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ENZYMATIC INACTIVATION OF SRS-CYS-GLY (LEUKOTRIENE D)

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SUMMARY

The peptidases in rat basophilic leukemia cells, guinea pig lung, peritoneal eosinophils and limpet arylsulfatase preparation were shown to inactivate SRS-Cys-Gly but not SRS-GSH and SRS-Cys. The SRS-Cys-Gly inactivating peptidases were resolved from arylsulfatases in these cell systems. We have shown that arylsulfatases do not inactivate SRS-Cys-Gly and that peptidases are probably the enzymes primarily responsible for modulating SRS-Cys-Gly levels *in vivo*.

INTRODUCTION

A variety of arylsulfatases were shown to inactivate the spasmogenic activity of SRS-A[1] (1-4). It was suggested that arylsulfatases may be responsible for the inactivation of SRS-A *in vivo* following antigen-antibody reaction (1). Moreover, susceptibility to arylsulfatase inactivation was used until recently as an important criterion in the positive identification of SRS-A.

In a previous paper (5), we defined the mechanism of SRS-A inactivation by limpet arylsulfatase. This enzyme preparation cleaved the peptide bond in the highly bioactive SRS-Cys-Gly to form the much less bioactive SRS-Cys. However, it was not known whether arylsulfatase *per se* or a contaminating peptidase was responsible for the hydrolysis of the peptide bond in SRS-Cys-Gly. Subsequent-

[1]Abbreviations: RBL, rat basophilic leukemia; SRS, slow reacting substance; HPLC, high pressure liquid chromatography; SRS-Cys, 5-hydroxy-6-S-cysteinyl-7,9,11,14-isocatetraenoic acid (LTE); SRS-Cys-Gly, 5-hydroxy-6-S-cysteinylglycyl-7,9,11,14-eicosatetraenoic acid (LTD or SRS-A); SRS-GSH, 5-hydroxy-6-r-glutamylcysteinylglycyl-7,9,11,14-eicosatetraenoic acid (LTC); leukotriene A, trans-5(S),6(S)-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid; SRS-NTFA-Cys-Gly, 5-hydroxy-6-S-N-trifluoroacetyl-cysteinylglycyl-7,9,11,14-eicosatetraenoic acid; p-NCS, p-nitrocatechol sulfate; p-NPS, p-nitrophenol sulfate; ECF-A, eosinophil chemotactic factor of anaphylaxis.

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ly, we reported (6) that L-cysteine altered the levels of SRSs indirectly by inhibiting an enzyme involved in the inactivation of SRS-Cys-Gly. It is the purpose of this report to show what types of enzymes are responsible for SRS-A inactivation in RBL-1 cells, guinea pig lung and peritoneal eosinophils, and that limpet arylsulfatase lacks intrinsic peptidase activity to cleave the R-S-Cys-Gly peptide bond of SRS-A.

MATERIALS AND METHODS

Type IV-S leucine aminopeptidase (batch 129C-80951, 11 units/mg), Type V sulfatase (Patella vulgata, 9.4 units/mg). Sephadex G-150 and G-200, DEAE-Sephadex (A-25), SP-Sephadex (SP-500-120) and Percoll were purchased from Pharmacia. Crystalline bovine albumin was obtained from Calbiochem. DEAE-cellulose (DE-52) was a product of Whatman. Purified rat renal dipeptidase and aminopeptidase M were kindly supplied by Drs. T. M. McIntyre and N. P. Curthoys, Department of Biochemistry, University of Pittsburgh. Synthetic SRS-GSH was prepared by reaction of (±)LTA methyl ester with GSH (5). SRS-Cys-Gly and SRS-NTFA-Cys-Gly were prepared by reaction of (±)LTA methyl ester with NTFA-Cys-Gly methyl ester. Treatment of the resulting SRS-NTFA-Cys-Gly dimethyl ester with pig liver esterase (7) gave SRS-NTFA-Cys-Gly. Complete deprotection to give SRS-Cys-Gly was achieved by reaction under argon with 0.13 M K_2CO_3 in water/methanol 3:1 at 25°C for 18 hrs.

Arylsulfatase Assay. The rate of p-nitrocatechol formation was determined by measuring the absorbance at 515 nm (8) with a Gilford spectrophotometer (Model 240). Specific activities were expressed in unit (1 μmole product per hour) per mg of protein. Protein was determined by the method of Lowry (9) and the Biuret method (10) using bovine albumin as standard.

SRS Inactivation Assay. The system contained: 0.3 nmoles of SRS-Cys-Gly and enzyme in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.2. The contents were incubated at 37°C and after 30 min incubation, an aliquot was removed and the residual SRS activity was bioassayed using the guinea pig ileum (5). One unit of SRS inactivating activity was defined as that amount of enzyme which destroys 0.1 nmole of SRS-Cys-Gly in 30 min.

