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Informational spectrum method applied to the investigation of the enzymatic activity of different α- amylases*

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A series of eight α-amylases (1,4-α-D-glucan-glucano-hydrolase: EC 3.2.1.1) of known primary structure were investigated using the Informational Spectrum Method (ISM), for the purpose of predicting new enzymes with increased efficiency. Through ISM, we transformed the primary structure of an enzyme into a numerical array, and were able to obtain and compare original spectra. Furthermore, using the multiple cross-spectrum function, we established only one mutual and well defined peak. The frequency of this peak is the characteristic frequency of all investigated α-amylases. There is a correlation between the value of the amplitude at the characteristic frequency and the activity of the biomolecule. By a further mathematical treatment, the method yielded information enabling the prediction of an enzyme structure with greater efficiency through minimum modification of the natural product. This minimum modification could be performed using the simplest method of genetic engineering, single point mutation. Mutations were suggested for α-amylases from the following sources: *Aspergillus oryzae, Bacillus amyloliquefaciens* and barley.

A series of α- amylases (1,4 - α - D - glucan- glucano-hydrolase; EC 3.2.1.1) originating from mouse liver and salivary gland,[1] from pancreas of mouse,[2] rat[3] and hog,[4] from barley,[5] fungus *Aspergillus oryzae*,[6] bacteria *Bacillus amyloliquefaciens*[7] and *Bacillus subtilis*,[8] of known primary structure were investigated by the Informational Spectrum Method (ISM). ISM is a numerical procedure for the analysis of proteins and nucleic acids[9-12] and in the present work it was used to determine the characteristic frequency of the above mentioned enzymes

The characteristic frequency is a parameter which appears to be common to proteins having the same biological function. Proteins of different origin and constitution, but with the same

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function, have the same significant peak frequencies, called characteristic frequencies.[12] By a further development of this method[9] it was proposed that it would be possible to design proteins with enhanced biocatalytic properties. In the present work, calculation of the changes required in the primary structure of an α- amylase molecule necessary to enhance its enzymatic activity was attempted. Because of their importance in biotechnological transformations of starch substrates, the calculations concerning structure changes were performed for α-amylases from barley, Bacillus amyloliquefaciens and Aspergillus oryzae.

METHOD

ISM is a theoretical method for the analysis of the informational content of protein and DNA sequences[11] using both mathematical and physical principles and computer techniques. There are two steps in the procedure. The first step is the transformation of genetic and protein sequences into a numerical array. Each nucleotide or amino acid is represented by a characteristic potential of electron-ion interaction.[13,14]

The potential of the electron-ion interaction (PEII), calculated from the appropriate model,[13] describes the energy levels of valence electrons in nucleotides or in the R groups of amino acids. These energy levels are closely related to the mechanisms of interaction between organic molecules.[10,12] According to the model proposed by Veljković and Slavić,[13] for single atoms, the PEII is a function of a valence number and two constants

where: z^* is the valence number - number of electron in the outer incomplete shell of a chemical element, α_1 and α_2 are constants dependant on the position of the atom in question in the periodic table.

$$PEII = \alpha_1 z^* \frac{\sin(2\pi \alpha_2 z^*)}{2\pi} \qquad \alpha_1 = -3.40 \text{ eV} \quad \alpha_2 = 0.52$$

Veljković,[14] further developed the method of PEII determination and extended it to the calculation of the potential for atomic groups, using instead of individual valence numbers, the "effective valence number" z^*_{ef}:

$$z^*_{ef} = \frac{\sum_{i=1}^{m} n_i z_i^*}{N}$$

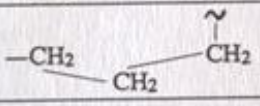
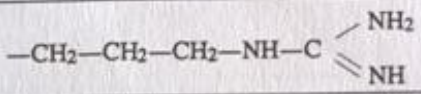
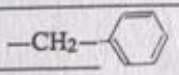
where: N is the total number of atoms in the R group, m the number of chemically different constituent atoms, n_i the number of the i-th constituent atom, and z_i the valence number of the i-th constituent atom.

