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Fluorometric Determination of Arylsulfatase A and B Activities

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Human lung arylsulfatase activity was determined by a fluorometric microanalytical method with 4-methylumbelliferyl sulfate as the substrate. By the use of a suitable deproteinizing agent after the enzymatic reaction, arylsulfatase activity in whole blood could be determined at the picomole level; without deproteinization, the blank value was higher because of turbidity due to protein. The present method is approx. 2000 times more sensitive than the conventionally used spectrophotometric method of arylsulfatase activity determination.

It was found in the present study that the optimum pH's of AS-A and AS-B were different when *p*-nitrocatechol sulfate was used as the substrate: optimum pH for AS-A = 4.2; that for AS-B = 5.5.

Keywords——arylsulfatase A; arylsulfatase B; 4-methylumbelliferyl sulfate; 4-methylumbelliferone; human lung enzymes

Since arylsulfatase A (AS–A) and arylsulfatase B (AS–B) [EC 3.1.6.1.] are present in minute amounts in biological systems, a sensitive and accurate analytical method for these activities is required to assay them in biological materials in order to clarify physiological significance. AS–A and AS–B are known to originate from lysosomal granules in mammals.

In the present study, the authors developed a sensitive fluorometric assay method, which is approx. 2000-fold more sensitive than the conventionally used spectrophotometric method.[1] The substrate for AS–A and AS–B is 4-methylumbelliferyl sulfate (4-MUS), and the product, 4-methylumbelliferyl sulfate (4-MUS), and the product, 4-methylumbelliferone (4-MU), is a fluorescent compound. In the conventional spectrophotometric determination, the substrate is *p*-nitrocatechol sulfate (*p*-NCS), and the product, *p*-nitrocatechol (*p*-NC), is determined spectrophotometrically.

Materials and Methods

Substrates and Standard Reaction Products——*p*-NCS, 4-MUS, *p*-NC and 4-MU were obtained from Sigma Chem. Co., St. Louis, Mo., U.S.A.

Protein Determination——Protein concentration was determined with a "Protein Assay Kit, Nippon Biorad Labs." and the standard protein was bovine serum albumin (BSA), Sigma Chem. Co.

Chromatographic Material——Diethylaminoethyl (DEAE) cellulose was Whatman DE-52, obtained from Nippon Biorad Labs., Tokyo, Japan.

Enzyme——Human lung was an autopsy sample. Blood as the enzyme source was collected from healthy volunteers. Control serum was the product of Boehringer Mannheim, Mannheim, W. Germany.

Instruments——A Hitachi 100-50 double-beam spectrophotometer and a Shimadzu RF-502 spectrophotometer were used for the determination of enzyme activities. A Hitachi Type 20 PR-50 refrigerating centrifuge and a Kubota Type KN 70 low speed centrifuge were used for centrifugations.

Separation of AS Activities into Two Fractions——Separation of AS-A and AS-B was performed according to the method described by Wasserman and Austin[2]: human lung, taken within a few hours of death, was homogenized with 20 ml of ice-cold 0.9% physiological saline per 10 g of the tissue for 5 min with a Waring blender. The homogenate was sonicated for 2 min. The homogenate was centrifuged at 15000 ***g*** at 4°C for 20 min, and the precipitate was sonicated and centrifuged once again. The combined supernatant was dialyzed against 0.01 M Tris-HCl buffer, pH 8.0, at 4°C for 18 h. The suspension was centrifuged at 15000 ***g*** at 4°C for 30 min, and the supernatant was subjected to DEAE-cellulose column chromatography on a 2.2 × 25 cm column previously equilibrated with 0.01 M Tris-HCl buffer, pH 8.0. The column was washed with

3 bed volumes of the buffer, then eluted with an NaCl linear gradient from 0 to 0.4 M. Each fraction, 7.5 ml, was analyzed for protein at 280 nm and for AS activities. For the determination of specific activities, protein content was measured by the use of Coomassie Brilliant Blue G-250 stain using BSA as the standard.

