CHROMATOGRAPHY OF PLANT AMINOACYL-tRNA SYNTHETASES ON ω-AMINOALKYL SEPHAROSE COLUMNS

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1. Introduction

Well known enzymological techniques used in purifying aminoacyl-tRNA synthetases, especially of plant origin, have often failed. Therefore, new methods based on affinity chromatography have been introduced to overcome these difficulties and to improve enzyme preparations. So far some of the above mentioned enzymes have been purified on modified Sepharose to which either amino acids [1–3] or tRNA's [4–6] were attached covalently.

During the adaptation of one of these methods it has been observed unexpectedly that the modified Sepharose containing aminoalkyl residues alone is able to bind aminoacyl-tRNA synthetases with a different mechanism from that of the ordinary ion exchangers. The binding of enzymes to aminoalkyl-Sepharose chiefly depends on the length of the alkyl chain as well as on ionic strength and kind of buffer, and differs for various synthetases. In the present report the purification of some aminoacyl-tRNA synthetases from yellow lupin seeds on aminohexyl-Sepharose is described.

Since this work was under investigation, we have learned that Er-el et al. [7] and Shaltiel and Er-el [8] have observed the same phenomenon and utilized those Sephadex derivatives as well as alkyl-Sepharoses to isolate two enzymes of glycogen metabolism. Similarly, Yon [9] and Hofstee [10] have investigated protein binding to Sepharose derivatives carrying hydrophobic groups. The term 'hydrophobic chromatography' has been introduced for this kind of protein fractionation because the retention of proteins on the Sepharose derivatives is in great measure, due to hydrophobic forces between the alkyl residues of absorbent and hydrophobic pockets of protein molecules.

2. Experimental

2.1. *Materials*

Sepharose 4B was purchased from Pharmacia Uppsala, Sweden. Cyanogen bromide was from Fluka AG Switzerland. ATP was a product of Reneal Budapest, Hungary and 2-mercaptoethanol of Loba-Chemie Wien-Fischamend, Austria. Tetramethylendiamine and hexamethylendiamine were products of Merck Darmstädt, West Germany and Rechim Moscow, USSR, respectively. Radioactive aminoacids: L-[U-^{14}C]valine, L-[U-^{14}C]tyrosine and L-[U-^{14}C]serine were obtained from the Institute for Research, Production and Application of Radioisotopes Prague, Czechoslovakia, and L-[^{14}C-methylene]tryptophan L-[2, 3-^{3}H]valine and DL-[G-^{3}H]phenylalanine from The Radiochemical Centre Amersham, England. The other reagents were from Polskie Odczynniki, Gliwice, Poland.

2.2. *Preparation of aminoalkyl derivatives of Sepharose 4B*

Sepharose 4B was activated with cyanogen bromide according to Cuatrecasas [11] 250 mg CNBr per ml of the packed gel was used. The activated Sepharose was coupled with 2 mmol of diamine per ml of the gel. Modified gels were stored in 0.02% sodium azide solution at 4°C.