Values for z^*_{ef} are from Ref. 14 and these values, together with the PEII for each amino acid, or more precisely, of its R group, are given in Table I.

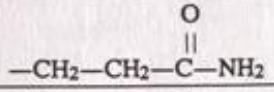
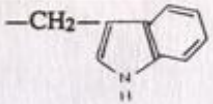
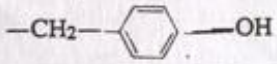
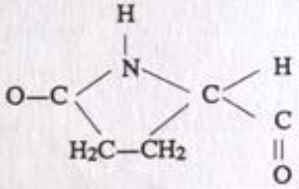
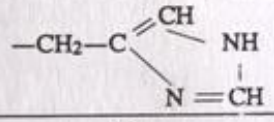
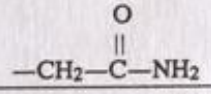
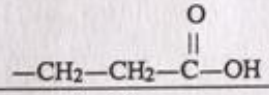
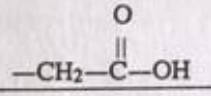
It has been shown previously[9,10,11,13,14] that such an approximation of the energy state of a molecule can be used for numerical analysis of the biological function of naturally occurring macromolecules. Thus, ISM has been used for the analysis of a number of proteins and regulatory DNA sequences.[10,15,16]

In all cited investigations, PEII is considered to be a basic parameter and each nucleotide or amino acid, regardless of its position in a sequence, is represented by a single specific PEII value.

Table 1. Values of PEII for the constituent amino acids of investigated enzymes

Amino acid	Symbol	R— group	Potential of the electron-ion interaction (PEII) eV
Glycine	G	—H	+0.06782
Alanine	A	$-CH_3$	+0.50741
Valine	V	$-CH(CH_3)_2$	+0.07745
Leucine	L	$-CH_2-CH(CH_3)_2$	0
Isoleucine	I	$-CH(CH_3)-CH_2-CH_3$	0
Proline	P		-0.26915
Lysine	K	$-CH_2-CH_2-CH_2-CH_2-NH_2$	-0.50562
Methionine	M	$-CH_2-CH_2-S-CH_3$	-1.11870
Threonine	T	$-CH(OH)-CH_3$	-1.27947
Arginine	R		-1.30464
Phenylalanine	F		-1.28660
Serine	S	$-CH_2-OH$	-1.12775
Cysteine	C	$-CH_2-SH$	-1.12775

able 1. (cont.)

mino acid	Symbol	R— group	Potential of the electron-ion interaction (PEII) eV
ilytamine	Q		-1.03436
ryptophan	W		-0.74542
yrosine	Y		-0.70167
-Pyrrollidone - -carboxylic acid	J*		-0.47867
listidine	H		-0.32841
sparagine	N		-0.04887
lutamic acid	E		+0.07885
spartic acid	D		+1.71809

ssignation for the purpose of this work for N-terminal amino acid of hog pancreas α-amylase.

The second step in the ISM procedure is the analysis of the numerical array to extract the information relevant to the biological function. The transformation of the numerical series into the corresponding spectral is done by the Discrete Fourier Transformation (DFT)[17] as follows:

$$x(n) = \sum_{m=0}^{N-1} X(\mathrm{m}) \exp(-\mathrm{j}\,(2\mathrm{P}/\mathrm{N})\mathrm{nm} \quad \mathrm{n} = 1,2 \ldots \mathrm{N}/2$$

where : $x(m)$ - *m -th member of a given numerical series,* N - total number of points of this series, $X(n)$ -DFT coefficients.

In a DFT treatment, an aperiodic discrete function can be represented by an array of sinusoidal functions, which are contained in the original signal. Amplitude of these functions constitute the amplitude spectrum of genetic or protein sequences, called the Informational Spectrum (IS). In order to determine frequency components which are mutual for a group of sequences, a multiple cross-spectral function is used. This is defined by :

$$M(n) = X1(n) * X2(n) * \ldots * XM(n) \quad n = 1,2,\ldots,N/2$$

where : $M(n)$ - multiple cross-spectral function, $Xi(n)$ - DFT coefficients of the ith signal, M - total number of signals.

