УДК 577.15.02

ДОКАЗАТЕЛЬСТВО КАТАЛИТИЧЕСКОЙ АКТИВНОСТИ ИСКУССТВЕННО СИНТЕЗИРОВАННЫХ ПЕПТИДОВ, СООТВЕТСТВУЮЩИХ КОНСЕРВАТИВНЫМ АМИНОКИСЛОТАМ

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Работа посвящена получению комбинаторных библиотек в воде и системах обращенных мицелл, а также оценке каталитической активности последних. На основании описанных биоинформационных подходов для синтеза полипептидов были выбраны аминокислоты: Gly, Asp, His, Arg и Leu. Полипептиды синтезировали карбодиимидным методом. Активность определяли по возрастанию величины оптического поглощения относительно контрольного образца, не содержащего аминокислот, через 15 мин после начала реакции. Использовали субстраты ABTS, катехин, пирогаллол (для оксидазной активности), ABTS/H_2O_2 (для пероксидазной активности) и NIPAB (для гидролазной активности) в присутствии и в отсутствие сульфатов Zn^{2+}, Cu^{2+}, Mn^{2+} и гемина. Получено доказательство каталитической активности в синтезированных комбинаторных библиотеках. Наличие полипептидных цепей подтверждено методом электрофореза.

INTRODUCTION

The highly effective catalysis by biological enzymes has inspired many a scientists to synthesize “artificial enzymes”. The objective of designing artificial enzymes is to mimic and extend the most exciting aspects of enzyme chemistry: high rate accelerations and high selectivity in aqueous solutions. The most interesting systems due to their catalytic properties are the ribonuclease, peroxidases, cytochrome P450 and cytochrome-c oxidase [1].

The design of artificial enzyme is based on the knowledge about the structure, architecture and functional properties of biological enzymes. It is well known that the enzymes contain a binding site and a catalytic site consisting of two or more catalytic amino acid groups. Using the catalytic groups, the reactions that would normally require two steps are carried out in one simultaneous step with an unstable intermediate.

There are at least two strategies to design artificial enzymes:

A) *Introduction of the functional catalytic groups in the macrocyclic structures.* For example, functionalized cyclodextrins as cyclic glucose oligomers, capable of forming inclusion complexes with various hydrophobic guest molecules (e.g., metalloporphyrins as coenzyme factor or imidazole groups as analog of histidine formed catalytic site), were prepared to mimic the catalytic systems such as ribonuclease A, cytochrome P450 and cytochrome-c oxidase [2]. A manganese porphyrin epoxidation catalyst, when placed in the cavity formed by the directed assembly of zinc porphyrin molecules acted as an artificial enzyme [3].

B) *Chemical and biological synthesis of peptide and protein analogues.* This line is a very interesting research area. Hellinga and his colleagues [4] used computational design methods to transform a non-catalytic, a ribose-binding protein into an enzyme known as triose phosphate isomerase [4]. Beginning with the ribose-binding protein, the biochemists used their computational methods to redesign the active site of the protein so that it would bind both the initial substrate and the product molecule. Computers are widely employed as tools in modern biochemistry for designing artificial enzymes and for various other purposes [6]. These computational methods involve basically “mutating” a protein *in silico* by altering its individual amino acids to work toward a protein whose active site has the three-dimensional shape and chemical binding properties that fit a specific target molecule. The methods not only generate an array of candidate mutated proteins, which can be vast, but also narrows down those arrays to a manageable number, which can be synthesized and tested in the laboratory. However, these artificial enzymes are frequently a thousand times less active than the natural enzyme.

Chemical synthesis which involves changes, deletions or substitutions may lead to potent new artificial enzymes. Today, the method of choice for synthesis of peptides and peptide analogues is the solid-phase method [5].

At present, bioinformatics and molecular modeling are the popular approaches used for the chemical and biological design of artificial enzymes. The bioinformatics methodology is based on the analysis of nucleotide and protein sequences [7]. The informational entropy (Shannon entropy, H_j) is a very convenient function for comparison of related proteins with distinct amino acid sequences[8].

