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Unnatural amino acids in enzymes and proteins

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Abstract

It is an intriguing question whether the proteins and enzymes could harbor the unnatural amino acid analogues and retain their activity. The present report reviews the published data on the enzymes harboring unnatural amino acids and presents the experimental results demonstrating the phenomenon of the cells' normal growth even if a substantial part of one amino acid is substituted by its element-organic analogue.

Alkaline phosphatase, aspartate transcarbamylase, β-lactamase, T4 lisozyme and glutathione transferase containing various unnatural amino acids are reviewed in this report.

The yeast cell growth in the presence of 4-fluorophenylalanine (FPA), being the element-organic unnatural amino acid, has been studied and the analogue content in the total cell protein determined. A significant part (35–40%) of phenylalanine was found to be replaced by FPA and the cells continued to function normally. FPA-resistant phenotype capable of growing in FPA presence has also been selected. The FPA content in the protein of the resistant strain has also been determined and it was found that 30–40% of phenylalanine was replaced by FPA.

Keywords: Unnatural amino acids; 4-Fluorophenylalanine; Alkaline phosphatase; Aspartate transcarbamylase; β-Lactamase; T4 lisozyme; Glutathione transferase; Analogue resistance; Analogue incorporation; Yeast growth

1. Introduction

All proteins are known to be made up of 20 amino acids and some of their biotic derivatives. It is an intriguing question whether the proteins and enzymes could harbor the unnatural amino acid analogues and retain their activity.

The interest to the unnatural amino acid-containing proteins is stimulated by at least three aspects as follows.

(i) A potential possibility of the unnatural amino acid incorporation into proteins infinitely widens the spectrum of the novel proteins to be synthesized. The elaboration of the effective methods to synthesize the proteins and enzymes containing element-organic amino acid analogues gives us hope to obtain the proteins and enzymes with the properties previously unknown.

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(ii) Novel classes of the anti-cancer and antiviral agents could be proposed using the amino acid analogues and their derivatives [1–6].

(iii) The replacement of any amino acid's significant part by their synthetic analogues is expected to lead to the formation of microorganisms and viruses with qualitatively novel properties. The question is: whether the normal functioning and development of the cell is possible if a significant part of any amino acid is replaced by its synthetic analogue.

The present report has two interconnected aspects:

1. A possibility of the amino acid analogues to be incorporated into the proteins and enzymes has been discussed.
2. A possibility to create the organisms containing a significant part of the specified amino acid to be replaced by its element-organic analogue is experimentally demonstrated here.

2. Methods of the unnatural amino acid incorporation into proteins

The key amino acid recognition enzyme in the protein biosynthesis is the amino-acyl t-RNA synthetase. It is evident that some analogues could not be incorporated into the proteins because of the specificity of the above synthetase. However, two main approaches to incorporate analogues into the proteins in a high yield have been elaborated to date. There are in vivo and in vitro approaches.

2.1. An in vivo analogue incorporation

In most cases, the harboring unnatural amino acid enzyme is produced by the administration of the analogue to the organisms producing the enzyme under investigation. This approach was applied in several works [7,8]. It is common to grow an amino acid auxotrophic *Escherichia coli* strain with a cloned enzyme gene under the inducible promoter or in the presence of a specified protein expression inhibitor in a medium containing all natural amino acids. After the biomass has been grown, the medium is replaced and an analogue is administrated in place of its natural analogue of that amino acid to which the strain is auxotrophic. After a short incubation period, the protein expression is induced by the inductor. The simplified method of the analogue incorporation is completely the same as the described above with the only difference: a non-auxotrophic *E. coli* strain is used. The yield of incorporation decreased but a major part of the specified amino acid can be replaced by the analogue.

2.2. An in vitro analogue site-specific incorporation

To incorporate an amino acid analogue into a specified position, Schultz et al. [9,10] elaborated a general method to insert virtually any analogue in any position of the protein. Some mutations are known to cease the translation of the protein encoded in the mutated gene. These mutations are the changes of the sense codons to the nonsense stop codons. Organisms found the way to overcome this problem. They have designed the so-called suppressor t-RNAs, which are charged with amino acids and recognize the stop codons. This fact is applied in the method described. The method implies chemical charging of the suppressor amino-acyl t-RNA with the amino acid analogue and an in vitro introduction of the analogue at the nonsense mutated positions of the enzyme gene under investigation in the cell-free translation system.

