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Sodium Sulfite as an Antioxidant in the Acid Hydrolysis of Bovine Pancreatic Ribonuclease A

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Treatment of hydrochloric acid with sodium sulfite prior to the acid hydrolysis of bovine pancreatic ribonuclease A has been found to suppress the oxidation of cystine, methionine, and tyrosine without adversely affecting the recoveries of other amino acids. Statistical analysis of the results indicated that the assumption of the independence of the mean and the variance, an assumption commonly used in the evaluation of the effects of various treatments, may not be valid in evaluating antioxidants used in the acid hydrolysis of proteins.

KEY WORDS: amino acid analysis; antioxidant; sodium sulfite; hydrochloric acid; hydrolysate.

Böhlen and Schroeder (1) have noted the need for a simple procedure for the acid hydrolysis of proteins and peptides that does not require a separate determination of tryptophan, tyrosine, methionine, cysteine, and cystine. These authors used thioglycolic acid to prevent the oxidation of the first three of these amino acid residues, but required a separate analysis under oxidizing conditions for the determination of the sum of cysteine and cystine as cysteic acid. Inglis and Liu (2) used posthydrolytic conversion of cysteine, cystine, and cysteic acid to *S*-sulfocysteine as a strategy in amino acid analysis, and Inglis (3) has coupled this method with prehydrolytic alkylation of cysteine as a procedure to differentiate between cystine and cysteine and to recover all amino acids, including tryptophan. Other authors (4–6) have used phenol as an antioxidant, and dimethyl sulfoxide has been used (7) to convert cysteine or cystine to cysteic acid during hydrolysis. Each of these methods has advantages and disadvantages. In particular, prehydrolytic derivatization of cysteine with 4-vinylpyridine or iodoacetate (3) requires the removal of the alkylating agent prior to hydrolysis, and would therefore be difficult to apply to very large or very small samples or samples of low molecular weight. For reasons of convenience, hydrochloric acid is often preferred as the hydrolytic agent, but extensive oxidation is sometimes observed. The present work explores the use of sodium sulfite to improve the recoveries of cystine, methionine, and tyrosine in the acid hydrolysis of bovine pancreatic ribonuclease A, a protein that does not contain cysteine or tryptophan. The practical benefits conferred by pretreatment with sodium sulfite are discussed, and the general requirements for a statistical study of the effects of an antioxidant are explored.

MATERIALS AND METHODS

Sodium sulfite (Na_2SO_3) was purchased from Fisher Scientific Company. Bovine pancreatic ribonuclease A, type I-AS, was purchased from Sigma, and was purified by ion-exchange chromatography on carboxymethylcellulose (8) purchased from Whatman. Hydrochloric acid (12 M) was either purchased from Mallinckrodt and purified by simple or fractional distillation by a procedure designed to produce a constant-boiling fraction (9), or was purchased from Baker Chemical Company (Ultrex) and diluted to 6 M.

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The Mallinckrodt HCl (lot KMPE) exhibited an elevated boiling point during the latter part of the simple distillation, and complete destruction of cystine, methionine, and tyrosine was observed with the use of this distilled acid in the absence of pretreatment with Na_2SO_3.

Eleven groups of replicate hydrolysates, each containing 4–6 samples, were prepared. The first four groups of replicates were prepared as 0.25-ml aliquots of simply or fractionally distilled Mallinckrodt acid which had been treated with 1 mg/ml of Na_2SO_3, or not treated, and into which ribonuclease A had been dissolved at a concentration of 1 mg/ml. The fifth group of replicates was prepared as 10-μl aliquots of fractionally distilled Mallinckrodt acid containing protein at a concentration of 27 mg/ml. The next two groups of replicates were prepared as 10-μl aliquots of Ultrex acid which had been treated with 7 mg/ml of Na_2SO_3, or not treated, and into which the protein had been dissolved at a concentration of 10 mg/ml. The final four groups of replicates were prepared as 10-μl aliquots of Ultrex acid with a protein concentration of 10 mg/ml. In two of these groups of replicates, the acid had been purged for 1 h with helium gas prior to sample preparation and, to two of the groups of replicates, 10 mg/ml of Na_2SO_3 had been added to the HCl prior to the addition of protein.