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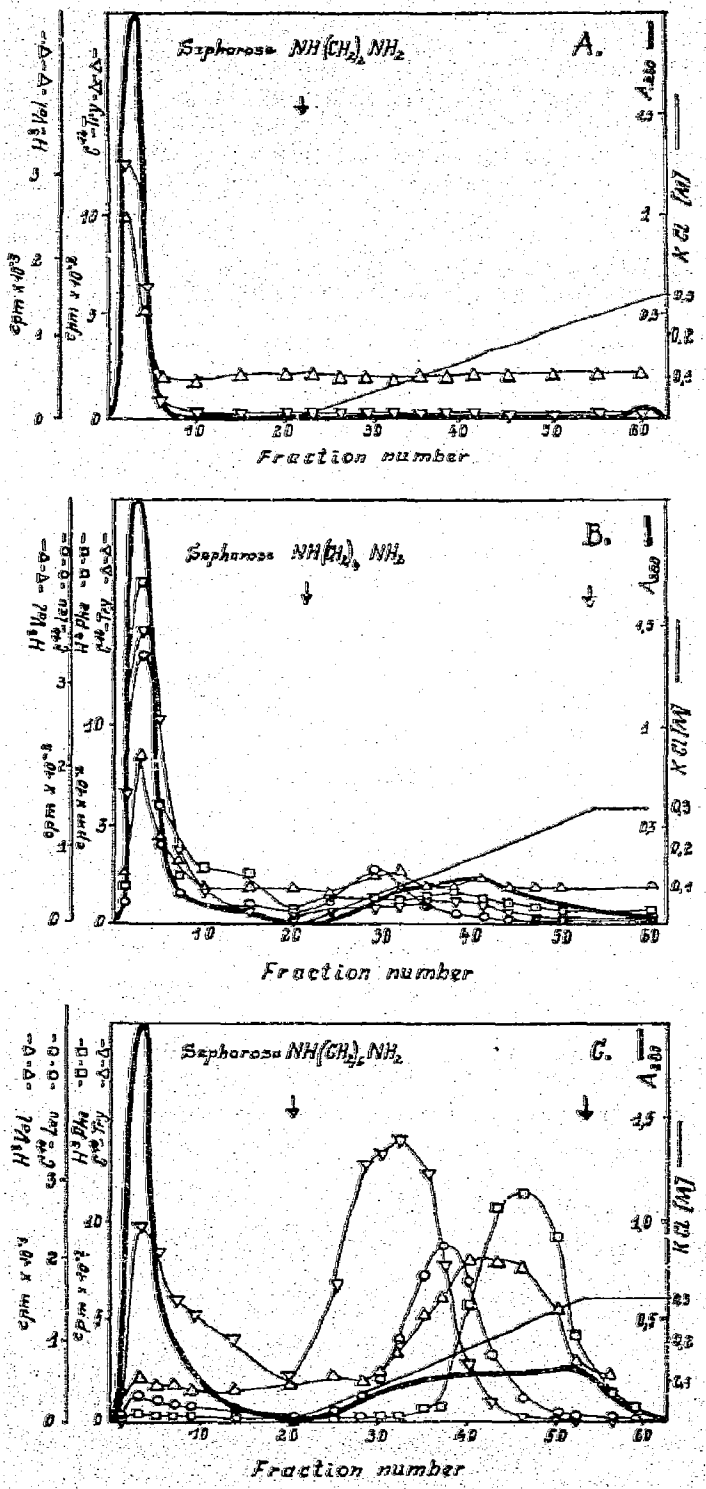

Fig. 1. Chromatography of aminoacyl-tRNA synthetases on modified Sepharoses containing: A) ω-aminoethyl- ; B) ω-aminobutyl- and C) ω-aminohexyl groups. 60 mg of protein 30–50% ammonium sulphate saturation fraction in 3 ml of buffer B was applied on each of three modified Sepharose columns (1 × 10 cm) equilibrated previously with buffer B at 4°C. The columns were washed first with buffer B (120 ml) and 6 ml fractions were collected. Then linear 0–0.3 M KCl gradient in buffer B (100 ml) was applied and 3 ml fractions were collected. The absorbance at 280 nm (——) and the synthetase activities for valine (— —), leucine (o—o—o), tryptophan (△—△—△), phenylalanine (□—□—□) were determined in each 10 μl fraction. (——) Indicates the linear NaCl gradient.

2.3. *Purification of aminoacyl-tRNA synthetases*

Meal of yellow lupin seeds (160 g) was extracted for 30 min at 4°C with 500 ml of 10 mM potassium phosphate buffer, pH 6.8, containing 1 mM 2-mercaptoethanol and 10% (v/v) of glycerol (buffer A). The extract was passed through miracloth and centrifuged for 30 min at 15 000 *g*. The supernatant was fractionated with ammonium sulphate. Fractions precipitated between 35–50% and 50–70% ammonium sulphate saturation were collected separately, dissolved in small volumes of 60 mM potassium phosphate buffer, pH 6.85, containing 1 mM 2-mercaptoethanol and 10% (v/v) of glycerol (buffer B) and dialysed overnight against two changes of this buffer. Then the fractions were applied to the aminoalkyl-Sepharose columns 1.5 × 30 cm equilibrated with (buffer B). The columns were washed with the same buffer to remove unadsorbed proteins and then eluted in linear 0–0.3 M KCl gradient in buffer B 300 ml. 6 ml Fractions were collected and the absorbance at 280 nm and aminoacyl-tRNA synthetase activities were determined.