Maybe the only drawback of the method is the protein must be synthesized in vitro. Correspondingly, there is no posttranslational modification of the enzyme and the folding of the enzyme does not seem to be supposed as proper in all cases.

The method appears to be potentially effective to overcome the above drawbacks of the

both methods is a mutation of the amino-acyl t-RNA synthetase. The mutagenesis of this enzyme may widen the enzyme specificity and enables to incorporate the substances from a larger analogue list than it is for in vivo method to date. An attempt to elaborate such method was reported [11].

3. Enzymes harboring unnatural amino acids

3.1. Alkaline phosphatase

3.1.1. Histidine analogues

There were a number of works dealt with the investigation of alkaline phosphatase properties influenced by incorporation of 1,2,4-triazole-3-alanine [12,13] and 2-methylhistidine [14]. In both cases, the authors failed to produce an active enzyme. In the first case, the enzyme subunits were unable to associate to form the active enzyme. In the last case, the subunits properly were associated but there was no enzyme activity. It seems to be strong structure aberrations, which emerged after the histidine analogue had been incorporated and caused the enzyme activity failure.

3.1.2. Phenylalanine analogues

The phenylalanine analogue 4-fluorophenylalanine (FPA) can be introduced into the cellular protein by the biosynthetic machinery. The FPA incorporation into the isoenzymes of the alkaline phosphatase from *Aspergilius nidulans* was investigated [15]. Some isoenzymes remained active after the analogue incorporation but isoenzyme III was inactive. It was proposed that 4-FPA incorporation into isoenzyme III caused strong structure perturbations and, hence, inactivation.

The problem of specific biosynthetic incorporation of the phenylalanine analogue 4-FPA was investigated [16]. The analogue was found to be incorporated into the enzyme statistically. So, as it looks like natural, there are no preferable positions for the analogue incorporation into protein by the cellular biosynthetic machinery.

3.1.3. Tryptophan analogues

The effect of quantitative incorporation of the tryptophan analogues 7-azatriptophan and tryptazan (α-amino-β-3-(indazole)propionic acid) on alkaline phosphatase properties was investigated [17]. The kinetic, spectral properties and the enzyme stability towards heating and acidic treatment were in the scope of this work. The stability of the substituted enzyme was found to be less than that of the native one. An acidic treatment of native enzyme at pH 2 for 5 min inactivated it by about 90%. The enzyme dissociated into inactive subunits, which can be reactivated by incubation with Zn^{2+} ions at 37°C [18]. The same observations were for the analogue-substituted enzyme. The rate and extent of the reactivation were identical for the native and the azatryptophan-containing enzyme, but less for the tryptazan-containing one.

The enzymatic properties of the substituted and native enzymes were the same. The Michaelis constants for *p*-nitrophenyl phosphate as substrate of these enzymes were equal ($K_m = 6$ μM). The K_I values for inorganic phosphate as inhibitor were equal for these three enzymes ($K_I = 3$ μM) too.

Alkaline phosphatase acts not only as a hydrolase, but also as a phosphoryl transferase [19,20]. This activity was found not to alter by the analogue incorporation. In fact, the ratios of the *p*-nitrophenol formed/inorganic phosphate released were the same for the three enzymes using Tris as phosphate acceptor at its various concentrations.

The activities of these enzymes with *p*-nitrophenyl phosphate, AMP, histidinol phosphate and β-glycerol phosphate were different. The azatryptophan-containing enzyme was 40–60% more active, whereas the tryptazan-containing enzyme was 5–15% less active than native enzyme in each case.

The spectrum alterations of the analogue-containing enzymes were also investigated. The

maximum absorption at 290 nm was found for tryptazan- and azatryptophan-containing phosphatases. Fluorescence emission spectrum maximum of the azatryptophan enzyme shifted from 345 nm for the native enzyme to 370 nm. The protein denaturation in guanidine hydrochloride caused the fluorescence intensity to be decreased and red-shifted for azatryptophan enzyme as well as for the native enzyme.

3.1.4. Proline analogues

Morris and Schlesinger [21] succeeded in the incorporation of two proline analogs: 3,4-dehydroproline and azetidine carboxylate. The incorporation of the first analogue resulted in the formation of the active enzyme. However, its stability was much less than that of the native one. Thus, the 30-min treatment of the mutated enzyme at 90°C reduced the activity by 85%, whereas the native alkaline phosphatase lost only 45% activity in the same conditions.

The azetidine carboxylate incorporation resulted in the formation of phosphatase subunits, unable to build the active enzyme.