Determination of AS-A and AS-B Activities——Spectrophotometric determination was performed according to the method described by Baum *et al.*,[1] using *p*-NCS as the substrate. Fluorometric determination was performed using 4-MUS as the sybstrate as originally described by Christomanou and Sandhoff.[3] The assay conditions of the spectrophotometric and fluorometric methods were made as similar as possible to permit a strict comparison of the two methods. The procedures i) through v) were used to optimize the fluorometric assay method.

i) Effect of pH on the Fluorescence Development: To 0.15 ml of 4-MU standard solution (10 nmol/ml), 4 ml of 0.2 M glycine–NaOH buffer, from pH 7 to 13, was added, and the resulting fluorescence intensities were determined.

ii) Deproteinizing Procedure: To 0.1 ml of 4-MU standard solution (10 nmol/ml), 0.05 ml of distilled water and 0.1 ml of deproteinizing agent [5, 10, 20 or 30% (w/w) trichloroacetic acid (TCA), or 5, 10, 20 or 30% (w/w) phosphotungstic acid (PTA), or 7, 17.5 or 35% (w/w) perchloric acid (PCA)], and 4 ml of 0.2 M glycine–NaOH buffer, pH 11.0, were added, and the fluorescence intensity obtained was determined against a control (to which 0.1 ml of distilled water had been added instead of the deproteinizing agent) on a fluorometer at 365 nm excitation and 450 nm emission wavelengths.

iii) Substrate Concentrations: To 0.05 ml of AS-A or AS-B solution (10 U/ml), 1.0, 2.5, 5.0, 7.5 or 10.0 mM 4-MUS dissolved in 0.1 M sodium acetate buffer, pH 5.5, was added, and the mixture was incubated at 37°C for 1 h. Then 4 ml of glycine–NaOH buffer, pH 11.0, was added and the fluorescence intensity was determined.

iv) Effect of Buffer Compositions: To 0.05 ml of human lung AS-A or AS-B fraction, 0.1 ml of 0.1 M sodium citrate buffer, pH 5.5, or 0.1 M or 0.05 M sodium acetate buffer, pH 5.5, containing 7.5 mM 4-MUS was added, and the assay was performed according to the procedure described in the preceding section.

v) Sensiticity of the Assay of *p*-NC and 4-MU: A standard solution (10 nmol/ml) of 4-MU was diluted with distilled water, and 3 ml of 0.2 M glycine–NaOH buffer, pH 11.0, was added to 0.1 ml of various concentrations of the standard solution. The fluorescence intensity was determined as described in ii).

vi) Comparison of the Spectrophotometric and Fluorometric Methods: For AS activity determination by the spectrophotometric method, 0.1 ml of 50 mM *p*-NCS in 0.5 M sodium acetate buffer, pH 5.5, and 0.1 ml of the enzyme solution of various concentrations were mixed, and the mixture was incubated for 1 h at 37°C, then 0.1 ml of 10% (w/v) TCA was added. The whole was centrifuged for 10 min, and 1 ml of 1.25 N NaOH was added to 0.2 ml of the supernatant. The absorbance was measured at 515 nm. For the determination of AS activity by the fluorometric method, a serial dilution of the standard enzyme solution was done, and the analysis was performed as described above.

vii) Examination of the Optimum pH for Both Procedures: Since AS's are enzymes having the optimum pH in the acidic region, solutions with 0.5 pH intervals were made from pH 4.0 to 7.0 with 0.5 M sodium acetate buffer, and the activity was determined.

viii) Definition of the AS Activities: For the spectrophotometric method, 1 U = 1.0 μmol *p*-NC/h/ml released from *p*-NCS. For the fluorometric method, 1 U = 1 nmol 4-MU/h/ml released from 4-MUS.

Results and Discussion

The substrate, 4-MUS, does not develop any fluorescence when excited at 350—365 nm, but the product, 4-MU develops strong fluorescence. Christomanou and Sandhoff[3] originally reported a fluorometric method using 4-MUS as the substrate for AS determination with leukocytes, but they did not report the effects of pH, deproteining agents, *etc.* They separated and purified the leukocyte AS–A and AS–B by an isoelectric focusing method, and then analyses were performed. In the present study, the original method was modified: the AS–A and AS–B fractions were partially purified for the analysis by chromatography, but a later experiment showed that such purification was not necessary. The analysis may be performed on whole blood, if protein is removed by PTA treatment after the enzymatic reaction. Thus in the present study we showed that the method is applicable to AS determination not only in leukocytes but also in whole blood. This is significant in that the procedure to isolate leukocytes may be omitted, so that the whole procedure is considerably simpler than the originally reported method.