In the multiple cross-spectral function the peak frequency indicates that all analyzed sequences have one mutual and characteristic frequency. Earlier studies [9-12,16] which included about 1000 protein and regulatory DNA sequences, showed that for the same, well defined biological function, a characteristic common frequency was detected for all investigated molecules. There is no relevant peak for unrelated sequences, in fact a "white noise" signal is produced.[12,15] It was also noted that the higher the amplitude of the characteristic frequency in the Informational Spectrum the greater the efficiency of the investigated biologically active natural macromolecule. It was therefore suggested that by using an inverse ISM procedure, it should be possible to propose a change in the primary structure for a calculated increase in the primary structure for a calculated increase in the amplitude at the characteristic frequency. The new protein should exhibit an increase efficiency in the specific biological function.[17]

RESULTS AND DISCUSSION

Figure 1 presents the stepwise procedure applied to one randomly chosen α- amylase out of the eight investigated. Figure 1.1 is the single letter notation of the primary structure of the α-amylase isolated from the fungus *Aspergillus oryzae*. This enzyme is popularly known as Taka amylase A and is active in the hydrolysis of rice starch. Figure 1.2 is the numerical array obtained by assigning the appropriate PEII value to each amino acid presented in Figure 1.1. This numerical array was then treated by DFT in the same way as signals are treated in telecommunications. This transformation leads to Figure 1.3, the Informational Spectrum is of Taka amylase A.

The transformation of the primary structure of all investigated α - amylases to IS was performed in the same manner. The IS for different α - amylases varied, as is evident in Table II.