Preparation of RBL-1 Cell Extract. Cells (2×10^9) were harvested and the cell extract was prepared (6,13) and dialyzed against 0.01 M Tris-HCl buffer, pH 8.0.

Guinea Pig Lung Extract. An extract of guinea pig lung (25 g) was prepared (2) and dialyzed against 0.01 M Tris-HCl buffer, pH 8.0. To the dialysate was added DEAE-cellulose (10 g), equilibrated with 0.01 M Tris-HCl buffer, pH 8.0. After 30 min, the mixture was filtered and the enzyme activity was eluted off the DEAE-cellulose with the same buffer containing 0.2 M NaCl. The eluate was again dialyzed against 0.01 M Tris-HCl buffer, pH 8.0.

Guinea Pig Peritoneal Eosinophil Cell Extract. The cells, collected by peritoneal lavage of ether-anesthetized animals with 50 ml of saline, were centrifuged (400 x G, 10 min). The cells were washed once with saline and suspended to $1\text{-}3 \times 10^7$/ml in a Percoll density gradient and centrifuged (11). The eosinophil-rich fraction (>83%, density 1.090-1.100 g/ml) was washed once with saline and the pellet was stored at -20°C. The cells (3.5×10^7), suspended in 2.5 ml of 0.1 M sodium acetate buffer, pH 5.7, were sonicated for 30 sec at 4°C.

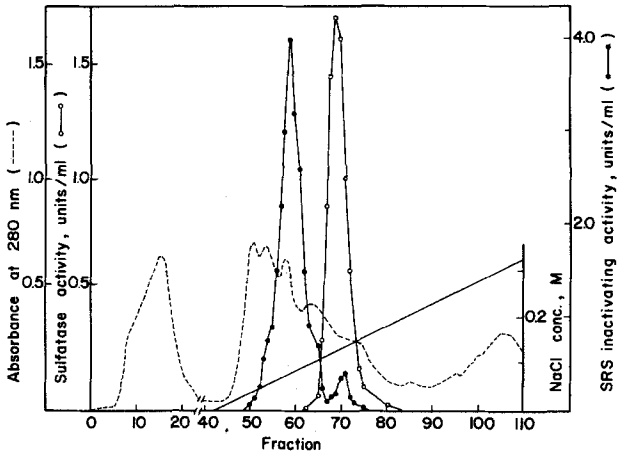


Fig. 1 The extract of RBL-1 cells was applied onto a DEAE-cellulose column (1.7 x 26 cm) and the column was eluted with a 400 ml linear salt gradient to 0.4 M NaCl at 1 ml/min. Fraction volume: 4 ml.

The cell extract was subjected to freezing and thawing (6 times), centrifuged (600 x G, 20 min) and then dialyzed against 0.01 M Tris-HCl buffer, pH 8.0.

RESULTS

RBL-1 Cells. After chromatography of the RBL-1 cell extract on a DEAE-cellulose column, two enzyme fractions possessing SRS-Cys-Gly inactivating activity were noted (Fig. 1). The major component (Fractions 54-64) was further purified by rechromatography on a Sephadex G-200 column to remove the trace of the minor component. This enzyme fraction readily converted SRS-Cys-Gly into SRS-Cys with a pH 7.0 optimum but had no effect on SRS-GSH and SRS-NTFA-Cys-Gly. L-cysteine (10^{-2} M) markedly inhibited the inactivation of SRS-Cys-Gly (Fig. 2A), but bestatin (10^{-4} M) did not. L-leucylglycine was hydrolyzed by this enzyme at a rate 90-fold faster than L-leucyl-p-nitroanilide. These properties resemble those of metallodipeptidases (12). The minor SRS-Cys-Gly inactivating peak (Fractions 69-73) has the same SRS-Cys-Gly inactivating properties, except that L-cysteine (10^{-2} M) didn't inhibit the inactivation of SRS-Cys-Gly by this enzyme.

The properties of the arylsulfatase (Fractions 66-71) corresponded to those of arylsulfatase A of RBL-1 tumor cells (13). The pH optimum of p-NCS hydroly-

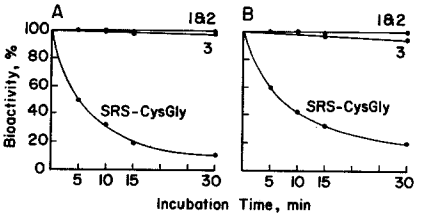


Fig. 2 The rate of inactivation of SRSs. The system contained 0.3 nmoles of SRSs and varying units of SRS inactivating activity [RBL-peptidase (5 units) or guinea pig lung peptidase (3 units)] in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.2. A. RBL-peptidase; B. guinea pig lung peptidase. 1. SRS-GSH; 2. SRS-NTFA-Cys-Gly; 3. SRS-Cys-Gly + 10 mM L-cysteine.

sis was at 5.2; its molecular weight estimated by gel filtration was approximately 100,000; it was inhibited to the extent of 56% by 5 mM ATP.

