_2N\text{-Asp-}\overset{15}{\text{Ser}}\text{-Ser-Thr-Ser-Ala-}\overset{20}{\text{Ala}}\text{-Ser-Ser-Ser-Asn-}\overset{25}{\text{Tyr}}\text{-CYSCH}_3\text{-Asn-Gln-}\begin{matrix}\text{HS}\\ \text{HSL}\end{matrix}$$

: Mixed disulfides with $HSCH_2CH_2OH$; Frgmts. 30-79 and 80-124 (I and II) ; Frgmt. 14-29 (A)
: S-Methylated Frgmt. 14-29 (A_1) + O-acyl intermediate [14-(Tyr-25)-O-Ser-26)] (A_2) + dehydroalanine (DHA) derivative [14-(DHA-26)-29] (A_3) + Frgmt. 14-25 (B) ; Frgmt. 26-29 (C)
: Frgmts. 14-29 (A_1 + A_3) ; 14-25 (B) ; 26-29 (C)
(Sephadex G 25 Fine: 1 and 2 ; Superfine: 3. 6 x 120 cm ; 0.2 N acetic acid
$CYSCH_3$ = S-methylcysteine ; HS = homoserine ; HSL = homoserine lactone

Forty milligrams of this peptide dissolved in 15 ml of 60% formic acid (2×10^{-3} M) which contained 31 mg BrCN (2×10^{-2} M) were incubated at 17°C. Aliquots were taken at intervals for amino acid analysis. The reaction follows the course shown in Figure 2 with the increase in serine content closely paralleling the decline of S-methylcysteine. On day 9, a further 31 mg of BrCN was added and the temperature increased to 37°C for one day. The reaction was terminated by lyophilization and the sample was chromatographed on a Sephadex G 25 Fine column (6 x 120 cm) in 0.2 N acetic acid both to remove bromide, which interferes with the subsequent formylation, and to determine the extent of cleavage of the serine O-peptide in the 60% formic acid solution.

Approximately 3 micromoles (10%) of the tetrapeptide (26-29; Fig. 2-2C) was found. The corresponding dodecapeptide (14-25) was not resolved from uncleaved material. This mixture was lyophilized after the addition of .05 ml/l of the relatively non-volatile octanoic acid. In preliminary experiments the omission of this addition resulted in considerably lower yields of final products. The freeze-dried product was dissolved in 15 ml of 98%

Table I. Amino Acid Analysis of BrCN-Fragments from the S-Methylated Peptide 14-29 of Bovine Pancreatic Ribonuclease A

Amino Acid	A_1		$A_1 + A_3$		B		C	
	μm	res	μm	res	μm	res	μm	res
Aspartic acid	.73	3.0	.69	2.9	.68	2.0	1.00	1.0
Threonine	.27	1.1	.26	1.1	.37	1.1	.06	
Serine	1.32	5.4	1.41	5.9[1]	1.98	5.5	1.02	1.0
Glutamic acid	.26	1.1	.26	1.1	.06		.95	1.0
S-Methylcysteine	.24	1.0	.05	.2				
Alanine	.53	2.2	.54	2.2	.76	2.2	.11	
Tyrosine	.24	1.0	.21	0.9	.31	0.9	.06	
Homoserine + Homoserine Lactone	.19	0.8	.19	0.8	.03		.82	0.8
S-Benzylcysteine[2]			.12	0.5				

A_1: S-Methylated fragment 14-29

A_3: Dehydroalanine (position 26) containing fragment 14-29

B : Fragment 14-25

C : Fragment 26-29

μm = micromole ; res = residue

[1] Extrapolated to zero time

[2] After the addition of benzyl mercaptan

The value for the amount of dehydroalanine present certainly represents an upper limit. Theoretically, Fraction A should contain 0.6 residues of S-methylcysteine on the basis of the ratio of unreacted S-methylcysteine to uncleaved material, which is 15:25. The lower value is possibly due to the formation and elimination of a derivative, such as sulfoxide, during exposure to alkaline conditions or acid hydrolysis. S-Methylcysteine itself is sensitive to air oxidation in dilute solution.

