ISOLATION, PURIFICATION, AND STUDY OF SOME PROPERTIES OF THE LYSL-, PHENYLALANYL-, AND ASPARTYL-tRNA SYNTHETASES FROM THE FUNGUS Verticillium dahliae

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Aminoacyl-tRNA synthetases (ARSs), which catalyze two successive reactions – the activation of amino acids and their transfer to transport ribonucleic acids (tRNA) – fulfill an important role in the complex process of the biosynthesis of protein in the cell [1, 2].

At the present time, lysyl-, phenylalanyl-, and aspartyl-tRNA synthetases have been isolated from various organisms [3-9, 10-17, 18-22], purified to various degrees, and characterized.

In our paper we give experimental results on the isolation, purification, and study of some physicochemical properties of the lysyl-, phenylalanyl-, and aspartyl-tRNA synthetases from the 12-day mycelium of a highly virulent form of the fungus Verticilium dahliae. The results of the purification of the ARSs investigated are given in Table 1.

The preparations obtained were homogeneous on electrophoresis in 7.5% polyacrylamide gel at pH 8.6 according to Davis [23] and on gel filtration in Sephadex G-200.

The molecular weights of the substances that we isolated were 98,000, 205,000, and 108,000, respectively, for the lysyl-, phenylalanyl-, and aspartyl-tRNA synthetases.

The amino-acid composition of the enzymes isolated (Table 2) is characterized by a predominance of aspartic and glutamic acids, glycine, and alanine. Some increase in the amounts of glycine and alanine is generally characteristic for the enzymes of the fungus V. dahliae.

The results of an investigation of the influence of the pH of the medium on the activity and stability of the ARSs has shown that the optimum activity of all the enzymes studied is found at pH 7.5 and falls sharply at higher and lower pH values of the medium. In order to determine whether the fall in activity of the enzymes outside the zone of the optimum is the result of reversible or irreversible inactivation, the enzymes were first incubated for 30 min at 37°C at various pHs of the medium (without substrate): 5.0, 9.0, 9.5, and 10.0 in 0.05 M tris-HCl buffer and their activities were then determined. It was found that the reduction of the activity of the enzymes previously incubated at pH 5.0 was very small – about 10-13% – while at pH 9.0 it was higher – about 90%.

The activity of the enzymes previously incubated at pH 9.5 was restored differently in the different ARSs. Thus, in the case of the lysyl- and aspartyl-tRNA synthetases, the activity was 55-60% restored, while for the case of the phenylalanyl-tRNA synthetase it was only 40-50% restored.

Thus, it has been found that the fall of the activity in an acid medium is accompanied by irreversible inactivation while in an alkaline medium this process is reversible to some degree.

In a study of the influence of the temperature on the activity of the ARSs it was established that the optimum temperature of enzymatic activation is 37°C; lowering it or raising it decreases the ARSs activity.

All the ARSs studied are heat-labile and are completely inactivated at 60°C for 1-3 min.

The Michaelis constants (K_M) of the enzymes isolated for ATP, a [^{14}C] amino acid, for tRNA, and Mg^{++} ions are, respectively: $2.9 \cdot 10^{-3}$ M; $0.31 \cdot 10^{-5}$ M; $0.75 \cdot 10^{-6}$ M; $3.3 \cdot 10^{-3}$ M for lysyl-tRNA synthetase;

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TABLE 1. Results of the Purification of the ARSs of the Fungus Verticilium dahliae Investigated

Fraction	Protein, mg	Activity		Yield of enzyme, % on the initial activity	Degree of purification	E_{280}/E_{260} ratio
		total	specific			
Lysyl-tRNA synthetase						
Crude extract	5000	6500	1,3	100	1	0,75
$(NH_4)_2SO_4$	1500	4620	3,08	71,8	2,25	0,90
DEAE-cellulose	35	877	25,08	13,5	19,3	1,15
Hydroxylapatite-72	2	788	394	12,1	303	1,50
Phenylalanyl-tRNA synthetase						
Crude extract	5000	2950	0,59	100	1	0,75
$(NH_4)_2SO_4$	1300	1950	1,5	66,1	2,54	0,90
DEAE-cellulose	49	951	19,4	32,3	32,88	1,15
Hydroxylapatite-72	2,2	510	232	17,3	393	1,48
Aspartyl-tRNA synthetase						
Crude extract	5000	8000	1,6	100	1	0,75
$(NH_4)_2SO_4$	1230	5560	4,52	69,50	2,83	0,95
DEAE-cellulose	34	1020	30	12,75	18,75	1,15
Hydroxylapatite-72	2,5	883	353	11,37	221	1,50