Partial purification of AS–A and AS–B of the human lung was performed in order to establish optimum conditions for the analytical procedure. Human lung AS–A and AS–B

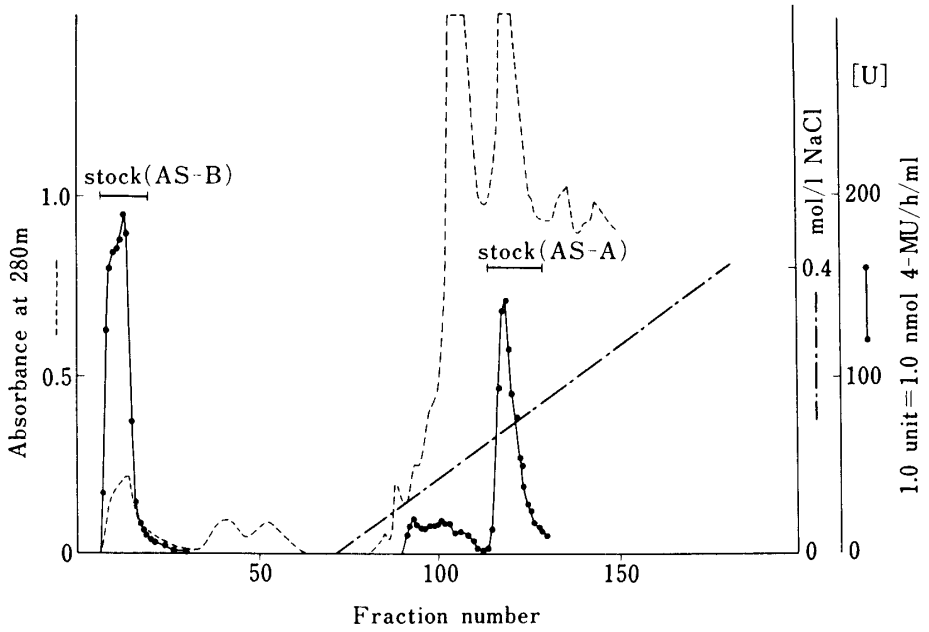

Fig. 1. DEAE-cellulose Chromatogram of Arylsulfatases in Human Lung

---- protein concentration.
—— arylsulfatase activity.
—— NaCl concentration.

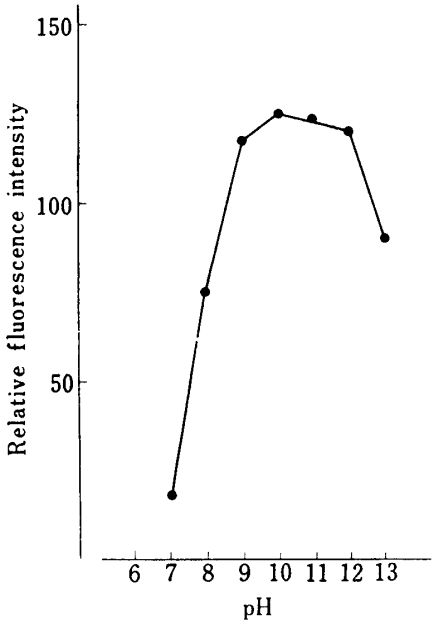

Fig. 2. pH Dependence of Fluorogenesis in the Assay Procedure

●——● 0.2 M glycine-NaOH buffer.

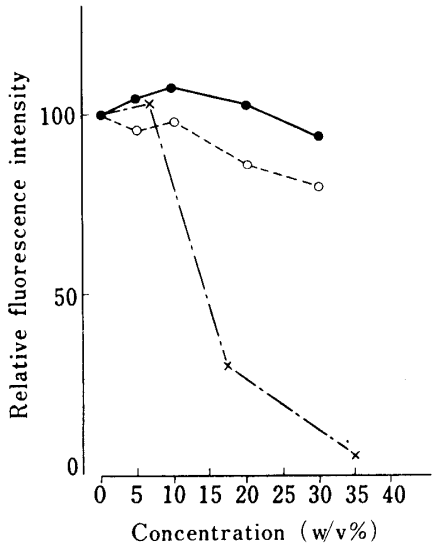

Fig. 3. Disturbance of Fluorogenesis in the Assay Procedure by Deproteinizing Agents, PTA, TCA and PCA

