A RAPID AND EFFICIENT METHOD OF RADIOACTIVELY LABELING L-ASPARAGINASE

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1. Introduction

L-Asparaginase (L-asparagine aminohydrolase, EC 3,5.1.1, ASNase), an enzyme that hydrolyzes the amide group of asparagine, inhibits the growth of certain neoplasms [1–4] and is used clinically for treating certain human acute leukemias [5,6]. Although ASNase sequentially inhibits protein, DNA and RNA synthesis [7], its primary cellular and molecular actions are not completely defined.

Initial work on the action of ASNase suggested that it acted by depleting L-asparagine from tumor cells which lacked asparagine synthetase (EC 6.3.1.1) and that this amino acid deprivation caused the tumor cell death [1–4]. Recent work suggests that the enzyme may have early effects on the cell plasma membrane topography, fluidity, and electrokinetic properties [8–12].

Because of the extreme clinical importance of this enzyme, its uniqueness as a cancer chemotherapeutic agent, and its possible importance as a tool for the investigation of the cell surface, it was deemed important to attempt to produce radioactively labeled ASNase. This report describes a method for the production of [^{3}H] acetyl–asparaginase ([^{3}H] Ac–ASNase) that is 100% active. It is suggested that this radioactively labeled ASNase will aid in the investigation of its cellular and molecular actions.

Examples of immediate uses of [^{3}H] Ac–ASNase are suggested by the work implicating the action of ASNase on cell plasma membrane topography. This action may be due to ASNase's preferential inhibition of protein and glycoprotein synthesis, causing inhibition of rapid membrane turnover [13,14]. It may also involve a physical interaction of ASNase molecules with the cellular plasma membrane. Use of ASNase molecules that have been gently acetylated with tritiated acetic anhydride may make it possible to determine the extent of such interaction between the enzyme and the external surface of the cell.

2. Materials and methods

2.1. *Materials*

L-Asparaginase ('Lyovac', 50 000 IU, 320 IU/mg protein, Merck Sharpe & Dohme, Rahway, N.J.) was kindly supplied by Dr. Martin R. Klemperer of the University of Rochester. [U-^{3}H] acetic anhydride (400 Ci/mole) was purchased from New England Nuclear Corp. L-Aspartic acid-β-hydroxamate and Sephadex G-25-300 were purchased from Sigma Chemical Co., St. Louis, Mo.

2.2. *Protein determination*

ASNase in the form of a lyophilized powder was solubilized in 15 ml of 0.01 M phosphate buffer, pH 7.5. The protein content of this ASNase solution was determined by a modified Lowry procedure [9]. This technique required that the original ASNase solution be diluted 1:100 with glass-distilled water. From this stock, serial dilutions were taken and made up to the final vol of 1 ml. 2.5 ml of 17.5% $Na_2C\Theta_3$ and 1 ml of 0.05% $CuSO_4 \cdot 5H_2O$, 0.1% K tartrate were added, and the solution was mixed and allowed to stand for

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10 min. At this point 0.5 ml of 1:3 Folin reagent: H_2O was added. After 30 min, the absorbance was monitored at 750 nm with a Gilford spectrophotometer. Bovine serum albumin (BSA) was used as the standard.

2.3. *Acetylating reaction*

Seven ml of the original ASNase solution were reacted with 62.5 μl of acetic-[U-^{3}H] anhydride (12.75 mg, 50 mCi) in an ice bath (0–3°C) for 1 hr. The reaction mixture was continuously mixed by means of a magnetic stirrer. This reaction acetylates free amine groups; i.e., free N-terminal ends of proteins and the ε-amino group of lysine residues (ASNase has 82 lysine residues) [15,16].

2.4. *Purification of reaction product*

A Sephadex G-25-300 column (2.75 cm × 40 cm) was prepared in 0.01 M phosphate buffer (pH 7.5), which was also used as the eluent. The flow rate was 10 ml/min. The spacing of the void volume (where the [^{3}H] Ac–ASNase appears) was determined by the use of Blue Dextran.

At the end of the 1-hr incubation, 3 ml of glycerol were added to the resulting reaction mixture containing [^{3}H] Ac–ASNase, [^{3}H] acetic acid, and unreacted acetic-[^{3}H] anhydride. The 10-ml solution was placed on the Sephadex G-25-300 column operating at room temperature, at which time 5-ml fractions were collected. The absorbance of the fractions was monitored at 280 nm (fig. 1) with the Gilford spectrophotometer, and fractions 13–18 inclusive containing the [^{3}H] Ac–ASNase were pooled.

2.5. *Test for radioactivity*

Four 100-μl aliquots of this pooled fraction were plated on glass fiber filters and counted in 5 ml of toluene-based scintillation fluid by a Nuclear Chicago liquid scintillation counter. Then to test whether the radioactivity was due to acetyl groups covalently bound to ASNase instead of free [^{3}H] acetic acid, two 100 μl aliquots were each precipitated with 300 μl of 5% trichloroacetic acid and centrifuged at 2000 *g* for 10 min. 100 μl ofthe supernatant was counted as described above.

