COLUMN CHROMATOGRAPHY OF PHOSPHOSERINE, PHOSPHOETHANOLAMINE AND S-SULFO-GLUTATHIONE AND THEIR IDENTIFICATION IN THE PRESENCE OF OTHER ACIDIC AMINO ACIDS

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In a previous paper in this journal[1] a method was described for the column chromatographic analysis of some acidic sulfur-containing amino acids. By using as eluent a solution containing 0.1 *M* citric acid and 0.2 *M* NaCl it was possible to separate on the long column (150 cm) of the Automatic Amino Acid Analyzer (Beckman-Spinco Model 120) some acidic compounds which emerge in the same fraction with the usual pH 3.25 citrate buffer[2]. Methods were also reported for the discrimination of some overlapping compounds.

In the present report we describe the behaviour of three other acidic compounds, namely phosphoserine, phosphoethanolamine and S-sulfo-glutathione*, which may interfere with the chromatographic analysis of the sulfur-containing amino acids previously examined.

MATERIALS AND METHODS

PS and PE were obtained from Cal-Biochem and oxidized glutathione from Boehringer & Soehne.

Sulfur-containing compounds were prepared by the methods referred to in the previous paper[1]. S-GSH was obtained by treating a 10^{-2} *M* solution of oxidized glutathione with sodium sulfite (final concentration 10^{-1} *M*; final pH *ca.* 7.4) for 2 h at room temperature.

After acidification with acetic acid the solution was taken to dryness to eliminate SO_2, and redissolved in the citric acid–NaCl solution used for chromatography. Alkaline phosphatase from calf intestine (acetone dry powder with specific activity of 8 units/mg AP-II 15437) was obtained from Boehringer & Soehne.

Chromatographic analyses were performed with a model 120 Beckman-Spinco Amino Acid Analyzer. The long column (150 cm) was used, equilibrated with 0.1 *M* citric acid/0.2 *M* NaCl (with addition of BRIJ and caprylic alcohol, see ref. 1) and the compounds were eluted with the same solution at 30°.

* The following abbreviations are used: PS = phosphoserine; PE = phosphoethanolamine; S-GSH = S-sulfo-glutathione (GS-SO_3H); ATS = alaninethiosulfonic acid; CSA = cysteinesulfinic acid; S-CYS = S-sulfo-cysteine (CYS-SO_3H); S-CYSA = S-sulfo-cysteamine.

RESULTS

PS and PE are well separated from each other, being eluted at 70 and 85 ml respectively (Fig. 1). S-GSH is eluted together with PS.

However, some other acidic compounds overlap in the same position, and the following procedures may be useful for discrimination.

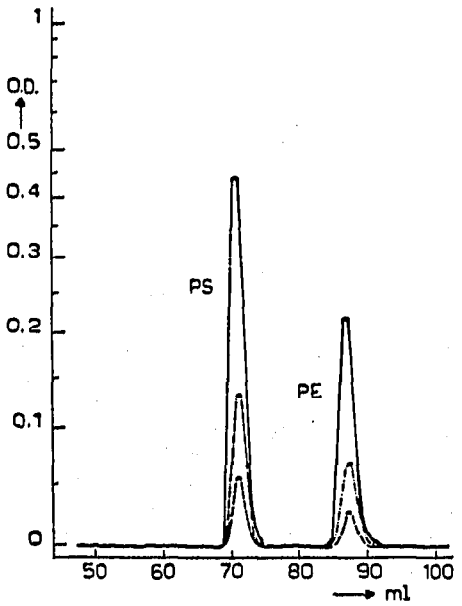

Fig. 1. 0.5 μmoles each of PS and PE chromatographed on the long column of the Beckman-Spinco Amino Acid Analyzer, using 0.1 *M* citric acid–0.2 *M* NaCl as eluent. Temp. 30°. ——— recording of the normal 570 mμ photocell; –·–·–· recording of the suppressed 570 mμ photocell; – – – recording of the 440 mμ photocell.

Phosphoserine

As well as overlapping with S-GSH, PS is eluted with cysteic acid, S-CYS and ATS[1]. Methods have already been reported for the identification of the latter three compounds[1].

To identify PS it has been shown that treatment with alkaline phosphatase leads to the complete disappearance of the peak due to PS, which may then be quantitatively recovered as serine, whereas all the other compounds are left unchanged.