TABLE 2. Amino-Acid Compositions of Some ARSs of the Fungus Verticilium dahliae, mole-%

Amino acid	Lysyl-tRNA synthetase	Phenylalanyl-tRNA synthetase	Aspartyl-tRNA synthetase
Lysine	8,07±0,10	7,88±0,11	6,77±0,55
Histidine	1,84±0,20	2,02±0,43	1,82±0,08
Arginine	4,29±0,50	3,88±0,50	4,20±0,02
Aspartic acid	11,74±0,36	11,73±0,58	13,07±0,26
Threonine	5,43∓0,15	5,93±0,05	5,81±0,52
Serine	6,12±0,22	6,94±0,17	6,44±0,15
Glutamic acid	13,67±0,55	12,69∓0,27	12,09±0,03
Proline	5,19±0,45	5,37±0,60	4,98±0,61
Glycine	9,91±0,60	10,35±0,20	9,61±0,34
Alanine	11,76±0,45	11,44±0,09	12,00±0,00
Valine	6,10±0,23	6,76±0,02	5,01±0,23
Methionine	0,72±0,20	0,51±0,10	0,65±0,05
Isoleucine	2,75±0,30	2,75±0,24	2,96±0,07
Leucine	7,45±0,15	7,80±0,96	8,94±0,09
Tyrosine	1,94±0,26	1,06±0,60	2,43±0,13
Phenylalanine	2,96±0,13	2,89±0,14	3,31±0,02

$3 \cdot 10^{-3}$ M; $1.1 \cdot 10^{-5}$ M; $0.95 \cdot 10^{-6}$ M; $2.6 \cdot 10^{-2}$ M for phenylalanyl-tRNA synthetase; and $3.1 \cdot 10^{-3}$ M; $2.5 \cdot 10^{-5}$ M; $0.90 \cdot 10^{-6}$ M; $1.9 \cdot 10^{-2}$ for aspartyl-tRNA synthetase, and the values of V_{max} (mμ mole/ml/min) were 40, 57, 50, and 42 for lysyl-tRNA synthetase; 36, 44, 33, and 45 for phenylalanyl-tRNA synthetase; and 50, 66, 50, and 50 for aspartyl-tRNA synthetase.

The observed differences in the value of K_M for each substrate to some extent confirms the assumption of the existence in the ARSs of different active centers for each substrate [24]. From the difference in the values of K_M it is possible to judge that ATP and Mg^{++} ions have the least affinity for the enzyme, and amino acids and tRNA the greatest, which is characteristic for the ARSs. This indirectly confirms the hypothesis of the sequence of the aminoacylation reaction of tRNAs catalyzed by ARSs.

The values of V_{max} of the ARSs studied are similar for all substrates; consequently, the aminoacylation reaction takes place independently of the order of addition of the substrates to the incubation mixture.

EXPERIMENTAL

Growth of the Fungus. The fungus was grown at 28°C for 12 days in a peptone-salt medium (pH 7.5) of the following composition: (in grams per liter); peptone – 30; KCl – 0.5; $MgSO_4$ – 0.5; NH_4NO_3 – 2.0; ammonium succinate – 2.0; KH_2PO_4 – 1.0; $FeSO_4$ – 0.1; $ZnSO_4$ – 0.1; streptocid album [sulfanilamide] soluble 0.1.

Isolation of the tRNAs. The cells of the mycelium of the fungus were destroyed by rapid freezing (liquid nitrogen) and thawing with subsequent grinding in the presence of quartz sand in a porcelain mortar. The isolation of the tRNAs was performed by extraction with aqueous phenol [26, 27], and purification from

polysaccharides, pigments, highly polymeric RNAs, and other impurities by chromatography on DEAE-cellulose [28], and deacylation of the tRNA by the method of Norton et al., [17].