Varfolomeev et al. [7, 8] used the bioinformatic approach to deduce the "conservative residues" in each of the related enzymes and ranked amino acid conservatism for overall enzymatic catalysis.Comparison and multiple alignments of amino acid sequences of a representative number of enzyme families demonstrated the existence of certain positions of amino acid residues which are permanently reproducible in all members of the whole family. For each amino acid, they determined its frequency as the conservative element (H_j 0), normalization on total number of conservative positions for all amino acid residues in these families. The comparison of enzyme amino acid sequences revealed that glycine (Gly) and aspartic acid (Asp) are the most frequently recognized absolute conservative residues [8]. Frequency of Gly as the most conservative residue is 37%. Varfolomeev et al. [8] observed that glycine is a unique amino acid possessing the highest possibilities for rotation along C–C and C–N bonds of the polypeptide chain. They proposed that conservative fixation of the glycine residues in polypeptide chains of related enzymes provided a possibility for directed assembly of amino acid residues into the catalytic subsite structure with a known conformational mobility of the protein and the active site.

Asp is the second important conservative residue and has a frequency of 12.9%. The sum of Gly and Asp represents about 50% of all conservative residues recognized. Varfolomeev et al. [7, 8] revealed that carboxyl group of aspartic acid is the most frequently employed nucleophilic (in deprotonated form) and electrophilic (in protonated form) agent involved in activation of molecules by the mechanism of general basic- and acidic- catalyses in the catalytic sites of enzymes.

Estimation of the frequency of amino acid residues as conservative elements during multiple alignment of amino acid sequences in enzymes reveled that Gly, Asp, Cys, His, Pro and Arg (in order of reported values) are the most frequently conservative residues in enzymes. They represented about 74% of all conservative residues in the enzymes, whereas Met and Ile represent the most variable elements in the amino acid sequences [7]. The most conservative residues are separated principally into two different groups: 1) residues involved in substrate activation and acting as acids and bases (Asp, His and Arg); 2) residues forming active site architecture (Gly, Cys, Pro). Thus, the bioinformatic approach allows recognition of side chains of amino acid residues forming a catalytic site of the enzymes, which are responsible for nucleophilic/electrophilic substrate conversion.

Further Varfolomeev et al. [7] analyzed the frequency of amino acid residues in proteins in the Swiss-Prot database. It was demonstrated that a total frequency of conservative residues in enzymes completely differed from total frequency of amino acid residues in proteins. Leu (9.53%) was the most frequent residue, whereas Gly (6.85%) and Asp (5.25%) were fourth and tenth frequent residues, respectively.

In this study, the amino acids for polypeptide synthesis were selected based on the above bioinformatic approaches [7, 8]. Gly was selected as it was the most important residue for active site architecture, Asp, His and Arg, were selected as they were involved in substrate activation and Leu as the most frequent residue in proteins. The chemical synthesis of polypeptides was performed by carbodiimide method [9]. The objective of this study was to obtain the combinatorial libraries in water and reverse micellar systems and to evaluate their catalytic activity using the reverse micelles as micro-reactors under size and conformational restrictions.

The reverse micellar system consisted of tiny water droplets stabilized by surfactants in a bulk water-immiscible, organic solvent [10]. The solutions are optically transparent and it is known that almost any enzyme can be solubilized in reverse micelles without loosing its specific activity [11]. Reverse micelles provide a unique microenvironment which may be exploited to achieve active conformation of synthesized polypeptides.

