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Determination of the glutaminase activity of asparaginase

The enzymic characteristics of glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) and asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) have been studied extensively in relation to their physiological functions, remission in human leukemia[1] and mechanism of ion transport and ATP formation in mitochondria[2–4]. Each enzyme possesses both glutaminase and asparaginase activities, although the enzymes are highly purified in the crystalline state[1,5–7].

Difficulties encountered in determining the activity of glutaminase, which catalyzes the hydrolysis of glutamine into glutamate and ammonia, were due mainly to the presence of an unstable amide in glutamine. The ammonia released spontaneously from glutamine at alkaline pH, so-called artifactural ammonia, affects the determination of ammonia produced by the enzymic action. The glutaminase activity has been determined either by a tedious procedure of measuring the amount of glutamate, by a paper chromatographic isolation followed by ninhydrin coloration[7], by an insensitive method of measuring the amount of ammonia released from glutamine with a cation-selective electrode[4], or by an indirect method of measuring the NADH oxidation at 340 nm[8] or measuring MTT [3-(4,5-dimethylthiazol-1-yl)-2,5-phenyltetrazolium bromide] reduction induced by glutamate dehydrogenase[9]. The Conway diffusion method[2,3] and the Serigson method[10,11] are not satisfactory for use in kinetic studies because of the production of the artifactural ammonia released from glutamine[3]. The present paper describes a simple and accurate method for determining ammonia produced from glutamine by enzymic action, by using anion-exchange chromatography, and the kinetics of the glutaminase action of asparaginase are discussed.

A crystalline asparaginase preparation from *Escherichia coli* A-1-3KY3598 was donated by Kyowa Hakko Kogyo Co. and the asparaginase activity of this enzyme was 220 μmole min^{-1} per milligram of protein. The molar concentration of the enzyme was determined spectrophotometrically, assuming the molar extinction coefficient, ε, to be $8.85 \cdot 10^4$ $mole^{-1}cm^{-1}$ at 278 nm. The measurement of glutaminase activity was carried out as follows. Amberlite IRA-400 (Type 2), a strongly basic anion exchanger, was washed with carbonate buffer solution of pH 9.2 (ionic strength 0.02) and poured into a column (0.9 × 24 cm) up to a height of 8 cm (5.0 ml of resin). To a mixture of 2 ml of glutamine solution plus 2 ml of a buffer solution was added 1 ml of the enzyme solution and the mixture was incubated at 37°. At a given time, a constant volume of the reaction mixture was diluted with an appropriate volume of water in order to make the concentration of glutamine less than 2 m*M* and the ionic strength less than 0.01. A 4-ml volume of the diluted solution was passed through the column of Amberlite IRA-400 and then 10 ml of carbonate buffer of pH 9.2 (ionic strength 0.02) was passed to elute ammonia completely from the column. The rate of flow was adjusted by the application of nitrogen gas at a low pressure (1.0 kg cm^{-2}). It is necessary to carry out the dilution and the elution of ammonia as rapidly as possible, in order to minimize the amount of ammonia formed by enzymic action. In this experiment, it takes about 3–4 min and the enzymic activity was reduced ten times during the elution, probably owing to blocking of the enzyme action by the resin.

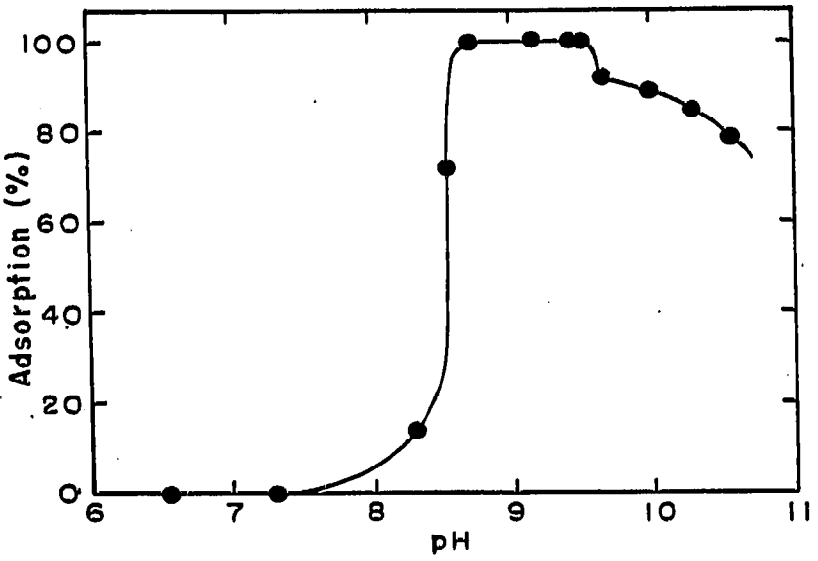

Fig. 1. Effect of pH on the adsorption of glutamine by Amberlite IRA-400. Glutamine, 8 μmoles; ionic strength, 0.02; height of resin in the column, 8 cm; temperature, 20°. Buffer solutions used: below pH 7.30, phosphate; pH 8.30–8.75, borate; pH 9.18–10.30, carbonate; above pH 10.56, $NaOH–Na_2HPO_4$.