1.1

```
AIPADWRSQSIYFLLIDRFARIDGSTTATCNTADQKYCGGTWQGIIDKLD
YIQGMGFTAIWITPVTAQLPQDCAYGDAYTGYWQTDIYSLNENYGTADDL
KALSSALHERGMYLMVDVVANHMGYDGAGSSVDYSVFKPFSSQDYFHPFC
FIQNYEDQTQVEDCWLGDNTVSLPDLDTTKDVVKNEWYDWVGSLVSNYSI
DGLRIDTVKHVQKDFWPGYNKAAGVYCIGEVLDGDPAYTCPYQNVMDGVL
NYPIYYPLLNAFKSTSGSMDDLYNMINTVKSDCPDSTLLGTFVENHDNPR
FASYTNDIALAKNVAAFIILNDGLPIIYAGQEQHYAGGNDPANREATWLS
GYPTDSELYKLIASANAIRNYAISKDTGFVTYKNPYIKDDTTIAMRKGTD
GSQIVTILSNKGASGDSYTLSLSGASYTAGQQLTEVIGCTTVTVGSDGNV
PVPMAGGLPRVLYPTEKLAGSKICSDSS
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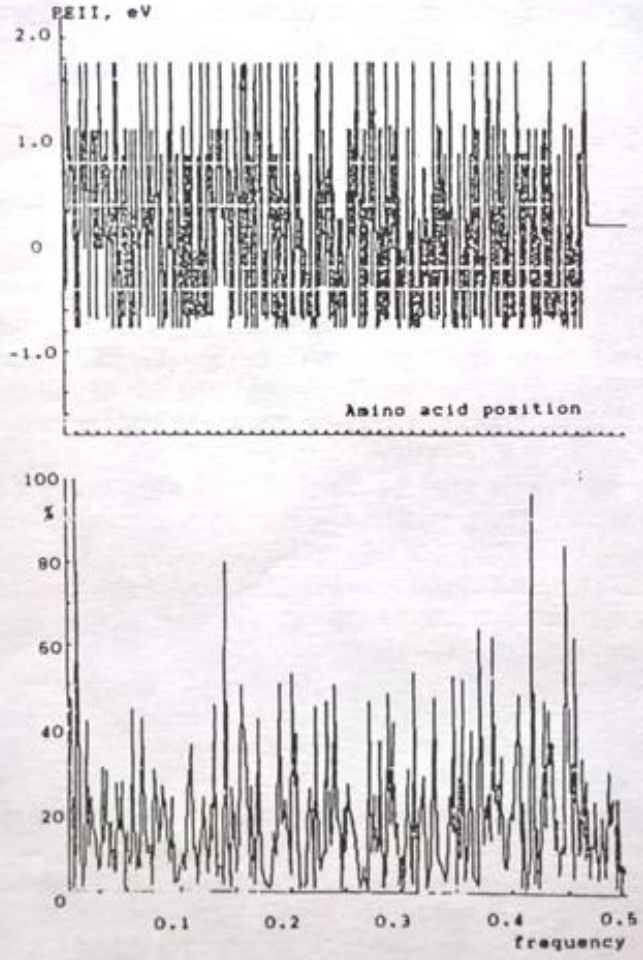

1.2

1.3

Fig . 1. The evolution of the informational spectrum (IS) for α-amylase of *Aspergillus oryzae* (Taka amylase A)

1. 1 Primary structure in single letter symbols of amino acids

1.2 Substitution of amino acids by PEII, the original spectrum

1.3 The informational spectrum of taka amylase A

TABLE II. Characteristic values obtained from informational spectra of investigated α - amylases

Source of α - amylase	Peak frequency	Amplitude of the highset peak frequency (eV)	Signal/ noise ratio
Mouse liver and silvary gland	0.05273	40.740	5.21
Pancreas of mouse	0.39648	44.365	6.14
Pancreas of rat	0.39648	47.239	7.15
Hog pancreatic isoenzyme I	0.41211	42.960	5.99
Barley	0.02734	35.204	4.60
Aspergillus oryzae (Taka amylase A)	0.00977	41.498	5.58
Bacillus amyloliquefaciens	0.31641	46.830	7.39
Bacillus subtilis	0.24316	43.957	9.55

The primary structure of the analyzed enzymes varies from approximately ten to ninety percent, according to the data of Refs.[1-8] but they all catalyze the random hydrolysis of the α 1 →4) glucoside bond in starch and glycogen.

A detailed survey of the primary structures of the α - amylases from animal sources showed that they are quite similar. Thus, amylases from two different mouse organs, the pancreas and liver, differ by 12.2%, while salivary gland amylase and liver amylases are practically the same, the mRNAs differ only in 5' non-translated sequences. Mouse and rat pancreatic amylases are most similar, considering the different species, differing only by 6.76% in the amino acid sequences. Among the mammalian amylases, those originating form mouse liver and hog pancreas differ the most, 19.15%. The other analyzed amylases differ considerably among themselves and are also bey different to those from mammalian sources. There is practically no similarity except ion the sequences given in Table III. As this amounts only to about 10% of the whole macromolecule, it is difficult to give a quantitative measure of similarity.

In Table III, the circles denote amino acids which are believed to participated in the active site of enzyme (on the basis of investigations performed on Taka amylase A).[6] The squares circles are the amino acids which appear in the sequences of maximum similarity in all the investigated α-amylases. The triangle is the only point where mutation is proposed in the neighborhood of the region of the similar sequence in Taka amylases A.

Regardless of the surprisingly different primary structure of enzymes from different origin and, consequently, the different informational spectra, the same biological function is expressed by a mutual characteristic frequency.

Table III. Similar sequences in investigated α-amylases

No.	Source of α-amylase	Number of amino acids	Amino acid sequence																