formic acid and 1 ml portions of acetic anhydride were added at 45 min intervals to a total of 5 ml. This reaction mixture was again freeze-dried and thereafter incubated for 48 hours in a solution 0.1 M in benzohydroxamic acid in 0.05 M phosphate buffer pH 8.5 (7). Following the addition of 1 ml of formic acid the mixture was passed over Sephadex G 25 Superfine (6 x 120 cm) with the result indicated in Figure 1 - 3. Amino acid analyses

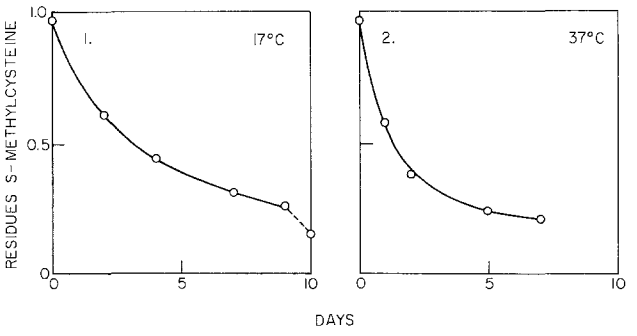


Figure 2

Bovine Pancreatic Ribonuclease A: BrCN-Fragment 14-29 Reduced and S-Methylated. 1. Treatment with cyanogen bromide at 17°C ; an additional 31 mg of BrCN were added on day 9. 2. Treatment with BrCN at 37°C. 1. and 2.: Peptide : BrCN = 2×10^{-3} M : 2×10^{-2} M.

presented in Table I show that fractions B and C are the dodecapeptide (14-25) and tetrapeptide (26-29), respectively, which are expected from the cleavage. One residue of tyrosine is released from fraction B upon incubation with carboxypeptidase A for 1 hour.

The unfragmented material of peak $A_1 + A_3$ was reacted with an excess of benzyl mercaptan at pH 9 under vacuum for two weeks. Amino acid analysis of the product revealed the formation of 0.5 residues of S-benzyl cysteine. Extrapolation based on a time study of destruction of serine during acid hydrolysis gave the expected six residues.

DISCUSSION: The reaction is of demonstrated value as a method of specific cleavage of peptide bonds, giving high yields and fragments which are easily subject to further study.

Certain precautions in dealing with solutions of serine O-peptides must be observed. The pH of such solutions must not be allowed to exceed 6. Small amounts of non-volatile weak acids must be added to prevent the N→O-acyl shift reaction during lyophilization. Strong acids should be avoided as their ammonium salts are resistant to formylation under the conditions employed.

The more rapid approach of the reaction curve to an asymptote

than expected on the basis of second order kinetics seemed to indicate the formation of an inhibitor. Since the most likely inhibitory product was bromide ion, the reaction was conducted as before except that the solution was made 2 x 10^{-2} M in ammonium bromide. Under these conditions, only 5% of the S-methylcysteine had reacted in 4 days. Evidently a concentration of bromide similar to that which would be formed from the reaction and from solvolysis has a strong depressing effect. Figure 2 shows that performing the reaction at 37°C results in a more rapid initial rate, but the same limiting inhibition is approached. The addition of another 31 mg of cyanogen bromide on day 5 results in very little further reaction, undoubtedly because of the greater solvolytic formation of bromide ion at the higher temperature.

By comparison, the reaction of methionyl peptides with BrCN is also inhibited by bromide ion, but the concentration required is more than 1000-fold greater than in the case of S-methylcysteine peptides (8).

This property may be useful in a case where a reactive cysteine is methylated in a structurally intact methionine containing protein. The methionyl peptide bonds may first be cleaved in the presence of bromide ion, the fragments separated, and the S-methylcysteine subsequently be allowed to react.

The use of the formyl group may not be considered ideal from the point of view of removability. Cleavage from serine, however, is facilitated by the neighboring hydroxyl group, being complete in 0.2 N hydrochloric acid at 25°C in 20 hours. The hydrolysis in the case of other H_2N-termini requires somewhat more stringent conditions.

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