MATERIALS AND METHODS

The following reagents, *viz.*, arginine, AOT (dioctyl sulfosuccinate sodium salt), ABTS (2,2′-azino-bis(3ethylbenzthiazoline-6- sulfonic acid), NIPAB (6-nitro-3-(phenylacetamido) benzoic acid), hemin were purchased from Sigma Chemical Company (USA). Histidine, glycine, hexane, catechol were purchased from Jalmek (Mexico). Leucine, hydrogen peroxide and sulfate salts of Zn^{2+}, Cu^{2+} and Mn^{2+} were from Analytyka (Mexico). Pyrogallol was from MP Biomedicals (Germany), 1,3-dicyclohexyl-carbodiimide (CC) was from Aldrich Chemical Company, Inc. (USA). Aspartic acid was synthesized by r. Yolanda

Table 1

Quantity of amino acids and DCC applied in different synthesis assays

Amino acid (AA)	Quantity used in the assays in water and micellar medium (solubility of AA in 5 ml of PBS) Assay A	Quantity used in the assay in micellar medium (solubility of AA in 10 ml of micellar medium) Assay B	Quantity used in the assay in micellar medium (solubility of AA in micellar medium at higher DCC concentration) Assay C
Glycine	5.36×10^{-4} mol	1.788×10^{-5} mol	1.788×10^{-5} mol
Aspartic acid	3.75×10^{-5} mol	3.759×10^{-5} mol	3.759×10^{-5} mol
Histidine	9.6×10^{-4} mol	2.577×10^{-5} mol	2.577×10^{-5} mol
Arginine	1.85×10^{-4} mol	9.492×10^{-5} mol	9.492×10^{-5} mol
Leucine	2.5×10^{-4} mol	5.586×10^{-5} mol	5.586×10^{-5} mol
DCC	1.968×10^{-3} mol	2.3202×10^{-4} mol	1.968×10^{-3} mol

Garza Garcнa, Biosynthesis Laboratory of the epartment of Biotechnology of this University. All regents were of analytical grade.

Peptide synthesis in water environment

To obtain the combinatorial library, the concentration of amino acids in this study was selected based on their solubility limit in 5 ml of 0.1 M phosphate buffer pH 8 (PBS) at ambient temperature (22°C). A solution of glycine, aspartic acid, histidine, arginine and leucine was prepared in 50 ml of PBS and equal moles of CC were added (Assay A). The moles quantities of amino acids and CC are presented in the Table 1. The reaction was carried out at ambient temperature (13–29°C) for 7 days under agitation at 100 rpm. After 7 days, the reaction mixture was precipitated with cold acetone.The obtained precipitate was completely dissolved in 50 ml of the reverse micellar medium containing hexane-AOT (0.05 M) with 0.5 ml of 0.1 M phosphate buffer (pH 8.0). This solution was employed for activity screening.

For the control activity measurements, same procedures without amino acids were used.

Peptide synthesis in reverse micellar medium

The conditions of the synthesis of polypeptides in reverse micelles are given in Table 1. Instead of PBS, 50 ml of reverse micellar medium was used. The resultant reaction mixture was not homogeneous. It was saturated with amino acids, which did not dissolve completely in applied media and equal moles of CC (Assay A). After 7 days of the reaction, the insoluble fraction was separated by filtration using 0.45 m m Millipore filter. The filtrate was used for activity screening.

The same assay was carried out using amino acids at their limit of solubility in 10 ml of AOT-hexane-PBS system at ambient conditions and equal quantity of CC moles was added (Assay B of Table 1). The obtained reaction mixture was optically transparent due to complete solubilization of all reagents.

The conditions of Assay C (Table 1) were almost similar to the Assay B, except that the CC concentration was same as in Assay A.

In all assays, the reverse micelle mixtures without amino acids were used as controls in activity screening.

Activity measurements

All spectrophotometric measurements were carried out in CINTRA-20 spectrophotometer.

To 2 ml of reverse micellar medium obtained in the previous step, 0.01 ml of buffer or 0.2 mM cofactor solution in PBS was added. The reaction was initiated with the addition of 0.01 ml of 2 mM substrate. The absorbance was read immediately. The measurement of absorbance was repeated 15 min after initiation. The activity was expressed as increase in absorbance with reference to control without amino acids.

Zn^{2+}, Cu^{2+} and Mn^{2+} sulfates were used as cofactors. Hemin was added as cofactor in ABTS/peroxide initiated reactions. ABTS, ABST/H_2O_2, NIPAB, catechol and pyrogallol were used as substrates, in the presence and absence of cofactors. The absorbance of the reaction mixtures was monitored at 405, 405, 420, 420 and 400 nm respectively for theses substrates. ABTS, catechol,

pyrogallol were used as the substrates for oxidase activity measurement [12–14] and NIPAB as the substrate for determination of hydrolase activity [15].