The eluted solution was subjected to the indophenol coloration reaction[2,10,12]; the amount of ammonia present was determined photometrically, assuming the molar extinction coefficient of the product to be $2.3 \cdot 10^4$ $mole^{-1}cm^{-1}$ at 635 nm and at pH 11.3.

To determine the experimental conditions for adsorbing glutamine by resin, the effect of pH on the adsorption of 8 μmoles of glutamine by resin was tested, and the result is shown in Fig. 1. The maximum adsorption capacity of resin for binding glutamine lies in the pH range 8.7–9.5 at an ionic strength of 0.02. The pH in the resin column is therefore maintained at pH 9.2. Under this pH, no liberation of the artifactural ammonia was observed. The ionic strength in the medium of the adsorbent significantly affects the adsorption capacity of glutamine by the resin; the capacity remains at 100% at ionic strengths between 0.01 and 0.05 and decreases gradually with increasing ionic strength (75% adsorption capacity at an ionic strength of 0.1). Table I shows the recovery of ammonia under the optimum condition of the resin column, pH 9.2 and ionic strength of 0.02, when ammonium bicarbonate solution containing glutamine was passed through the column containing resin to a height of

TABLE I

RECOVERY (%) OF AMMONIA UNDER THE OPTIMUM CONDITIONS FOR THE RESIN COLUMN

Amberlite IRA-400 resin; pH 9.2; ionic strength 0.02; height of resin in column, 8 cm (5 ml of resin).

NH_4HCO_3 (μmole)	Glutamine (μmole)				
	0	2.0	4.0	6.0	8.0
0.4	97	98	94	92	94
0.6	93	99	96	93	94
0.8	95	95	95	93	100

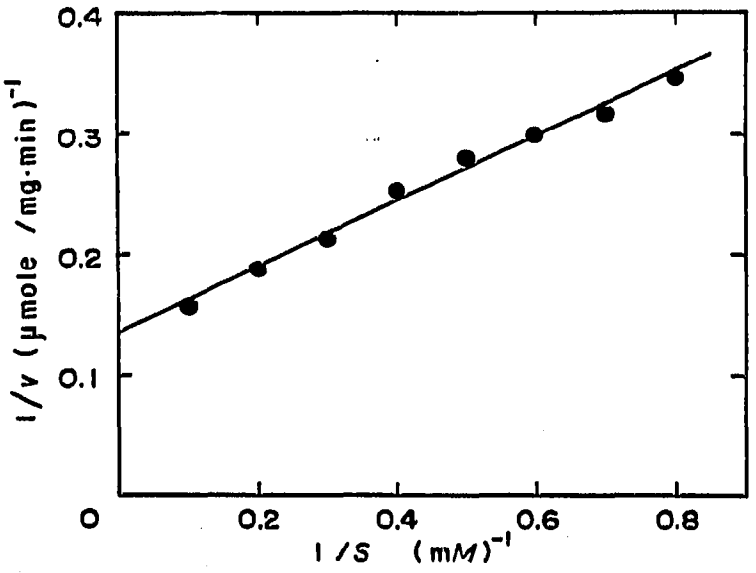

Fig. 2. Lineweaver–Burk plots of the glutaminase action of asparaginase. pH 7.50 (phosphate buffer); ionic strength, 0.02; temperature, 37°.

8 cm. It is clear from Table I that the recovery of ammonia is satisfactory within experimental error (96 ± 4%).

Based on the above experimental conditions, the kinetic study of the glutaminase activity of asparaginase was carried out with L-glutamine as the substrate at the optimum pH of 7.5, and the results are shown in Fig. 2. The plots of $1/v$ and $1/S$, gave a straight line and the maximum velocity V and the Michaelis constant, Km, were calculated from the line to be 7.4 μmole min^{-1} of ammonia per milligram of protein and 2.0 mM, respectively.

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Laboratory of Biological Chemistry,
Tokyo Institute of Technology,
Ookayama, Meguro-ku, Tokyo (Japan)

TSUTOMU ABE
YUTAKA TAMAURA
YOSHIHISA NISHIMURA
OSAMU TAKENAKA
YUJI INADA

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