1	Mouse liver and salivary gland	511	107 R	108 I	109 Y	110 V	111 D	112 A	113 V	114 I	115 N	116 H	117 M	118 C	119 G	120 V	121 G	122 A	
2	Pancreas of mouse	508	107 R	108 I	109 Y	110 V	111 D	112 A	113 V	114 I	115 N	116 H	117 M	118 C	119 G	120 V	121 G	122 A	
3	Pancreas of rat	503	102 R	103 I	104 Y	105 V	106 D	107 A	108 V	109 I	110 N	111 H	112 M	113 C	114 G	115 S	116 G	117 N	
4	Hog pancreatic isoenzyme I	496	92 R	93 I	94 Y	95 V	96 D	97 A	98 V	99 I	100 N	101 H	102 M	103 C	104 G	105 S	106 G	107 A	
5	Barley	438	108 Q	109 A	110 I	111 A	112 D	113 I	114 V	115 I	116 N	117 H	118 R	119 C	120 A	121 D	122 Y	123 K	
6	*Aspergillus oryzae*	478	113 Y	114 L	115 M	116 V	117 D	118 V	119 V	120 A	121 N	122 H	123 M	124 G	125 Y	126 D	127 G	128 A	
7	*Bacillus amyloliquefaciens*	433	94 Q	95 V	96 Y	97 G	98 D	99 V	100 V	101 L	102 N	103 H	104 K	105 A	106 G	107 A	108 D	109 A	
8	*Bacillus subtilis*	660	134 K	135 V	136 I	137 V	138 D	139 A	140 V	141 I	142 N	143 H	144 T	145 T	146 S	147 D	148 Y	149 A	
1	Second similar sequence		205 G	206 V	207 A	208 G	209 F	210 R	211 L	212 D	213 A	214 S	215 K	216 H	217 M				
2			202 G	203 V	204 A	205 G	206 F	207 R	208 L	209 D	210 A	211 A	212 K	213 H	214 M				
3			197 G	198 V	199 A	200 G	201 F	202 R	203 L	204 D	205 A	206 A	207 K	208 H	209 M				
4			190 G	191 V	192 A	193 G	194 F	195 R	196 I	197 D	198 A	199 S	200 K	201 H	202 M				
5			136 T	137 S	138 D	139 G		140 R	141 L	142 W	143 G	144 P		145 H	146 M				
6			119 S	200 I	201 D	202 G	203 L	204 R	205 I	206 D	207 T	208 V	209 K	210 H	211 V				
7			224 S	225 L	226 D	227 G	228 F	229 R	230 I	231 D	232 A	233 A	234 K	235 H	236 I				
8			219 G	220 A	221 D	222 G	223 F	224 R	225 F	226 D	227 A	228 A	229 K	230 H	231 I				
1	Third similar sequence		308 L	309 V	310 F	311 V	312 D	313 N	314 H	315 D	316 N	317 Q	318 R						
2			305 L	306 V	307 F	308 V	309 D	310 N	311 H	312 D	313 N	314 Q	315 R						
3			300 L	301 V	302 F	303 V	304 D	305 N	306 H	307 D	308 N	309 Q	310 R						
4			293 L	294 V	295 F	296 V	297 D	298 N	299 H	300 D	301 N	302 Q	303 R						
5			308 A	309 T	310 E	311 V	312 D	313 N	314 H	315 D	316 T	317 G	318 S						
6			290 G	291 T	292 E	293 V	294 E	295 N	296 H	297 D	298 N	299 P	300 R						
7			321 V	322 T	323 F	324 V	325 E	326 N	327 H	328 D	329 T	330 Q	331 P						
8	No similar sequence																		

Using the multiple cross-spectral function, Fig. 2, shows the procedure for the definition of the mutual frequencies for seven out of the eight investigated α-amylases. Figure 2.7 depicts a clearly defined peak frequency at 0.41211. This is the most significant frequency for all signals obtained as a mathematical representation of the primary structure of liquefying α-amylases. The only one that did not fit into this analysis was the saccharifying α-amylase from *Bacillus subtilis*.

The α-amylase from *Bacillus subtilis* was not included in further treatment as the multiplication of its spectrum with the spectra of other α-amylases during multiple cross-spectral analysis caused a decrease instead of an increase in the peak amplitude at the characteristic frequency and so decrease the accuracy of the method used.

The possible reason for this is the number of amino acids residues in this enzyme, which is 660.