There are several other works describing the influence of the analogue incorporation on the alkaline phosphatase properties [22–24].

3.2. Aspartate transcarbamylase

Several amino acid analogues were tested to produce active aspartate transcarbamylase from *E. coli* [8]. This is a first pyrimidine pathway enzyme. It is made up of two trimeric catalytic subunits and three dimeric regulatory ones. The subunits association requires six zinc ions per molecule [25–28]. These amino acid analogues were azatriptophan (tryptophan analogue), etionine (metionine analogue), 1,2,4-triasolalanine and 2-methylhistidine (histidine analogues), 2-, 3- and 4-FPAs (phenylalanine analogues).

Etionine and azatriptophan incorporation into the aspartate transcarbamylase does not seem to alter significantly the catalytic and regulatory properties of the enzyme.

2-Methylhistidine and 1,2,4-triazolalanine incorporation damages the enzyme catalytic activity. This phenomenon could be explained by a supposition of the catalytic role of histidine in this enzyme [8].

The replacement of phenylalanine with 2-, 3- or 4-FPAs leads to the formation of the active enzyme. The activity of the substituted enzyme depends on a fluorine position in the analogue. The enzyme produced lacks its regulatory subunits and therefore cannot be inhibited by CTP. The thermal stability of the 4-FPA-containing enzyme is much lower than that of the native enzyme stability.

The 2-FPA harboring aspartate transcarbamylase was purified to homogeneity. Its properties were thoroughly tested. It was shown that about 60% of the phenylalanine was replaced by 2-FPA. It was supposed that the lack of the homotropic and heterotropic cooperative interactions was due to incorporation of the analogue into the regulatory subunits. To support this supposition, the hybrid enzyme with analogue-altered catalytic and normal regulatory subunits was investigated. The hybrid enzyme was found to represent a normal regulation.

3.3. β-Lactamase

The general method of the enzymes harboring unnatural amino acids production at specified positions was applied to study β-lactamase [9].

Phenylalanine at the 66th position based on the X-ray analysis data [29] was chosen as a target of the mutagenesis. Seven phenylalanine analogues were incorporated into the β-lactamase by this technique, namely D-phenylalanine (D-Phe), (*S*)-4-fluorophenylalanine (*p*-FPhe), (*S*)-*p*-nitrophenylalanine (*p*-NO_2Phe), 2-amino-phenylbutanoic acid (HPhe), (*S*)-3-amino-2-benzylpropionic acid (ABPA) and (*S*)-2-hydroxy-3-phenylpropionic acid (PLA).

The replacement of Phe 66 with *p*-FPhe, *p*-NO_2Phe and HPhe yielded the active enzyme.

The remaining analogues, being inserted in place of Phe, inactivated or made impossible to synthesize the enzyme. The influence of tyrosine replacement on the β-lactamase properties was also investigated. The kinetic properties of the altered enzyme are presented in Table 1.

The failure to produce enzymes harboring PLA, ABPA, D-Phe might be due to the failure at the ribosomal synthesis stage or the protein could not be folded. PLA may be out of function at the P-site [30]. The stability of the ternary complex D-Tyr-tRNA-EF-Tu is 25-fold lower than that of L-Tyr-tRNA-EF-Tu [31].

3.4. T4 lisozyme

Ellman et al. [10] applied their general method for incorporation of unnatural amino acids into the T4 lisozyme. Alanine 82 was chosen as a target for mutation. The mutation of Ala 82 to Pro was reported to increase the enzyme thermal stability [32]. Of interest was to investigate the thermal stability altered by the unnatural analogue. Furthermore, the Ala 82 is far from the catalytic site and the enzymatic activity was not supposed to be perturbed.

Five mutants containing the analogues were tested on their thermal stability. The structures of the acids used are presented in Fig. 1 [10].

The thermal stability of the altered enzymes is presented in Table 2.

3.5. Glutathione transferase

Parsons et al. [7] have succeeded in the incorporation of 5-fluorotryptophan into the rat M1-1 glutathione transferase. This enzyme has two polypeptide chains with 2 Trp residues per chain.

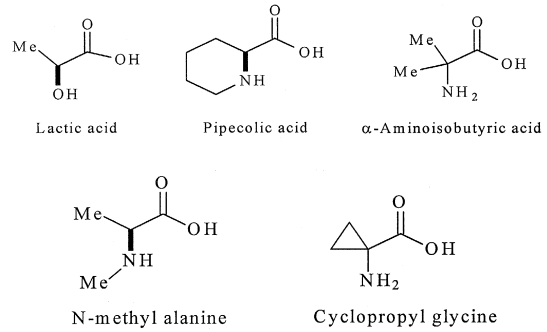

Fig. 1. Amino acid analogues tested in the regard of their incorporation into the T4 lisozyme.