Guinea Pig Lung. L-cysteine was used to enhance SRS-A levels in guinea pig lung tissue (14), which suggested the presence of a thiol-sensitive peptidase in this system. However, the only SRS-A inactivating enzyme was ascribed to the action of arylsulfatase type II-B (15). When guinea pig lung extract was chromatographed over a DEAE-Sephadex column, the SRS-Cys-Gly inactivating activity did not coincide with the arylsulfatase activity (Fig. 3). The peptidase peak (Fractions 48-60) as revealed by its ability to cleave leucyl-p-nitroanilide was further purified on a Sephadex G-200 column. This partially purified enzyme also hydrolyzed leucylglycine and like serum cystine aminopeptidase (16) it

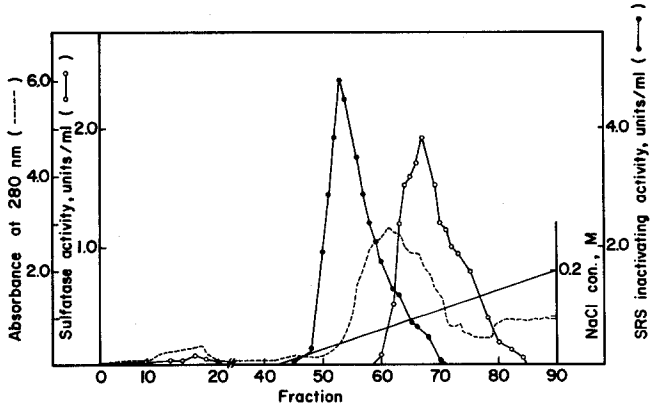


Fig. 3 The extract of guinea pig lung was applied over a DEAE-Sephadex column (1.7 x 30 cm). The column was eluted with a 100 ml linear salt gradient to 0.2 M NaCl at a flow rate of 0.5 ml/min. Fraction volume: 2 ml.

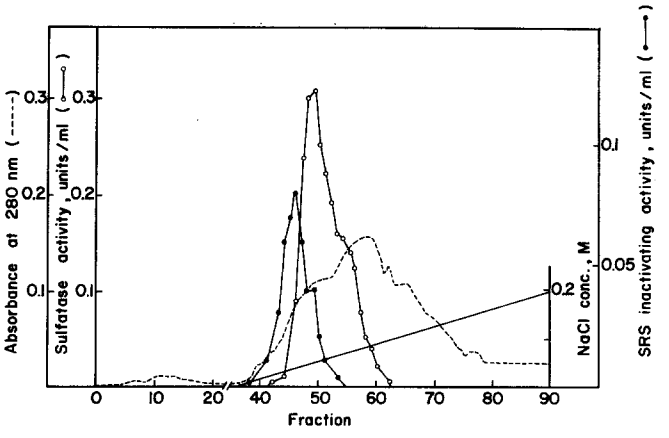
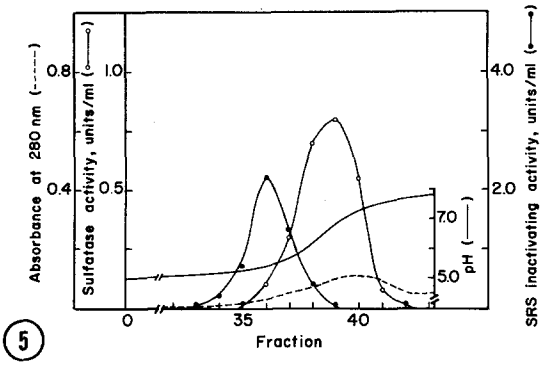


Fig. 4 The extract of eosinophil cells was applied onto a DEAE-cellulose column (1.7 x 10 cm). The column was eluted with a 100 ml linear salt gradient to 0.2 M NaCl at a flow rate of 0.6 ml/min. Fraction volume: 2 ml.

cleaved S-benzylcysteine-p-nitroanilide. Fig. 2B shows the inactivation of SRSs by this enzyme. Like the RBL-1 peptidase, this enzyme didn't attack SRS-GSH and SRS-NTFA-Cys-Gly and was inhibited by 10^{-2} M L-cysteine. The arylsulfatase activity (Fractions 64-78) exhibited a 5.7 pH optimum for p-NCS hydrolysis, and was more active in cleaving p-NCS than p-NPS. These properties resemble those of arylsulfatase type II-B (15).