It should also be noted that being the only saccharifying amylase and different in some of its characteristics from the others,[18-20] the *Bacillus subtilis* α-amylase could have a somewhat different mechanism and therefore a different characteristic frequency.

TABLE IV . Values of amplitude at mutual frequency (0.41211)

Source of α - amylase	Amplitude on mutual frequency (eV)	Square of the amplitude (eV^2)
Mouse liver and salivary gland	34.19	1169.0
Pancreas of mouse	36.42	1326.2
Pancreas of rat	32.64	1065.4
Hog pancreatic isoenzyme I	42.96	1845.9
Barley	24.06	578.9
Aspergillus oryzae (Taka amylase A)	19.42	377.3
Bacillus amyloliquefaciens	22.88	523.4

Table IV gives the results of the ISM procedure applied to the seven α-amylases. The amplitude of the mutual frequency is the significant parameter for comparison of enzymatic activity, but the square of the amplitude was used for comparison. This is similar to what was done for other biomolecules. [10,15] It is well known that it is very difficult to correlate kinetic data of various enzymes. The data available in literature usually concern a single amylase and a single substrate. However, we were able to find, in the literature comparable activity parameters for pairs of α - amylases which have been successfully analyzed by ISM in the present work.

These were compared using the values of the squares of the amplitudes given in Table IV.

Bernfeld [21] gives Km values for hog pancreas (1.8×10^{-4} g/cm ^{3}) and barley α-amylase (6.3×10^{-4} g/cm ^{3}). The ratio of the Km constants for these two enzymes is 3.50 and the ratio of the squares of the amplitudes at the mutual frequency is 3.19. We considered this to be very good agreement.

Tauber [22] states that, at optimum pH, the activity of α-amylase from barley (1200 units) is 1.84 times higher than the activity of α-amylase from *Aspergillus oryzae* (650 units). The respective ratio of the squares of the amplitude of the investigated α-amylases is 1.53. Data for the activity of crystalline α-amylases were given by Barman.[23] According to this source, the ratio between the activity of porcine pancreatic amylase (1360 IU, pH 6.9) and that from *Aspergillus oryzae* (438 IU, pH 5.9) is 3.11. The ratio of the values given in Table IV is 4.89. Although the agreement between the last two ratios is only fair, we consider that the obtained comparison indicates the possibilities of using ISM for the study of enzyme activity. This has not been done till now.

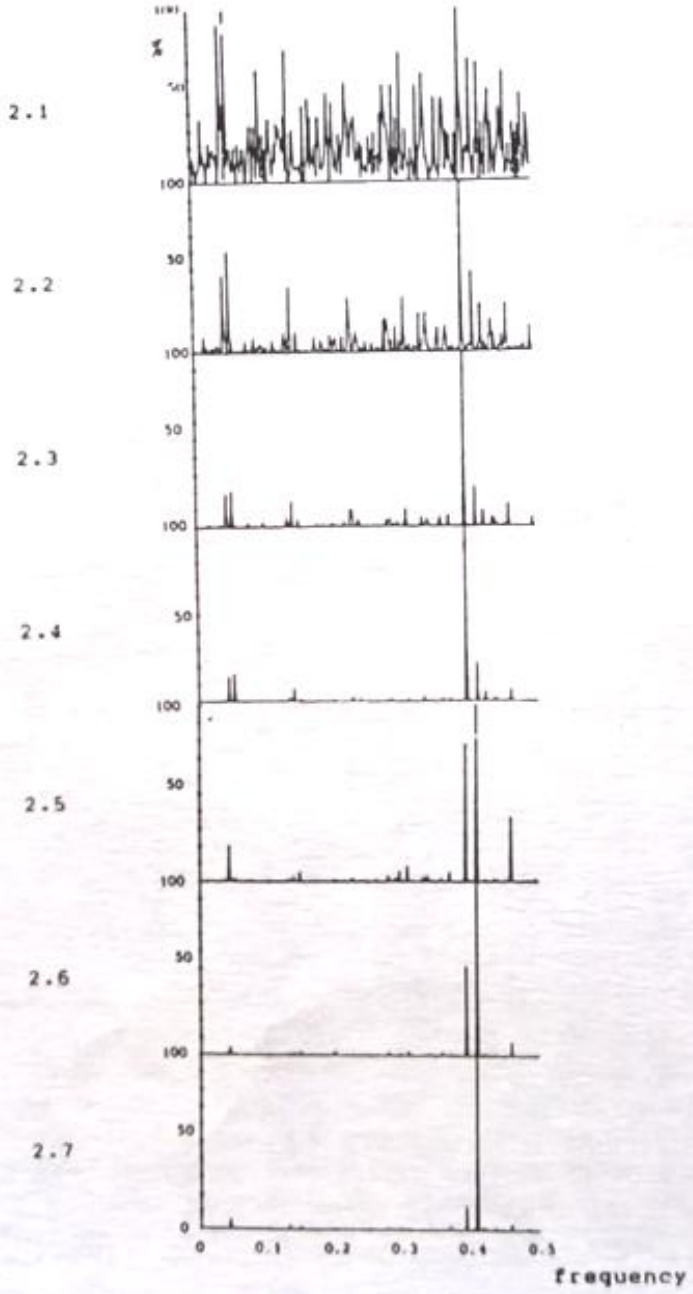

Fig. 2. The procedure for the extraction of the mutual frequency by multiple cross-spectral function of investigated α- amylases:

2.1 The Informational Spectrum of α-amylases from mouse liver and salivary gland

2.2 The cross-spectral function of α-amylases from two different tissues of mouse liver and pancreas

2.3 The multiple cross-specrtal function of two mouse α-amylases and one from rat

2. 4 The multiple cross-spectral function of tree rodent α-amylases and one from hog

2. 5 The multiple cross-spectral function of four mammal α-amylases and one from barly

2. 6 The multiple cross-spectral function of mammal, barley and *A. oryzae* α-amylases

2.7 The multiple cross-spectral funstion of all seven liquefying α-amylases