●——● phosphotungstic acid (PTA).
○----○ trichloroacetic acid (TCA).
×——× perchloric acid (PCA).

were purified 7- and 106-fold, respectively, from the crude homogenate. AS–A and AS–B fractions shown in Fig. 1 were used in this experiment. The effect of pH with 0.2 M glycine–NaOH buffer on the fluorescence developed by 4-MU is shown in Fig. 2, and the influences of various protein precipitating agents are shown in Fig. 3.

As–A and AS–B activities depended on the compositions and ionic strength of the buffer,

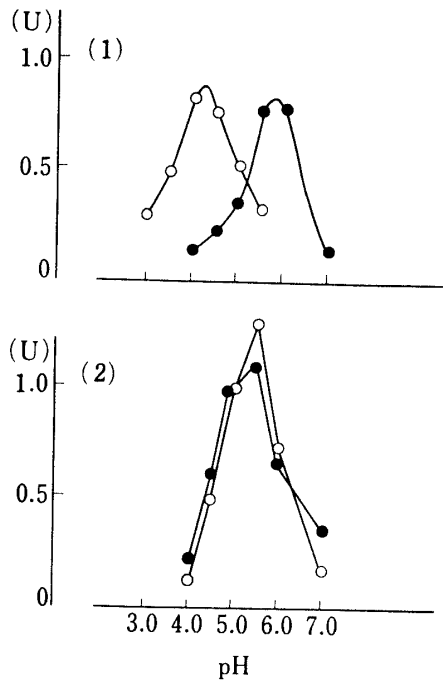

Fig. 4. pH Optima of Human Lung Arylsulfatase Activities

○—○ lung AS–A.
●—● lung AS–B.
(1) Spectrophotometry: 1 U=1.0 μmol *p*-NC/h/ml.
(2) Fluorometry: 1 U=1.0 nmol 4-MU/h/ml.

mix, { 0.05 ml, fractionated lung homogenate
0.10 ml, 10 mM 4-MUS[a)]
↓
incubate, at 37°C for 60 min
↓
add, 0.10 ml PTA[b)]
↓
centrifuge, at 3000 rpm for 10 mn
↓
take, 0.20 ml supernatant
↓
add, 4.00 ml, 0.27 M glycine–NaOH buffer, pH 11
↓
read fluorescence intensity, Ex λ_{365} nm and Em λ_{450} nm

Chart 1. Assay Procedure for Arylsulfatase A and B Activities

a) 10.0 mM 4-MUS in 0.5 M Na acetate buffer, pH 5.5.
b) 10.0% phosphotungstic acid.

pH 5.5. With 0.1 M acetate buffer, the activities were not altered, but with 0.5 M acetate, AS–A activity was increased (to 104%) and AS–B activity was decreased (to 96.3%). With 0.1 M citrate the AS–A (to 5.0%) and AS–B (to 2.0%) activities were both decreased considerably.

The detection limits of AS activities corresponded to 5 nmol of *p*-NC and 2.5 pmol 4-MU, that is, the fluorometry is 2000 times more sensitive than the conventionally used spectrophotometric method.

As shown in Fig. 4, the apparent optimum pH's for the spectrophotometric and fluorometric methods are different. This is a new finding.

The AS activities determined by spectrophotometry (using *p*-NCS as the substrate) and fluorometry (using 4-MUS as the substrate) were compared; the results of the two methods were well correlated with a correlation coefficient $\gamma=0.99$ ($p<0.001$). The coefficient of variation (CV) of the AS–A and AS–B activity determinations was approx. 10.0%. Thus, the fluorometric method of determination of AS activity has been established, and the procedures are summarized in Chart 1.

Conventionally used spectrophotometric AS determination requires 0.1 ml of concentrated urine[1)] or 0.4 ml of serum,[4)] and even in fluorometry,[3)] 0.05—0.1 ml of leukocytes is required, while the method presented here requires only 0.05 ml of whole blood. This very sensitive method should be useful to determine AS–A and AS–B activities in the body constituents in studies to clarify their physiological significance.

References and Notes

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