The activity measurements were carried out at different time of the polypeptide synthesis reaction by means of ($ABTS/Zn^{2+}$) system.

The kinetic measurements were performed for 25 min under same conditions using combinatorial library of assay A and ($ABTS/Zn^{2+}$) or ($ABTS/Cu^{2+}$).

All measurements were done twice. The average values of absorbance change are reported. The standard deviations were not more than 15%.

Electrophoretic separation of the combinatorial library synthesized in assay A by means of reverse micellar system

The reaction mixture was first concentrated by hexane evaporation at room temperature. The residue was dissolved in 2 ml of water and then precipitated with a final concentration of 2% trichloro-acetic acid. The precipitate was washed twice with water and then dissolved in 0.5 ml phosphate buffer (0.1 M; pH 8). Twenty microliters of the sample were applied on 10% sodium dodecyl sulphate-polyacrylamide (SS-PAGE) gel electrophoresis system [15] employing Tris-glycine buffer at pH 8.5. After 3 hours of electrophoresis at 100 V constant voltage, the gel was removed from the cassette, fixed in acetic acid: methanol: water (10:45:45) mixture and stained with Coomassie brilliant blue R dye for 2 h. After detaining overnight, the gel was scanned in a flatbed scanner and the molecular weight of the bands was approximated basing on color bands of standard protein markers (K-12 Marker, Invitrogen, CA, USA).

RESULTS AND DISCUSSION

In the present study, five amino acids glycine, aspartic acid, histidine, leucine and arginine were selected by bioinformatic approaches for chemical synthesis of polypeptides by carbodiimide method. The solubility of selected amino acids in 5 ml of PBS was estimated to define the concentrations that may be used for obtaining of the combinatorial libraries. These results corresponding to assay A are presented in the Table 1.

Initially, the synthesis was carried out in the PBS (aqueous medium) as well as in reverse micellar medium (assay A). In both cases, the activity was determined in reverse micelle systems.

In the present study, amino acids cysteine and proline were not employed, because of these conservative amino acids are responsible for formation of bonds to maintain the enzyme conformation [7, 8]. It has been proposed that the micellar system is an appropriate substitute for these amino acids to restrict the size and conformation of synthesized polypeptides.

The characteristics of micellar medium employed during the entire study in the synthesis and activity measurements are described in the Table 2. The W_0 (the molar ratio of water to surfactant concentration in reverse micelle system) values were different in the synthesis process and in the activity assay. In the synthesis PBS was added for regulation of pH of the medium at 8, whereas in activity assay, the substrate and cofactors were also dissolved in aqueous medium.

Based on the data reported for reverse micelles of AOT in different organic solvents (cyclehexane, heptane, isooctane, hexane), Mat (1994) correlated the hydrodynamic radius of the water nucleus (r_c) as a function of W_0: r_c (nm) = 0.389 + 0.155 W_0. Using this equation, the radius (r_c) of water nucleus for both micellar systems was estimated. It increased from 2.11 to 3.83 nm, respectively for synthesis and activity assay (Table 2).

Considering that the surfactant is located in the water/hexane interface and that the surfactant and water are homogeneously distributed in reverse micelles, the aggregation number (the number of surfactant molecules for each micelle, N_{agg}) was determined using the polynomial function $N_{agg} = 32.1 - 1.25\ W_0 + 0.873\ W_0^2$ [18]. The N_{agg} value increased from 126 to 435 (Table 2).