Bearing in mind the methodology of th ISM procedure outlined above, it should be possible, theoretically, to determine the points in the sequence which are related to the characteristic frequency which are related to the characteristic frequencies of the group of molecules having the same biological function. Therefore, using inverse ISM, it should be possible to predict changes in the primary structure which should influence the biological action. In a recent publication,[15] this procedure has been tested on SV40 enchancers, and the results were successfully compared with experimental data.

In this work, we used inverse ISM to propose the changes in the primary structure of selected biotechnologically important α-amylases, which should produce a more efficient enzyme. Employing inverse DFT, actually its more convenient form FFT (Fast Fourier Transformation), and the well known algorithm,[24] a preselected percentage increase of amplitude at the characteristic frequency was effected.

It is well known[25] that an amplitude change at only one frequency in a spectrum causes changes at each point in the numerical sequence. Therefore, the percentage increase in the amplitude at the characteristic frequency is selected in such a manner (trial and error method) that when IFT, (Inverse Fourier Transformation) is applied only minimal changes are effected. This means that in the new numerical sequence, only in few points (2-3) are the PEII increased up to the next nearest full PEII value, corresponding to a different amino acid indication, for example, a change of glycine to valine.

Thus in α-amylase from *Bacillus amyloliq*uefaciens it was predicted that using single point mutation, the simplest method of genetic engineering, changes in the primary structure of the enzyme should be made at positions 43, 53 and 63. At these points in the protein glycine should be replace by valine, thus producing a more efficient enzyme with minimum changes in the primary structure.

In α-amylases from barley, glycine should be replaced, by valine at positions 58 and 75 and phenylalanine replaced with threonine at position 272. It was also predicted, that Taka amylases A would be more efficient if glycine was replaced with valine at positions 290, 438, and 448 ; and phenylalanine with threonine at position 301.

Mutations where glycine is replaced by valine are among the most frequent in nature. Therefore, the small proposed changes in the primary structure should not cause any dramatic perturbations of the other structural and bonding characteristics of the protein.

It should be noted, however, that at least for Taka amylase A, the proposed changes in the primary structure do not concern the activity centre. According to Kakudo[17] in Toda's paper,[6] proposed from X-ray crystallographic analysis of Taka amylase A, That His-210, Gly-230 and Asp-297 are located in the active site cleft and that His-122, Asp-168, His-296, Asp-297, and Asp-340 participate in the substrate binding. Therefore, the mutations proposed in the present work should not affect either the active centre nor the substrate binding position but, more likely, the general energy level of the molecule. According to our findings, this is important for the biological function of the protein investigated.