2.4. *Assay of enzymatic activities*

The reaction mixture contained in a total volume of 50 μl: 5 μmol of Tris–HCl buffer, pH 8.05, 250 nmol $MgCl_2$, 50 nmol ATP, 50 nmol 2-mercaptoethanol, 400 μg of lupin tRNA, $2–5 \times 10^{-5}$ M ^{14}C- or ^{3}H-labelled amino acid and enzyme fraction (1–30 μg of protein or 10 μl of chromatographic fraction). Samples were incubated for 10 min at 36°C and then 30 μl aliquots were withdrawn and pipetted on 3 MM Whatman filter paper discs 2.2 cm in diameter. The discs were washed three times in 5% trichloroacetic acid, once in ethanol–ether 1:1 mixture and finally in ether, then air-dried. They were counted in the Packard Tri-Carb scintillation counter with the efficiency of 55% for ^{14}C and of 5% for ^{3}H.

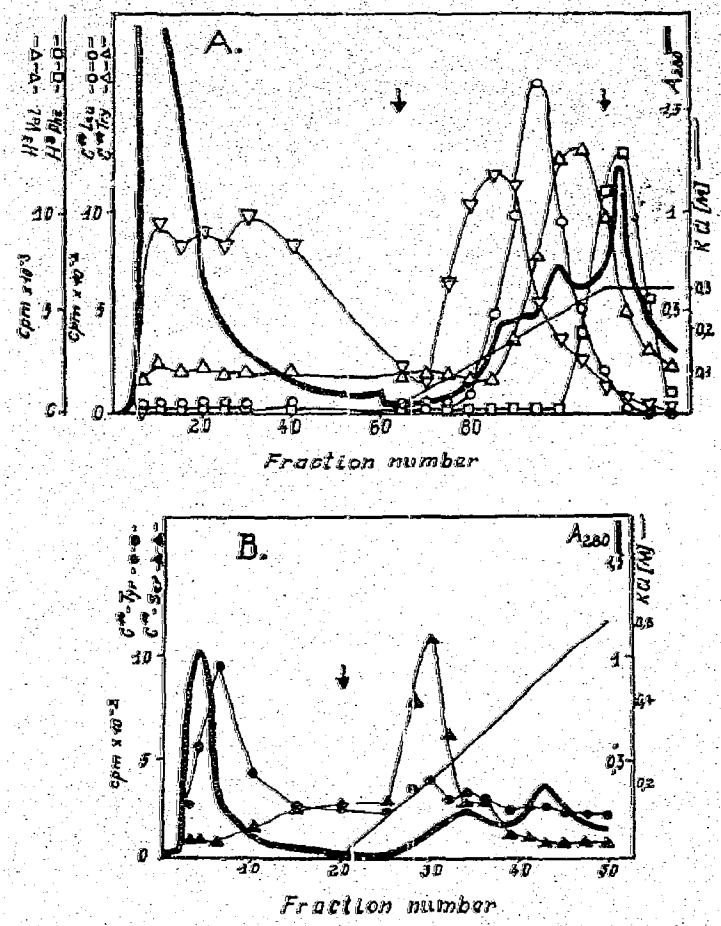

Fig. 2. Distribution of aminoacyl-tRNA synthetases chromatographed on an aminohexyl-Sepharose column: A) 700 mg of protein of 30–50% ammonium sulphate saturation fraction was applied on 1.5 × 30 cm column and washed with 850 ml of buffer B. Adsorbed proteins were then eluted in 350 ml linear 0–0.3 M KCl gradient in buffer B. Finally the column was washed with 150 ml of 0.3 M KCl in buffer B. 7.5 ml fractions were collected. Symbols as in fig. 1; B) 100 mg of protein of 50–70% ammonium sulphate saturation fraction was applied on 1.5 × 30 cm column and washed with 300 ml of buffer B. Adsorbed proteins were eluted in 0–0.6 M KCl in buffer B 300 ml of 10 ml fractions were collected. (▲—▲—▲) Indicates seryl-synthetase and (●—●—●) tyrosyl activities. Other symbols as in fig. 1.