It is known that the reverse micelles are kinetically active systems, which may exchange the entrapped ma-

Table 2

Characteristics of micelle systems applied in synthesis reaction and in activity measurements

Assay	$W_0 = H_2O/AOT$	Hydrodynamic radius of the watery nucleus, nm $r_c = 0.389 + 0.155\ W_0$	Number of surfactant molecules for each micelle $N_{agg} = 32.1 - 1.25\ W_0 + 0.873\ W_0^2$
Polypeptide synthesis	11.11	2.11	125.97
Activity	22.22	3.83	435.35

Table 3

Change of absorbance (Δ A) after 15 min in the activity screening assay using combinatorial libraries obtained in water and micellar medium in assay A

Substrate	Cofactor	Δ A in library from water medium		Δ A in library from micellar medium	
		In the presence of AA	Control	In the presence of AA	Control
ABTS	Zn^{2+}	0	0	0.250	–0.059
	Cu^{2+}	0.04	0	–0.037	–0.085
	Mn^{2+}	0	0	0.065	–0.143
	Hemin	0	0	–0.309	–0.076
	Without cofactor	–0.103	0	–0.143	–0.073
ABTS/ H_2O_2	Zn^{2+}	–0.008	0	–0.065	–0.15
	Cu^{2+}	–0.063	0	–0.033	–0.102
	Mn^{2+}	–0.007	0	0.063	–0.016
	Hemin	0.025	0	–0.251	–0.0845
	Without cofactor	0	0	–0.164	0.0705
Catechol	Zn^{2+}	0.031	–0.003	–0.094	–0.009
	Cu^{2+}	0.014	0.016	0.071	0.326
	Mn^{2+}	0.002	0.029	–0.194	–0.088
	Hemin	0.028	0.048	–0.096	0.082
	Without cofactor	0.027	0.014	0.044	–0.167
NIPAB	Zn^{2+}	0.013	0.063	–0.114	0.002
	Cu^{2+}	–0.005	0.003	–0.001	0.006
	Mn^{2+}	0	0	0.060	–0.007
	Hemin	0	0	–0.006	–0.118
	Without cofactor	–0.009	0	0.016	–0.048
Pyrogallol	Zn^{2+}	ND	ND	0.088	–0.166
	Cu^{2+}	ND	ND	–0.084	0.165
	Mn^{2+}	ND	ND	–0.070	0.143
	Hemin	ND	ND	–0.044	0.098
	Without cofactor	ND	ND	–0.0705	0.088

ND – not determined. Values in bold and underlined correspond to the positive response.

terials among them. This depends on the micelle size (which is proportional to W_0) and temperature. The second order constant for the kinetics of material exchange is 10^6 to 10^8 $M^{-1}\cdot s^{-1}$, which is two to four orders of magnitude lower than the collision rate among the particles [18].

Thus, in the first step of the present study, two different combinatorial libraries (assay A) were obtained using water and micellar medium. The primary evaluation of activity was performed visually. Change in color was observed only in the library synthesized in micellar system using high concentration of ABTS as substrate and Zn^{2+} as the cofactor. Spectrophotometric measurements were used for accurate estimation of the catalytic activity.

The catalytic activity of both libraries in the presence of different substrates and cofactors are presented in Table 3. The presence of activity was defined as an increase in absorbance compared to control performed under the same conditions of activity assay (i.e. substrate and cofactor concentrations, temperature, pH, W_0, etc.) using the systems obtained on the synthesis procedure but without amino acids.

Table 4

Evaluation of oxidase activity in the combinatorial library obtained in micellar medium (repetition of assay A)*

Substrate	Cofactor	Δ A in library from micellar medium	
		In the presence of AA	Control
ABTS	Zn^{2+}	<u>0.076</u>	<u>–0.005</u>
	Cu^{2+}	–0.005	–0.006
	Mn^{2+}	<u>0.153</u>	<u>0.013</u>
	Hemin	–0.086	–0.002
	Without cofactor	–0.112	0.002
Pyrogallol	Zn^{2+}	0.070	–0.021
	Cu^{2+}	0.133	–0.002
	Mn^{2+}	0.003	–0.026
	Hemin	– 0.339	–0.022
	Without cofactor	–0.345	–0.006

*Values in bold and underlined correspond to the positive response.