This finding is, to a certain extent, supported by predictions given earlier in the literature. It was suggested that, in enzyme engineering, initial successes may result from modelling changes which are not directly related to the mechanism of catalysis and, therefore, with the structure of the active centre. According to the same reference[26] enhanced characteristics for industrial catalysis may be provided by a catalyst with an altered substrate specificity or sensitivity to product inhibition.

From the point of view of a biochemist, it may be argued that ISM does not take into account the higher levels of the protein structure. However, secondary, tertiary and quaternary structures, taken individually or together, ar a function of the primary structure. At present the mathematical expression of this function is unknown, but this does not mean that it does not exist.

CONCLUSION

We consider that the treatise put forward in this work presents, at least, a possibility for predicting changes in the primary structure of α-amylases, which maybe expected to result in increase in the efficiency of the enzyme. One may conclude from a literature survey of texts on enzyme engineering that guiding design principles for changes in enzyme structure are required.[26-28] It is further believed that, considering that recombinant DNA methodology for protein synthesis exists today,[26] *de novo* enzyme design and synthesis will be limited only by the ability to select the proper amino acid sequences for engineering proteins.

The main advantage of the results obtained by use of ISM is that the proposed changes in the primary structure can be performed without drastic genetic manipulations, *i.e., via* recombinant DNA methods. Single point mutation is a method which is constantly being developed and it is possible that it will gain in importance in the future.

Enzymes of specific and maximum efficiency are necessary because in a bioreactor a single biochemical reaction is important. It is not certain that the proposed changes will be suitable for processes using live cells, as these are complex and not sufficiently known.

It should also be pointed out that all propositions put forward in the present work need to be verified experimentally. We are not in the position to do that now, but possibly somebody else is.

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ИЗВОД

ПРИМЕНА МЕТОДЕ ИНФОРМАЦИОНИХ СПЕКТАРА У ИСПИТИВАЊУ ЕНЗИМАТСКИХ РАЗЛИЧИТИХ α-АМИЛАЗА

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Низ од 8 α-амилаза (1,4-α-D-глукан-глукано-хидролазе; EC 3.2.1.1) познате примарне структуре испитивано је методом информационих спектара (ISM), с циљем да се предложе нови ензими веће ефикасности. Помоћу ISM-а трансформисана је примарна структура ензима у низ бројева, а затим је било могуће добити и упоредити оригиналне спектре. Коришћењем мултиспектралне функције утврђен је само један заједнички, добро дефинисан пик. Фреквенца овог пика представља карактеристичну фреквенцу свих испитиваних амилаза. Постоји зависност између вредности амплитуде на карактеристичној фреквенцији и активности биомолекула. Даљом математичком обрадом, метода је дала предлог за минималне промене у структури природног производа којима се омогућава повећана ефикасност ензима. Ове минималне промене могуће је извести најједноставнијом методом генетског инжењерства - методом тачкасте мутације. Мутације су предложене за α-амилазе из следећих извора: *Aspergillus oryzae, Bacillus amyloliquefaciens* и јечам.

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