3. Results and discussion

The comparison of sorption properties of three Sepharose derivatives containing aminoethyl-, aminobutyl-, or aminohexyl-active groups, respectively, shows that Sepharose–$NH(CH_2)_2NH_2$ does not retain aminoacyl-tRNA synthetases or other proteins under the conditions used. Sepharose–$NH(CH_2)_4NH_2$ binds those enzymes, if at all, very weakly. On the other hand synthetases are retained on Sepharose–$NH(CH_2)_6NH_2$ column (fig. 1).

Table 1
Purification of aminoacyl-tRNA synthetases of yellow lupin seeds.

Step	Specific activities nmol of amino acid/mg of protein/10 min					
	Trp	Phe	Ser	Val	Leu	Tyr
1. Crude extract	0.43	0.07	0.53	2.6	1.1	1.4
2. Ammonium sulphate fraction						
35–50%	0.65	0.2	–	7	0.5	–
50–70%	–	–	2	–	–	2
3. Aminohexyl-Sepharose	9	26	18.5	106	2	2

The chromatographic properties of the last adsorbent enable us to purify several aminoacyl-tRNA synthetases from yellow lupin seeds (*Lupinus luteus*) in a simple manner. For this purpose the protein fraction precipitated at 35–50% saturated of ammonium sulphate and containing valine, leucine, tryptophan, and phenylalanine activating enzymes, was poured into an aminohexyl-Sepharose column (1.5 × 30 cm) and washed with buffer B to remove unadsorbed proteins. Enzyme activities were then eluted in 0–0.3 M KCl gradient in the same buffer. In a similar manner seryl- and tyrosyl-tRNA synthetases were purified from 50–70% ammonium sulphate saturation fraction in a gradient from 0–0.6 M KCl (fig. 2). The results of the purification procedure are given in table 1.

The chromatographic method together with the preliminary ammonium sulphate fractionation gave 20 to 370-fold purification of synthetases with the exception of leucine- and tyrosine enzymes that were probably unstable under the conditions used. The lupin valyl-tRNA synthetase preparation obtained had a specific activity over 100, the value unattainable by other methods, e.g. ion exchange or hydroxyapatite chromatography which are much less efficient. The effect of pH, in the range of 5.85–7.75, on the binding of valine enzyme was negligible (table 2). The most

Table 2
pH dependence of the binding of valyl-tRNA synthetase to aminohexyl-Sepharose.

pH	5.85	6.85	7.75
u[x]	0.145	0.165	0.145

[x] Strength of binding is expressed in terms of the ionic strength at which the synthetase is eluted.

important fact is that, by this method, some of the synthetase preparations could be obtained free of activities of other synthetases. Thus, valine, serine, and phenylalanine activating enzyme preparations free of other synthetase activities were prepared.

Considering the mechanism of synthetase binding to modified Sepharose a question has been raised whether aminoalkyl groups attached to Sepharose matrix do not imitate amino acid moieties. It is well known that the amino group of an amino acid does contribute to the binding of the amino acid with its synthetase. On the contrary, the carboxyl group of amino acid seems to be less essential to the binding [12]. In that case the retention of aminoacyl-tRNA synthetases on aminoalkyl-Sepharose columns could be considered as a kind of affinity chromatography. To examine this suggestion two new Sepharose derivatives were synthetized by the treatment of aminohexyl-Sepharose with nitrous acid or with dimethyl