Guinea Pig Peritoneal Eosinophils. It was proposed that eosinophils were attracted to the site of an immediate-type hypersensitivity reaction by ECF-A, and SRS-A was assumed to be inactivated by eosinophil arylsulfatase B. Since published data (1) implied that arylsulfatase type II-B was the only SRS-A inactivating enzyme in eosinophils, we decided to examine the validity of this supposition. Again, Fig. 4 shows that the SRS-Cys-Gly inactivating peptidase activity (Fractions 41-48) as manifested by the hydrolysis of leucine-p-nitroanilide did not coincide with the arylsulfatase activity (Fractions 48-60) as assessed by the hydrolysis of p-NCS. Unfortunately, we were unable to conduct more in depth studies due to the small number of eosinophils.

Limpet Arylsulfatase. When limpet arylsulfatase was chromatographed over an SP-Sephadex column, most of the SRS-Cys-Gly inactivating activity (Fractions



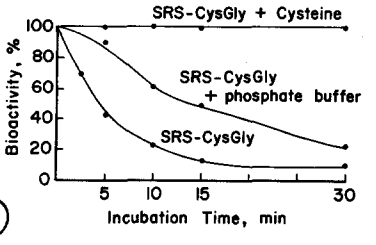


Fig. 5 Limpet arylsulfatase (5 mg), dissolved in 15 ml of 0.01 M sodium acetate buffer, pH 5.0 was applied onto a SP-Sephadex column (1.7 x 26 cm). The column was washed with a 100 ml pH gradient to pH 8.0 (0.01 M Tris-HCl buffer) at a flow rate of 12 ml/hr. Fraction volume: 2 ml. SRS inactivating activity was measured using 0.1 M sodium acetate buffer, pH 5.0.

Fig. 6 Inactivation of SRS-Cys-Gly bioactivity by purified rat renal dipeptidase (0.15 units). The system contained 10 mM L-cysteine or 50 mM sodium phosphate buffer, pH 7.2, where indicated.

34-37) was found not to coincide with arylsulfatase activity (Fractions 38-41) (Fig. 5). The SRS-Cys-Gly inactivating peptidase had a pH 5.0 optimum and did not attack SRS-GSH but hydrolyzed SRS-NTFA-Cys-Gly slowly. This peptidase didn't hydrolyze leucyl-p-nitroanilide and was not inhibited by L-cysteine (10^{-2} M). The arylsulfatase having a pH optimum of 5.0 for p-NCS hydrolysis was inhibited by 70 μM-ATP to the extent of 50%.

Kidney Brush Border Membrane Peptidases. We previously demonstrated (6) the conversion of SRS-Cys-Gly into SRS-Cys by a crude kidney particulate peptidase (Sigma aminopeptidase M). Recently, another particulate kidney enzyme, dipeptidase, was shown to hydrolyze cystinylglycine (17). Because we observed wide variations in SRS-Cys-Gly inactivation rates among different Sigma kidney aminopeptidase M preparations, we surmised that a contaminating dipeptidase may be primarily responsible for SRS-Cys-Gly inactivation. Fig. 6 shows that SRS-Cys-Gly is rapidly inactivated by 0.15 units of purified rat renal dipeptidase; L-cysteine (10^{-2} M) and phosphate ions (50 mM) inhibited this inactivation. On

the other hand, purified rat renal aminopeptidase M (0.15 units) did not significantly inactivate SRS-Cys-Gly.

DISCUSSION

In all the cell systems studied, the SRS-Cys-Gly inactivating peptidase activities were resolved from the arylsulfatase activities. Further, the arylsulfatase (Fractions 75-80, Fig. 3 and Fractions 40-41, Fig. 5) did not inactivate SRS-Cys-Gly. These results suggest that both type A and type B arylsulfatases lack intrinsic peptidase activity to cleave the R-Cys-Gly peptide bond. These SRS-Cys-Gly inactivating peptidases were considerably more active than those reported for cytosol leucine aminopeptidase and carboxypeptidase A, which required high enzyme to substrate ratios and prolonged incubation (18). Although the precise nature of these peptidases were not defined, their properties more closely resembled those of dipeptidases and aminopeptidases. The kidney brush border dipeptidase was highly active in SRS-Cys-Gly inactivation. Consequently, the peptidases, especially dipeptidase, may play an important role in regulating the level of SRS-A *in vivo*.

Other possible mechanisms of reducing the spasmogenic activities of SRSs could involve the isomerization of double bond (11-*cis* → 11-*trans*) of SRSs (19) catalyzed by RS• in cells. In addition, soybean lipoxygenase (6,20) has been shown to render SRSs inactive by C-15 hydroperoxidation and 15-hydroxyeicosatetraenoic acid has been isolated from several cell systems (21-23). Hence, the significance of mammalian lipoxygenase in SRS inactivation warrants further investigation.

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