Supposedly, 5-fluorotryptophan is a better hydrogen-bond donor than tryptophan. The pK_a of the indole NH is 17, whereas that of 5-fluoroindole is 16.3. The length of the aromatic C–F bond is 30% longer than the corresponding C–H bond. These properties of 5-fluorotryptophan were investigated in Armstrong's work in regard of its influence on the enzyme structure and function.

It was obtained completely 5-FTrp-substituted enzyme. The steady-state kinetic constants of the substituted enzyme, compared to the native one, are presented in the Table 3.

The activity of fluorotryptophan-substituted enzyme is evidently higher towards 1-chloro-2,4-dinitrobenzene (CDNB), whereas the catalytic constants are virtually unchanged in the case of phenantrene 9,10-oxide (PO) and 4-phenyl-3-buten-2-one (PBO). The rate-limiting step for CDNB is a product release [33], but the rate-limiting step for PO and PBO is a chemical conversion. In addition, the K_a of the enzyme-

Table 1
Characterization of native and mutant β-lactamases [9]

Amino acid	K_m (μM)	k_{cat} (s^{-1})
Phe	59 ± 6	870
Tyr	49 ± 3	420 ± 40
p-FPhe	59 ± 2	1120 ± 290
p-NO_2Phe	57 ± 4	370 ± 70
HPhe	72 ± 14	150 ± 60

Table 2
Thermal stability of the mutated T4 lisozyme

Protein	T_m (°C)
Wild-type	43.4 ± 0.25
Ala^{82} → lactic acid	39.7 ± 0.25
Ala^{82} → pipecolic acid	41.3 ± 0.25
Ala^{82} → α-amino isobutyric acid	44.6 ± 0.25

Table 3
Steady-state kinetic constants for native and $(5\text{-FTrp})_4$ enzyme [7]

	Enzyme	
	Native	5-FTrp
k_{cat} (s^{-1})	18 ± 2	73 ± 3
K_m^{GSH} ($\pm$M)	36 ± 4	55 ± 3
k_{cat}/K_m^{CDNB} ($M^{-1}\,s^{-1}$)	$(1.0 \pm 0.1) \cdot 10^6$	$(2.1 \pm 0.2) \cdot 10^6$
K_m^{CDNB} ($\pm$M)	41 ± 4	55 ± 4
pK_a (kinetic)	6.2 ± 0.3	6.5 ± 0.2
pK_a (spectroscopic)	6.6 ± 0.2	6.9 ± 0.2
k_{cat}^{PO} (s^{-1})	0.19 ± 0.02	0.21 ± 0.02
k_{cat}^{PBO} (s^{-1})	0.11 ± 0.01	0.11 ± 0.01

bound GSH is the same for the substituted and the native enzyme.

It was supposed that the fluorotryptophan incorporation altered the rate of the product release, which is the rate-limiting step in the reaction involving CDNB. This supposition was considered by the X-ray analysis of the fluorotryptophan-substituted enzyme. The overall structures of the mutated and native enzyme are very similar. The structure of GSH binding domain of the enzyme has some changes. The two FTrp residues (7 and 45) occupy the same positions as in the native enzyme. These residues are hydrogen bond donors. In the case of their fluorinated substitutes, the hydrogen bonds are also maintained. Most changes in the position are observed for the neighboring to the Trp7 residue, the Ala8. The Ala8 residue is in a much more different orientation compared to the native structure.

The structure of the enzyme domain II is more perturbed by fluorotryptophan incorporation. There are FTrp146 and FTrp214 in domain II. According to the X-ray analysis, there are discrepancies in crystallographic parameters of the native and mutated enzymes in the domain. The native enzyme has a hydrogen bond between Ser 209 and Tyr 115, whereas this bond is absent in the mutated one. Despite the distance between the Ser209–Tyr115 location and the fluorotryptophan incorporated, the polypeptide chain transmits the influence of the amino acid analogue on the above residues. The lack of this hydrogen bond results in the product release acceleration as was proposed by Johnson et al. [33].