For this test 10 μmoles of PS were incubated with 1 mg of alkaline phosphatase in 5 ml of 0.1 *M* borate–NaOH buffer pH 10.5 at 38°. Analyses for phosphates by the method of FISKE AND SUBBA-ROW[6] showed that hydrolysis was complete in 60 min. The incubation was nevertheless continued for 90 min, then 0.2 ml of 100 % trichloroacetic acid were added, the slightly turbid solution was centrifuged at high speed, and 0.25 ml of the supernatant was put on the column.

After 90 ml of the citric acid eluent have been passed through the column, the eluting solution was changed to pH 3.25 citrate buffer[2] and the temperature was raised to 50°, to detect serine. The chromatogram obtained showed only the presence of serine.

Fig. 2 shows the results obtained by treating a solution containing PS and S-CYS in the same way with phosphatase. The relative control incubated without

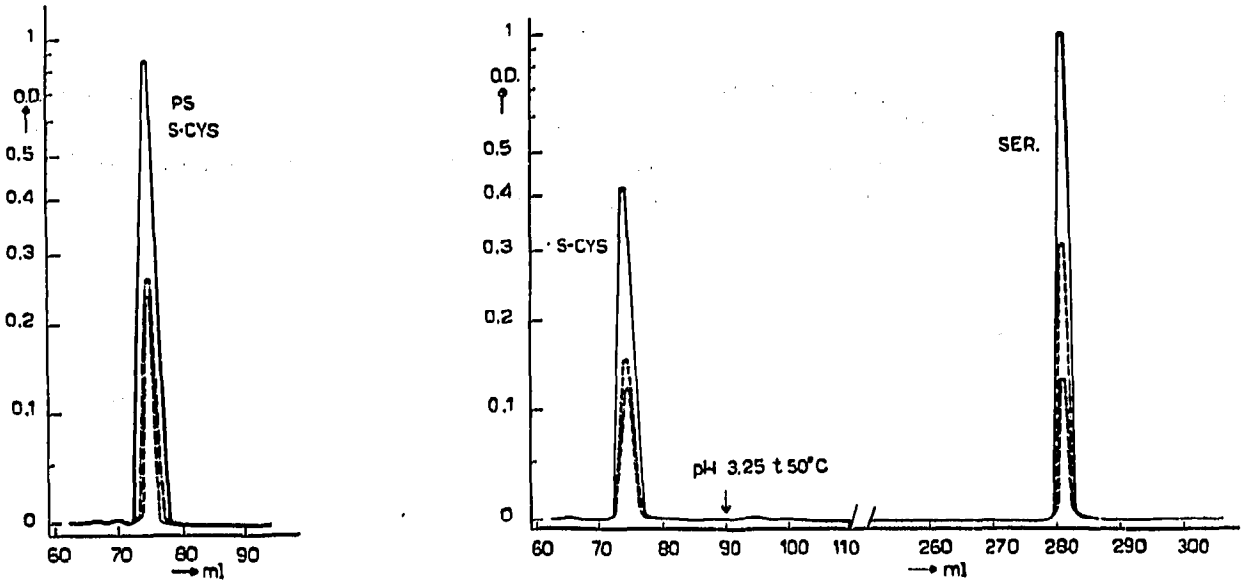

Fig. 2. Chromatography of PS and S-CYS before and after alkaline phosphatase treatment. Experimental conditions as in Fig. 1. Left = 0.5 μmoles each of PS and S-CYS from an incubate at pH 10.5 made as described in the text, without phosphatase added. Right = 0.5 μmoles each of PS and S-CYS from an incubate made as described in the text with phosphatase added. The arrow indicates that the citric acid eluent has been changed to pH 3.25 citrate buffer, and the temperature raised from 30° to 50°, to detect serine (SER). Note that in the peak of S-CYS the absorbancy recorded by the 440 mμ photocell is higher than that of the suppressed 570 mμ photocell. This results in a high absorbancy at 440 mμ, and in a low 570:440 mμ absorbancy ratio in the peak due to PS + S-CYS. ——— recording of the normal 570 mμ photocell; –·–·–· recording of the suppressed 570 mμ photocell; – – – recording of the 440 mμ photocell.