Hydrolyses were performed in 8-mm-i.d. glass tubes. The solutions were degassed by the freeze–pump–thaw method (10) at 0.03 mm Hg. The freeze–pump–thaw cycle was repeated three times. The additional precaution of decreasing the pressure in the tube to about 2 mm Hg prior to freezing the sample in liquid nitrogen was observed for the final four groups of replicates; this should prevent the condensation of oxygen. All samples were hydrolyzed at 110°C for 24 h, and the HCl was removed under vacuum. Samples were analyzed on a Technicon TSM amino acid autoanalyzer.

In the following section, groups of replicates have been labeled with the symbols M or U to indicate Mallinckrodt or Ultrex acid, with the symbols s or f to indicate that the HCl was distilled by simple or fractional distillation, and with the symbol He if the HCl had been purged with helium. The symbol ϕ indicates that no sulfite was added, and the symbol S indicates the addition of Na_2SO_3.

Statistical analysis was performed according to the technique of variance analysis (11). This technique apportions the variance that exists between groups of replicates to random error or to the effects of the various treatments, i.e., to the method of distillation, to the effect of purging with helium, or to the effect of addition of Na_2SO_3. In the technique of variance analysis, the statistical significance of the effect of a particular treatment is determined by an F test. To evaluate the effects of various treatments in the first four groups of replicates, a two-sided F test was used, i.e., it was assumed that addition of Na_2SO_3 could *either* increase or decrease the yields of the oxidizable residues. The results demonstrated unambiguously that Na_2SO_3 does not decrease the yields; hence, one-sided F tests were used in all subsequent analyses of variance. One cannot combine groups of replicates for the purpose of variance analysis unless it is known that the mean and the variance are approximately independent of one another. For the purpose of investigating the dependence of the variance on the mean, a phenomenon formally known as heteroscedasticity, Bartlett's test (11) was used. A simpler, but qualitative technique involves graphing the variance or standard deviation against the mean.

RESULTS AND DISCUSSION

The principal results of this work are presented in Table 1. Under conditions of high sample dilution, extensive destruction of the oxidizable residues occurred in the absence

TABLE 1

HYDROLYSES OF BOVINE PANCREATIC RIBONUCLEASE IN MALLINCKRODT HCl

Method of treatment	Amino acid residue	Theoretical	No Na_2SO_3 added: Replicate group number and mnemonic[a]	No Na_2SO_3 added: Number of residues recovered[b]	Na_2SO_3 added: Replicate group number and mnemonic	Na_2SO_3 added: Number of residues recovered
			1 Msϕ		3 MsS	
Simply distilled HCl	Half-Cys	8		0.0 (—)		4.84 (0.51)
	Met	4		0.0 (—)		3.25 (0.45)
	Tyr	6		0.0 (—)		1.88 (1.50)
			2 Mfϕ		4 MfS	
Fractionally distilled HCl	Half-Cys	8		1.48 (1.28)		5.07 (0.19)
	Met	4		1.59 (0.96)		3.56 (0.18)
	Tyr	6		1.76 (0.83)		3.31 (1.31)

[a] See text for the nomenclature of the replicate groups. Each group contained five replicates. All samples were prepared with a protein-to-HCl molar ratio of 1:80,000. In samples MsS and MfS, the protein-to-Na_2SO_3 molar ratio was 1:100. In a fifth group of four replicates, the protein-to-HCl molar ratio was increased to 1:3,000, but no sulfite was added. The observed recoveries were: Cys 5.45 (σ = 0.97), Met 3.86 (σ = 0.12), and Tyr 4.61 (σ = 0.42) residues.

[b] Values reported are the number of residues recovered, based on an assumed recovery of Ala plus Glu equal to 24. Values in parentheses are standard deviations.

of Na_2SO_3. In a footnote to Table 1, it is noted that, at a higher concentration of protein, fair recoveries of the oxidizable residues were observed. Hence, although the quality of the HCl as purchased was undoubtedly poor, perhaps due to the length of time between production and purchase, it is evident that recoveries of the oxidizable residues are improved by distillation of the acid, by reduction of the ratio of HCl to protein, and by the addition of Na_2SO_3. Two-factor analysis of the variance, using a two-sided F test as described in the previous section, showed that addition of Na_2SO_3 increased the recovery of the oxidizable residues with a confidence level greater than 99%. Fractional distillation of the HCl also improved the recoveries with a confidence level greater than 97.5%.