2.6. *Test for enzyme activity*

Enzyme activity of the [^{3}H] Ac–ASNase was tested against an equal amount of unlabeled ASNase from the same original ASNase solution, by a modification of the colorimetric procedure of Frohwein [17]. In this assay, L-aspartic acid-β-hydroxamate (AHA) is used as the substrate for ASNase since it is the only substance in the assay that will produce a red chromogen upon reacting with acidic ferric chloride. The technique involves addition of 100 μl (1 μmole) of AHA at 37°C to 50 μl of 0.1 M Tris–HCl (pH 8.0) and the enzyme sample; the final volume is brought up to 900 μl with glass-distilled H_2O. After 5 min the reaction is terminated by addition of 1 ml of acidic ferric chloride [17]. After 2 ml of H_2O is added, the absorbance is monitored at 500 nm, the wavelength that gives the maximum absorbance difference.

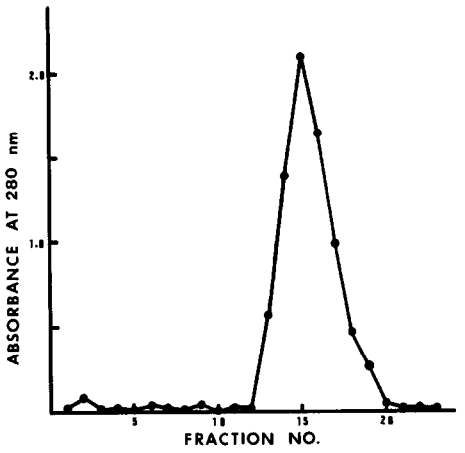

Fig. 1. Gel filtration of the reaction mixture of ASNase and acetic [U-^{3}H] anhydride (60 min, 0–3°C) on Sephadex G-25-300 at room temperature. Eluent was 0.01 M phosphate buffer, pH 7.5. Column size was 2.75 cm × 40 cm. Flow rate was 10 ml/min. Fraction volume was 5 ml.

3. Results and discussion

The protein determination by the modified Lowry method of the ASNase solution gave a concentration of 10.4 mg/ml. From this calculation it can be determined that 72.8 mg of ASNase was acetylated and that the specific enzymatic activity of the ASNase was 320 IU/mg protein. From the absorbance curve in

fig. 1 it can be seen that approximately 94% of the original [^{3}H] Ac–ASNase was recovered (68.4 mg) in 30 ml (230 μg/100 μl).

The 100-μl aliquots of the [^{3}H] Ac–ASNase were found to be highly radioactive – 2.01 X 10^6 cpm; 99.6% of this radioactivity was trichloroacetic acid precipitable, showing that the radioactive acetyl label is covalently bound.

Since the Nuclear Chicago liquid scintillation counter displayed an overall efficiency of 40%, the 2.01 X 10^6 cpm can be translated into 11.2 nmoles. Since ASNase has a molecular weight of 141 000, 230 μg is 1.6 nmoles. This results in 7 acetyl residues per ASNase or 8% of the maximum amino groups, using this mild acetylation procedure, but not all the ASNase lysine groups may be chemically free to react with the acetic anhydride under the stringently stipulated conditions.

The test for unaltered enzymatic activity of the [^{3}H] Ac–ASNase was crucial in the light of reports of conformational changes in proteins following acetylation. These changes were especially found to occur in BSA [18,19]. Also acetylation produced loss of anti-BSA antibody's ability to precipitate BSA [20]. But under those acetylation conditions, approximately 95% of the free amino groups were acetylated (18–20). As is evident from fig. 2, under the gentle

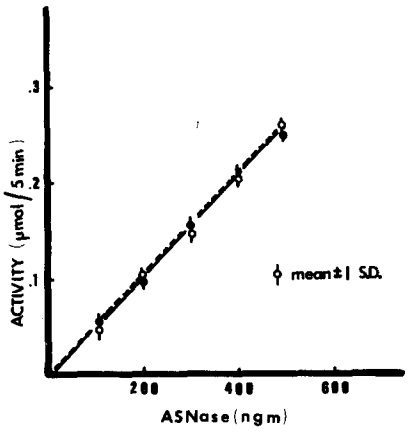

Fig. 2. Determination of ASNase activity of [^{3}H] ASNase (•) and unlabeled ASNase (○) by measurement of remaining substrate (AHA) after exposure to various amounts of ASNase (5 min, 37°C) by the method of Frohwein. Only AHA in this assay produces a red compound upon reaction with acidic ferric chloride. The maximum absorbance was 500 nm.

acetylation conditions described above, the enzymatic activity of the [^{3}H] Ac–ASNase is almost identical to that of unlabeled ASNase.

The simple, efficient procedure described here results in highly labeled ASNase that retains 100% of its enzyme activity. This [^{3}H] Ac–ASNase should be invaluable for the study of the action of asparaginase, from both clinical and molecular standpoints.

Acknowledgements

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