The evidence of the oxidase catalytic activity was obtained in the combinatorial library synthesized in the aqueous medium. Oxidase activity was demonstrated in the presence of ABTS and Cu^{2+}, catechol with Zn^{2+} and without ions, and peroxidase activity in the presence of ABTS, H_2O_2 and hemin as cofactor. In the other reactions, the absorbance decreased or remained unchanged and sometimes was greater in control without amino acids than in the system with polypeptides. The increase in absorbance in reactions with control implied the existence of unspecific transformation of substrates under the conditions of experiment.

Oxidase activity was also demonstrated in the reverse micelle synthesis. In this case, a greater number of positive responses were obtained (Table 3). In addition to the substrates listed above, pyrogallol was also used to extend the variety of the substrates. In comparison with the first library, the positive responses exhibited by the ions were different for same substrates. Moreover, the hydrolase activity was detected using NIPAB as substrate.

Higher increase in absorbance was observed in the reaction with ABTS and Zn^{2+}, which corresponded to the visual evaluations performed previously. Hence this reaction was chosen as an indicator for the presence of oxidase activity in the subsequent experiments.

Table 4 shows the results obtained after repetition of the synthesis under the similar conditions of reverse micelle system. The response obtained in the presence of ABTS was similar to the values described in Table 3. In the case of pyrogallol, the oxidase activity was also observed in the presence of other ions (Cu^{2+} and Mn^{2+}) and actual values differed from the reported values in Table 3. This could be attributed to the partial reproducibility of synthesis conditions (temperature, agitation, etc.).

The kinetic curves obtained in the reaction ABTS/Zn^{2+} and ABTS/Cu^{2+} are presented in Fig. 1. An initial lag period was observed in the ABTS/Zn^{2+} reaction, which subsequently showed an increase in absorbance, while it was decreased in control. The yield of substrate transformation was calculated considering the major increase of absorbance, the coefficient of extinction was equal to 36800 M^{-1} cm^{-1} [13] and at an initial concentration of 2 mM of ABTS. The yields were from 60% to 81% in different assays performed for 25 or 60 min with repeatedly synthesized libraries. In the presence of Cu^{2+}, absorbance decreased both in the control without amino acids and in the library synthesized from amino acids.

The effect of storage time of the micellar system at ambient conditions was studied by monitoring oxidase

Table 5

Monitoring of oxidase activity during different days of storage of reaction mixture at room temperature

Reactor	Day of reaction									
		0	3	5	7	9	19	21	28	40
With AA	ΔA	–0.198	–0.326	–0.197	0.18	0.267	0.076	0.079	0.035	–0.098
Control	ΔA	–0.136	–0.225	–0.234	–0.056	–0.067	–0.005	–0.093	–0.010	–0.096

activity using the ABTS/Zn^{2+} reaction and the results are presented in Table 5.

The higher increase in absorbance of the library synthesized from amino acids than that of control was detected only on the seventh day. On this day the reactors were removed from agitator and stored under ambient conditions without agitation. An increase in activity was subsequently observed on 9th day, and continued to be detected until 28th day. Thereafter, a decrease in activity was noticed and no activity could be detected after forty days (Table 5). Formation of white precipitate was observed during the storage and the samples were filtered at the time of assay.

The obtained results demonstrated that the presence of activity is related to the process of the synthesis, which require a minimum of one week. The activity was variable and depended on the synthesis reaction time. The CC or unreacted soluble amino acids were not removed from the medium and the reaction of the synthesis of peptide bonds or other lateral reactions may have taken place continuously.

The combinatorial library obtained in assay A by means of reverse micelle system was separated by SS-PAGE electrophoresis. It demonstrated the presence of 2 separated polypeptides bands and the wide peptide band (Fig. 2). The molecular weights of separated polypeptides were between 55.4 and 66.3 ka, and a peptide band was also located between 6 and 14 ka molecular weight markers (Fig. 2). The aqueous solution of amino acids or mixture obtained in control synthesis without amino acids did not demonstrate any bands. The obtained results confirmed the presence of polypeptides in the performed reaction.