Additional tests, using a higher grade of acid, were also performed, and these results are contained in Table 2. Groups 6 and 7 could not be combined with the remaining groups because of heteroscedasticity. A one-sided F test on groups 6 and 7 demonstrated that the addition of sulfite improved the recovery of methionine at a confidence level of 99.9%, and the recovery of cystine at a confidence level of 97.5%. The confidence level on the increased recovery of tyrosine is below 90%.

Two-factor analysis of the variance of groups 8–11 was not conclusive because of the limiting effect of the protein composition; as one approaches quantitative yield in the absence of any treatment, increases in percentage yield due to the effects of the various treatments necessarily become small. The improvements of the yields of cystine and methionine, attributable either to purging of the HCl with helium or to the addition of Na_2SO_3, are not statistically significant, although it is germane to note that addition of Na_2SO_3 qualitatively seems to improve the yields of these residues. For tyrosine, however, a one-sided F test indicated at the 90% confidence level that the recovery is increased by purging with helium, by addition of

TABLE 2

HYDROLYSES OF BOVINE PANCREATIC RIBONUCLEASE A IN ULTREX ACID

Replicate group number and mnemonic[a]	Number of replicates in group	Molar ratio of protein:HCl:Na_2SO_3	Number of half-cystine residues (theory = 8)		Number of methionine residues (theory = 4)		Number of tyrosine residues (theory = 6)	
			Mean	SD	Mean	SD	Mean	SD
6 Uϕ'	6	1:6800:0	6.26	0.99	3.61	0.03	4.61	0.12
7 US′	6	1:8200:80	7.12	0.40	3.76	0.14	5.05	0.09
8 Uϕ	5	1:5400:0	7.57	0.41	4.02	0.16	5.22	0.22
9 US	5	1:5400:72	7.64	0.21	4.00	0.14	5.40	0.27
10 Uϕ-He	5	1:5400:0	7.68	0.21	4.04	0.13	5.48	0.16
11 US-He	5	1:5400:72	7.83	0.19	4.14	0.06	5.70	0.30

[a] See text for nomenclature. Uϕ' and US′ differ from Uϕ and US, respectively, because, in the former, the pressure inside the hydrolysis tubes was not reduced prior to freezing the sample in the freeze–pump–thaw cycle. Statistical analysis, described in the text, precludes the combination of Uϕ' with Uϕ or US′ with US.

Na_2SO_3, and by a synergistic interaction of these treatments. Additional experiments, not presented in this paper, demonstrated that the addition of up to 40 mg/ml of Na_2SO_3 to the hydrolysis acid did not reduce the yields of the other amino acids of ribonuclease.

We have not been able to establish the mechanism by which pretreatment of HCl used in acid hydrolysis improves the yields of cystine, methionine, and tyrosine. In acidic solution, Na_2SO_3 is converted to SO_2 gas, most of which is slowly evolved from solution prior to addition of the sample, and the remainder of which is presumably removed as the pressure in the hydrolysis tube is reduced under vacuum. The apparent synergism of purging with helium and adding Na_2SO_3, observed in the recoveries of tyrosine in groups 8–11, suggests that Na_2SO_3 reduces trace oxidants present even in freshly prepared acid, and does not simply purge the HCl of dissolved molecular oxygen. One interesting observation, perhaps relevant to the elucidation of the mechanism by which antioxidants function, is the linear relationship obtained if the mean recovery of a given amino acid in the absence of Na_2SO_3 is plotted against the mean recovery in the presence of sulfite, i.e., if the result of Msϕ is plotted against that of MsS, that of Mfϕ against that of MfS, and so on. Hence, although we do not know the antioxidative mechanism, the effect of addition of Na_2SO_3 appears to be regular and predictable.

Successful amino acid analysis depends on many factors, including the composition of the protein, the molar ratio of HCl to protein, the freshness of the hydrolysis acid used, and the experimental details of sample preparation. The present work demonstrates that improved yields of the oxidizable amino acids can be obtained by pretreatment of the HCl with sodium sulfite. The practical applications of this observation are many. First, in some applications, it may be possible to substitute a lower grade of hydrolysis acid. Second, it may be possible to extend the effective shelf life of hydrochloric acid used in amino acid analysis. Third, Na_2SO_3 may serve as a replacement for phenol, which is difficult to purify. Finally, as amino acid analysis is extended to samples of smaller size, reduction of the volume of hydrolysis acid becomes increasingly difficult; hence, the use of an antioxidant becomes necessary. In many ap-

plications, Na_2SO_3 may prove to be the antioxidant of choice.

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