4. The cells are able to function normally if a part of the amino acid is substituted by its unnatural analogue

The restricted number of the reported works on the problem of the analogue incorporation showed that several elaborated methods produce proteins that are harboring unnatural amino acids. In some cases, there are significant stability and activity alterations.

The possibility of the cell normal functioning with such amino acid-specific multi-mutagenesis still remains undefined. We have investigated the yeast cell growth in the presence of the unnatural amino acid analogue 4-FPA and determined the analogue content in the whole cell protein. The aim of the experiment was the answer to the question about a possibility of the cells to function normally and to grow in the presence of the element-organic analogue.

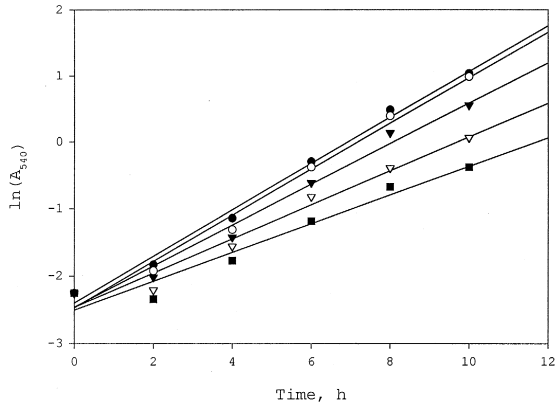

Fig. 2. The growth curves of the *Saccaromyces cerevisae* strain 232 at the various FPA concentrations (mg/ml) in the liquid medium: ● — 0, ○ — 0.05, ∇ — 0.5, □ — 1.0. The *Saccaromyces cerevisae* strains 230, 232 and 233 were purchased from Microbiology Collection of Moscow State University, Biology Faculty. The yeast strains were grown in the rich media containing glucose — 50 g/l, DIFCO yeast extract — 2 g/l, NaCl — 1 g/l, KH_2PO_4 — 1 g/l, $MgSO_4 \cdot 7H_2O$ — 500 mg/l, $(NH_4)_2SO_4$ — 500 mg/l and FPA in corresponding concentration in distilled water. 4-D,L-fluorophenylalanine was purchased from Sigma.

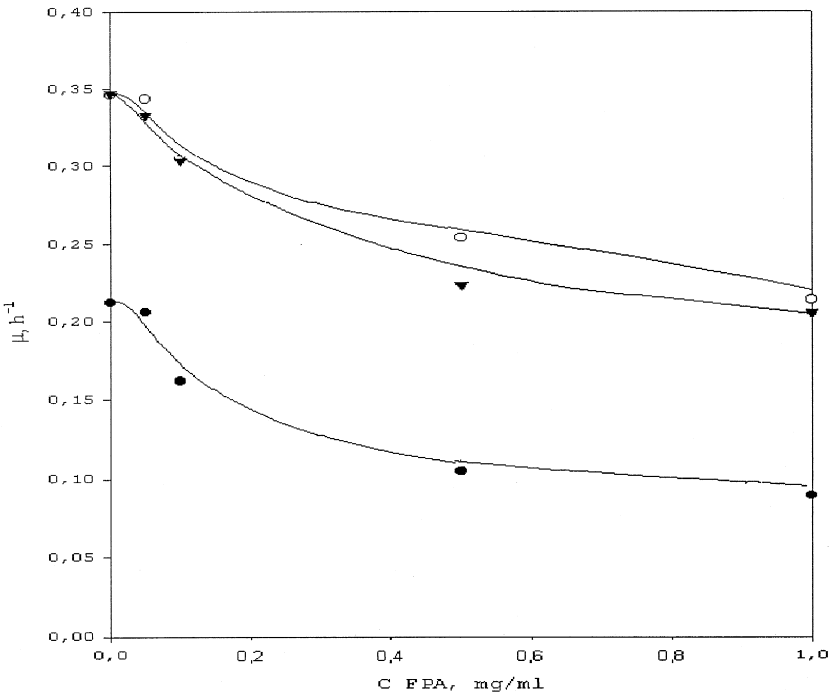

Fig. 3. The growth rates (μ) of various *Saccaromyces cerevisae* strains as a function the FPA concentration ● — 230, ⬡ — 232, ▲ — 233.