To exploit the advantage of combinatorial chemistry, assay B and assay C were carried out parallely to assay A (Table 1). The results of activity measurements are given in Table 6. The results of the system corresponding to assay A are similar to that reported in the Tables 3 and 4. However, decrease of amino acid concentrations in the assay B and C prompted a difference in the positive responses with the applied ions. These combinatorial libraries in the assays B and C demonstrated activity in presence of Cu^{2+} ions, which was not detected

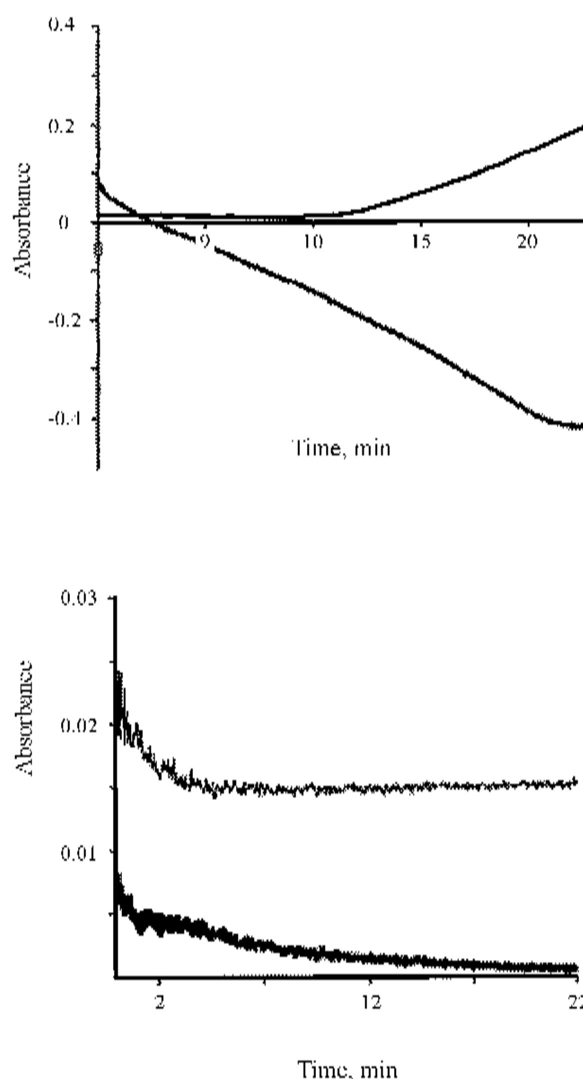

Fig. 1. Kinetics of ABTS oxidation by combinatorial library of assay A synthesized in micellar medium: Top – in the presence of Zn^{2+} as cofactor; Bottom – in the presence of Cu^{2+} as cofactor (The thin top lines correspond to sample with amino acids and the thick bottom lines to control without amino acids)

Table 6

Comparison of oxidase activity in the combinatorial libraries obtained in the micellar system under conditions of assay A, B and C

Substrate	Cofactor	Δ A in library from micellar medium (Assay A)		Δ A in library from micellar medium (Assay B)		Δ A in library from micellar medium (Assay C)	
		With AA	Control	With AA	Control	With AA	Control
ABTS	Zn^{2+}	0.024	0.013	0.02	0	0.109	0.013
	Cu^{2+}	0.005	0.028	0.244	0	0.065	0.028
	Mn^{2+}	0.038	0.030	–0.103	0.008	–0.031	0.030
	Without cofactor	0.009	0.128	–0.028	–0.019	0.019	0.128
Pyrogallol	Zn^{2+}	0.135	–0.019	–0.014	–0.064	0.127	–0.019
	Cu^{2+}	0.097	–0.090	–0.013	0.021	–0.007	–0.090
	Mn^{2+}	0.222	–0.086	–0.248	0.149	0.172	–0.086
	Without cofactor	–0.006	0.002	0.362	–0.014	0.074	0.002

in the same reaction with the library obtained in assay A. The activities were not observed in the presence of Mn^{2+}. There was difference in pyrogallol oxidation in both assays as well as in comparison with assay A. The greater unspecific oxidation of this subtrate was detected using library from assay B in the presence of Cu^{2+} and Mn^{2+}. Zn^{2+} did not provoke the oxidation of pyrogallol and only without ions the increase in absorbance was determined.