The isolation of the total preparations of the ARSs and the determination of their activity were performed by the method of Berg and Bergmann [29]: 500 g of freshly frozen fungal mycelium was ground as described above and homogenized with 1000 ml of 0.05 M tris-HCl buffer at pH 7.5 containing 0.001 M EDTA, 0.005 M $MgCl_2$, and 0.005 M 2-mercaptoethanol. All the operations were performed at 0-4°C. The largest particles were separated by centrifuging for 40-50 min at 6000 rpm. Then, to free it from the smaller subcellular particles (ribosomes, etc.), the supernatant liquid was centrifuged at 105,000 × g for 2 h. To eliminate the endogeneous RNAs, the supernatant was treated with a 0.25% solution of streptomycin sulfate and centrifuged. The resulting supernatant (crude extract of the total enzymes) was used to isolate the individual ARSs preparations.

The protein content was determined by Lowry's method [30] and spectrophotometrically from the absorption at 280 mμ, taking the extinction $E_1^{0.1\%}_{cm}$ as 1.0. The activities of the ARSs were determined by Berg's method [31]. The time of incubation at 37°C was 13 min. The radioactivity was counted on a BFL-25 counter with an efficiency of about 15%.

The experiments were performed with carbon-labeled amino acids having the following specific activities (mCi/mmole): lysine 120, phenylalanine 225, and aspartic acid 100. Until activity of the enzyme was taken to be that amount of protein catalyzing the formation of 1 mμmole of amino acyl-tRNA at 37°C in 1 min under standard conditions. The specific activity was expressed in units of enzyme activity per milligram of protein.

Isolation and Purification of the Individual ARSs. The individual ARSs from the total extract were isolated by fractional precipitation with $(NH_4)_2SO_4$ at various percentage saturations of the extract. The activities of the individual ARSs were determined from the inclusion of the corresponding [^{14}C] amino acid in the tRNA. The highest activities for the lysyl-, phenylalanyl-, and aspartyl-tRNA synthetases was found at the following percentage saturations with $(NH_4)_2SO_4$: 40-50, 50-65, and 67-70, respectively.

The further purification of the enriched ARSs was performed by column chromatography on DEAE cellulose and by chromatography on hydroxyapatite-72 [32, 33]. The chromatography of the synthetases was performed by published methods – lysyl-tRNA synthetase [4], phenylalanyl-tRNA synthetase [13], and aspartyl-tRNA synthetase [19, 20].

The fractions showing activity were combined, and their protein contents were measured. The yield amounted to about 2-3 mg of protein.

The molecular weights of the ARSs were found by gel filtration on Sephadex G-200 [34], using as standards pepsin, albumin, and pyruvate kinase with molecular weights of 35,000, 68,000 and 160,000, respectively.

Columns (2.5 × 50) were filled with Sephadex G-200 and equilibrated with 0.05 M tris-HCl buffer, ph 7.5, containing 0.1 M KCl. Then a sample of the enzyme or of a standard protein containing 2-3 mg of protein in 2 ml of the same buffer was deposited on the column. The density of the buffer solutions was increased by adding 5 mg of sucrose to the sample. Elution was performed with 0.05 M tris-HCl buffer, 7.5, containing 0.1 M KCl. The rate of elution was 20 ml/h. The volume of a fraction was 3.3 ml.

The kinetic constants K_M and V_{max} of the enzymes isolated were determined by Lineweaver and Burk's method of double reciprocal values [35] from the formation of the [^{14}C]aminoacyl-tRNA in a balance aminoacylation reaction according to Berg [31]. The time of incubation was 10 min.

The amino-acid compositions of the ARSs isolated were determined after acid hydrolysis in 5.7 N HCl at 105°C for 24 h on a Hd-1200 (Czechoslovakia) automatic amino-acid analyzer.

CONCLUSIONS

Relatively highly purified preparations of lysyl-, phenylalanyl-. and aspartyl-tRNA synthetases have been isolated from the 12-day mycelium of a strongly virulent form of the fungus V. dahliae and some of their physicochemical properties have been studied.

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