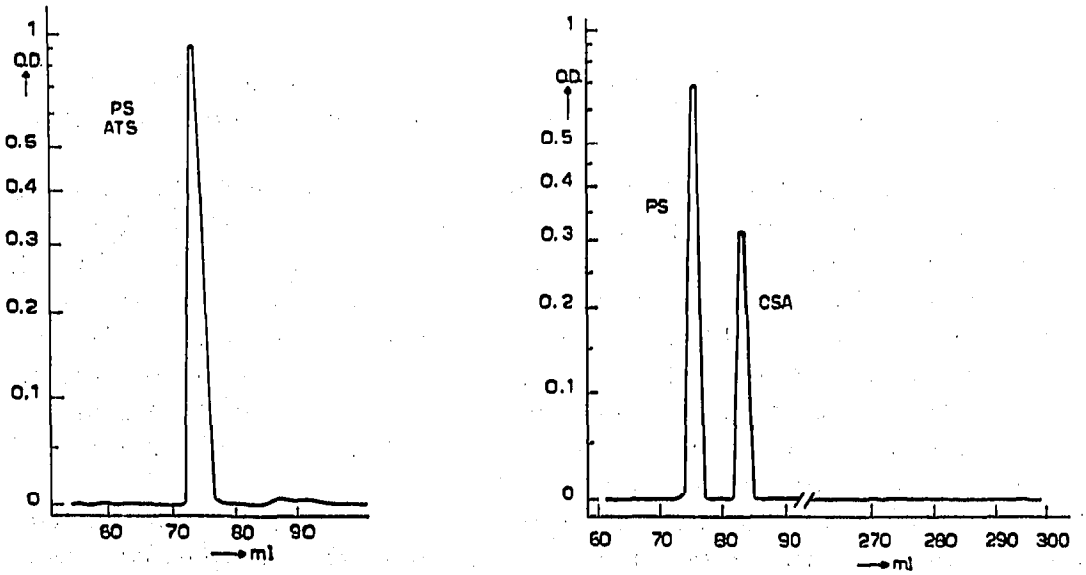

Fig. 3. Chromatography of PS and ATS before (on the left) and after (on the right) the cyanolysis reaction (see text). Experimental conditions as in Fig. 1. Only the recording of the normal 570 mμ photocell is reported.

phosphatase is also shown. Quantitative analysis of S-CYS showed that this compound is unaffected by the incubation in alkaline solution with or without added phosphatase.

The same test was made on a solution containing PS and ATS; in this case, also, it was observed that ATS remains unchanged. The chromatographic patterns obtained with ATS could be superimposed on those reported in Fig. 2, except that the 570:440 mμ absorbancy ratio of the peak at 70 ml, when both compounds are present (control solution), is lower in the case of PS + S-CYS than in the case of PS + ATS.

Since, as described previously, the method for identifying ATS is based on its conversion into CSA by cyanolysis[1], the experimental conditions in which this reaction is allowed to take place were checked as to whether they could affect PS. For this purpose a solution containing 10^{-3} moles PS and 10^{-3} moles ATS per l was chromatographed before and after the cyanolysis reaction. The results obtained (Fig. 3) showed that whereas ATS is completely changed into CSA, PS is left unchanged.

Phosphoethanolamine

This compound is eluted together with taurine, thiotaurine and S-CYSA. Also, under the experimental conditions described for PS, it is completely hydrolyzed by alkaline phosphatase.

In this case the product of the enzymatic hydrolysis was detected and identified by descending paper chromatography in phenol–NH_3, in which solvent R_F values of 0.28 and 0.90 for PE and ethanolamine were obtained.

It was also checked that S-CYSA and thiotaurine are unaffected by incubation in alkaline solution with or without phosphatase, and it was noted that PE is unchanged after cyanolysis reaction.

S-Sulfo-glutathione

This compound is eluted together with other acidic amino acids at 70 ml. To distinguish between S-GSH and the other compounds eluted at the same position, advantage may be taken of the spontaneous reaction of the S-sulfo-compounds with an excess of thiol, as previously adapted to the identification of S-CYS and S-CYSA[1]. Cysteamine has been used for this purpose and the complete disappearance of S-GSH has been checked by column chromatography.