Assay C differed from assay A only on amino acid concentrations, but not in CC, the obtained combinatorial library demonstrated the tendencies to transform ABTS as observed in the case of assay B. However, with pyrogallol, the response was more similar to that observed in assay A, except in the reaction carried out without ions and in the presence of Cu^{2+}. These results demonstrated a perspective on the application of combinatorial chemistry approaches to enrich the possible combination of polypeptides to give a variety of responses.

Combinatorial chemistry or "high-throughput experimentation", if applied in a smart way can lead to a wide range of highly reproducible and comparable results. A new line of polymer science can be developed, eventually leading to some kind of "material informatics" in combination with theory, prediction and molecular modeling. This could allow "design" of new catalyst materials, based on a detailed understanding of polymerization processes, catalyst function, structure-prop-

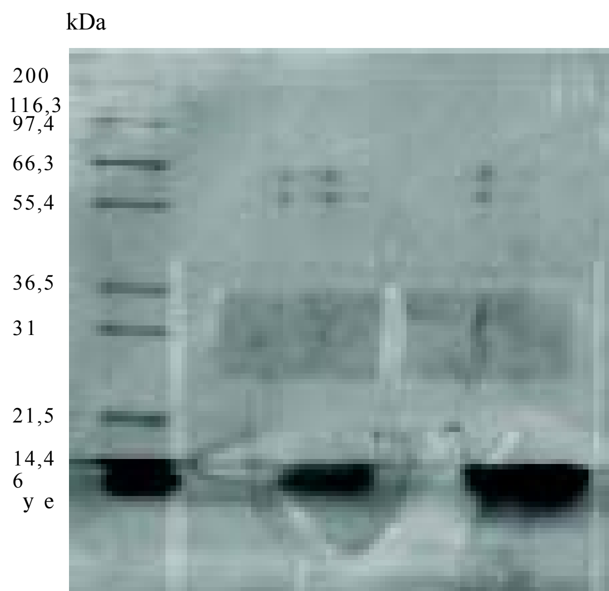

Fig. 2. Electrophoretic separation of the polypeptides of combinatorial library synthesized by means of reverse micelle system in assay A

erty relationship and correlation of molecular and nano structure and function with macroscopic features. From the academic standpoint the obtained results represent a new step in the understanding of protein structure function relationships as well as experimentally demonstrated the important role of conservative amino acids in the principles of catalytic polypeptide properties.

ACKNOWLEDGMENTS

The authors wish to express their sincere appreciation to Dr. Varfolomeev S.D. for his interesting conference during his visit to Mexico and as well as for his helpful suggestions during the development of this project; to Dr. Jorge Romero García and Dr. Yolanda Garza García for technical assistance. The present research was supported by the grant SEP-CONACyT 42522.

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Поступила в редакцию 01.12.05

EVIDENCE OF CATALYTIC ACTIVITY OF POLYPEPTIDES ARTIFICIALLY SYNTHESIZED FROM CONSERVATIVE AMINOACIDS

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The objectives of this study were to obtain the combinatorial libraries in water and reverse micellar systems and to evaluate their catalytic activity applying the reverse micelles as micro-reactors defined the restrictions of size and conformation. The amino acids, viz., Gly, Asp, His, Arg and Leu were selected for polypeptide synthesis based on the reported bioinformatic approaches. The polypeptides were synthesized by carbodiimide method. The activity was defined as increase in absorbance after 15 min of reaction with reference to control without amino acids using ABTS, catechol, pyrogallol as the substrates for oxidase activity, ABTS/H_2O_2 as the substrates for peroxidase activity and NIPAB as the substrate for hydrolase activity, with and without Zn^{2+}, Cu^{2+}, Mn^{2+} sulfates and hemin. The evidence of catalytic activity was observed in the synthesized combinatorial libraries. The presence of polypeptide chains was demonstrated by electrophoresis.