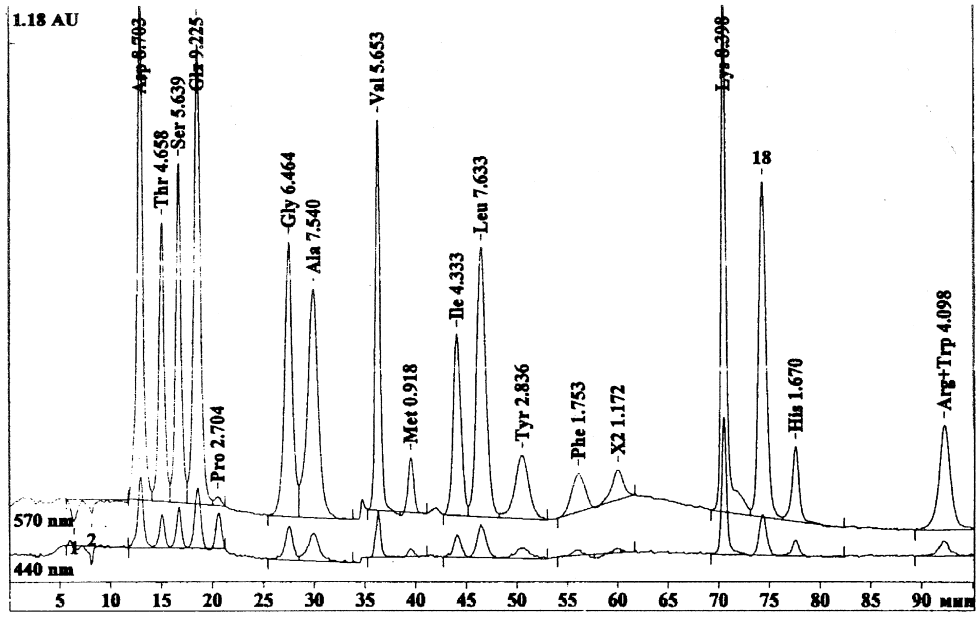

Fig. 4. Amino acid analysis of the total yeast protein. X2-peak corresponds to the FPA. The yeast was grown in 250 ml of the liquid medium for 22 h and centrifuged. The cells were diluted in water and sonicated at 22 kHz (10×10-s sonication). The insoluble cell fragments were removed by centrifugation. Trichloroacetic acid was added to the supernatant contained, all soluble proteins to 5% final concentration. Denatured proteins were isolated by centrifugation and lyophilized. This protein was then analyzed in an amino acid analyzer.

The investigation of several yeast strains growth kinetics in the media containing various concentrations of the 4-FPA was carried out. It was shown that the yeast could grow in the presence of the FPA and it was observed that the growth rate decreased at high FPA concentrations. The linearised exponential growth phase curves of strain 232 are presented in Fig. 2. Similar lines were obtained for other strains investigated.

It is seen from Fig. 2 that the FPA is a growth inhibitor and its influence on the growth rates is complicated. The dependence of the growth rate on the FPA concentration is presented in Fig. 3. The same curves were obtained for other yeast strains examined.

It is possible to determine at least three concentration ranges of FPA influence on growth rates. There is no effect on the growth rates at FPA concentrations less than 0.1 mg/ml in the medium. In the 0.1–1 mg/ml FPA range, the rate is decreased approximately 1.5 times. At higher concentrations, the rate seems to be independent of FPA concentration again. After this experiment, we obtained FPA-tolerant yeast strain 232 using several generations.[1] This strain is able to grow on the agar plates containing 1 mg/ml FPA.

It was determined that the relative content of the FPA in the whole yeast protein from the cells grown in the medium contained 1 mg/ml FPA. The amino acid analysis data are presented in Fig. 4.

It is necessary to outline that a significant part (35–40%) of a phenylalanine is replaced by FPA. The same picture was obtained for the FPA-tolerant strain grown in the liquid medium at 5 mg/ml FPA concentration. In this case, the same FPA/Phe ratio was observed.

To describe the phenomenon of amino acid replacement in the proteins, we propose a new term — amino acid-specific multimutagenesis. This way of mutagenesis opens a new protein investigation branch. The data obtained show that amino acid multi-mutagenesis has no dramatic effect on the yeast growth physiology. This investigation illustrates a possibility to create the living systems with a significant part of their natural amino acid replaced by the element-organic analogue.

[1] The yeast were grown in the liquid medium containing 1 mg/ml FPA for 14 h. Then the cells were recultivated in the same conditions. The growth had a much more prolonged lag-period. The cells were then seeded in the plates with the solid medium after the optical density of the cell suspension reached approximately 1. The solid growth medium contained the same components at the same concentrations as the liquid media and agar of 1.5% w/w.

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