The usefulness of another spontaneous reaction has also been tested for the identification of S-GSH, namely the exchange reaction between S-sulfo-compounds and disulfides, which has been previously studied[4,5]:

$$2\ \text{R-S-SO}_3\text{H} + \text{R'-S-S-R'} \rightleftharpoons 2\ \text{R'-S-SO}_3\text{H} + \text{R-S-S-R}$$

By using cystamine the corresponding S-sulfo-derivative, S-CYSA which is easily detectable on the long column of the Amino Acid Analyzer, would be formed in the above reaction. The reaction was performed as follows. S-GSH obtained as described, was dissolved in 0.01 *M* phosphate buffer pH 6 to give a solution $2 \cdot 10^{-3}$ *M*, and cystamine was added to a final concentration of 10^{-2} *M*. The solution was allowed to stand for 2 h at room temperature and was then chromatographed. As shown in Fig. 4 the peak at 70 ml completely disappeared and was substituted by a peak at 90 ml. This latter was identified as S-CYSA from its position as well as by the 570:440

mμ absorbancy ratio[1]. Also evident from Fig. 4 is that the O.D. recording of the 440 mμ photocell is higher than that of the suppressed 570 mμ photocell in the case of S-CYSA, which is contrary to what happens in the case of S-GSH and other compounds. The results obtained therefore showed that the exchange reaction with cystamine may be usefully adapted to identify S-GSH. It may be observed also that

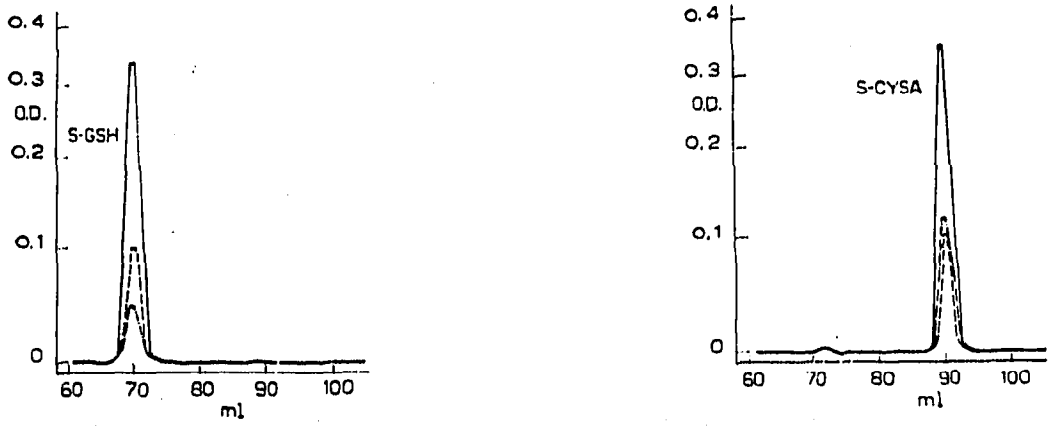

Fig. 4. Chromatography of S-GSH before (left) and after (right) reaction with cystamine (see text). S-GSH is completely transformed into S-CYSA. Note that in the peak due to S-CYSA the absorbancy recorded by the 440 mμ photocell is higher than that of the suppressed 570 mμ photocell. ——— recording of the normal 570 mμ photocell; – – – recording of the suppressed 570 mμ photocell; –·–·–· recording of the 440 mμ photocell.

S-CYS, which is eluted together with S-GSH, may give rise to S-CYSA by reaction with cystamine[4,5]. However, the presence of both S-GSH and S-CYS may be suspected by recording the 570:440 mμ absorbancy ratio of the peak at 70 ml (see also Fig. 2). Whereas S-CYS (as well as S-CYSA) gives a value of 2,5 to 3 for this ratio, S-GSH gives a normal value of 7 to 8.

In any case S-GSH and S-CYS may also be differentiated by treatment with cysteamine and detection of the resulting thiol (reduced glutathione and cysteine, respectively).

CONCLUSIONS

The data reported here and in the previous papers on this subject[1,3] emphasize the usefulness of the 0.1 *M* citric acid solution as the first eluent in the column chromatographic analysis of amino acids.

Some acidic compounds can be easily separated from each other, and some others which overlap can be differentiated with the aid of simple chemical or enzymatic reactions. Moreover the use of citric acid as the first eluent does not affect the chromatographic behaviour of the other amino acids later displaced from the column by the other usual buffers.

SUMMARY

The column chromatographic behaviour of phosphoserine, phosphoethanolamine and S-sulfo-glutathione using 0.1 *M* citric acid as eluent is described.

Treatment with alkaline phosphatase has proved useful for hydrolyzing phosphoserine and phosphoethanolamine, which can thus be differentiated from some other overlapping compounds, and then estimated by difference or as the free amino compounds.

S-Sulfo-glutathione may be identified by treatment with an excess of cysteamine which leads to its degradation into free GSH, or by treatment with cystamine, which leads to production of S-sulfo-cysteamine.

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