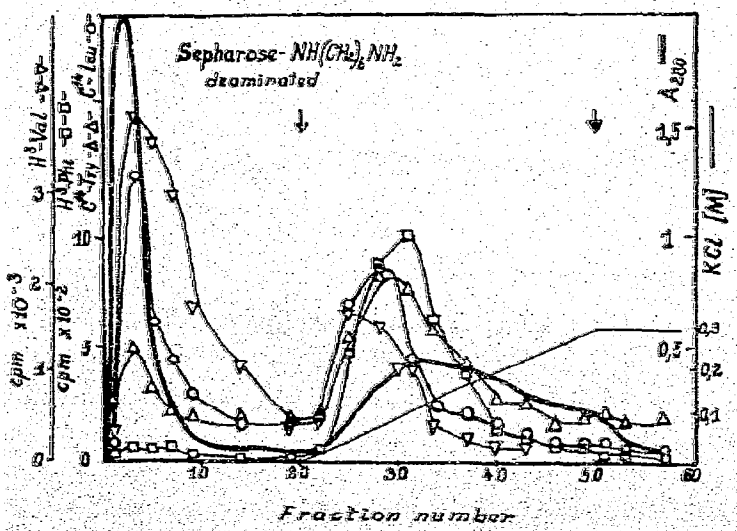

Fig. 3. Fractionation of aminoacyl-tRNA synthetases on a deaminated aminohexyl-Sepharose column. 60 mg of protein was fractionated under conditions given in the legend to fig. 1. Symbols as in fig. 1.

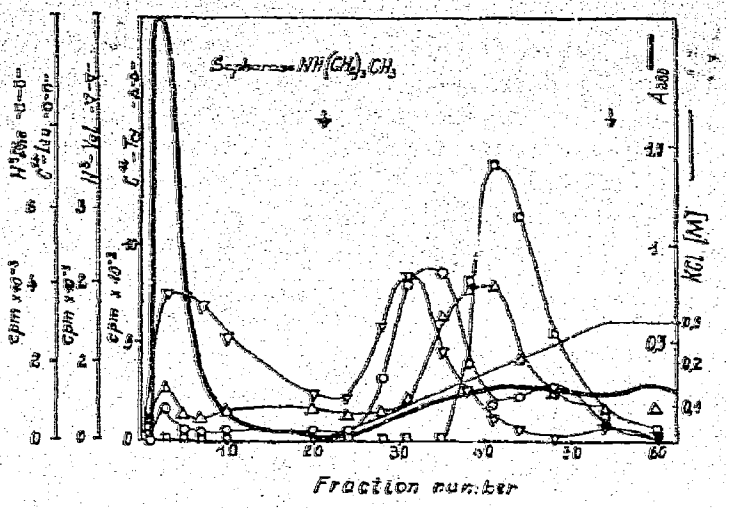

Fig. 4. Fractionation of aminoacyl-tRNA synthetases on a butyl-Sepharose column. Conditions and symbols as in fig. 1.

sulphate. The former derivative, deprived of primary amino groups, has preserved the binding ability of aminoacyl-tRNA synthetases although their binding capacity is weaker than that observed for aminohexyl-Sepharose column as is indicated by the lower ionic strength required for their elution. The chromatographic resolution of the deaminated derivative is also worse than that of the unchanged adsorbent (fig. 3). On the other hand methylated aminohexyl-Sepharose shows analogous chromatographic properties as does the unmethylated product (not shown here). These experiments establish that hydrophobic forces are the major ones which are responsible for the binding properties of aminoalkyl-Sepharose. This conclusion was reinforced by the observation that butyl-Sepharose which does not contain primary amino groups is also able to bind the enzymes mentioned above (fig. 4).

It seems reasonable to assume that the specificity of aminoacyl-tRNA synthetases binding in the hydrophobic chromatography method may depend, at least in part, on their complex structure, when the occurrence of hydrophobic pockets is more frequent in enzyme molecules. Indeed, enzymes with more complex structure and higher molecular weights are eluted at higher ionic strength from the modified Sepharose column than those having lower molecular weights and eluted at lower ionic strength. The mol. wt. of valine activating-enzyme is 145 000 daltons as judged by Sephadex G-200 filtration. It is eluted from amino-

hexyl-Sepharose column at 0.1 M KCl whereas tryptophan enzyme (mol. wt. about 280 000) is not released until at 0.2 M KCl.

The data presented in this report indicate that hydrophobic chromatography offers new possibilities in the purification of aminoacyl-tRNA synthetases.

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