

---

REVIEW

---

## D-Amino Acid Oxidase: Structure, Catalytic Mechanism, and Practical Application

V. I. Tishkov\* and S. V. Khoronenkova

Department of Chemical Enzymology, Faculty of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russia;  
fax: (7-095) 939-2742; E-mail: vit@enz.chem.msu.ru; tishkov@pochta.ru

Received July 15, 2004

**Abstract**—D-Amino acid oxidase (DAAO) is a FAD-dependent enzyme that plays an important role in microbial metabolism, utilization of endogenous D-amino acids, regulation of the nervous system, and aging in mammals. DAAO from yeasts *Rhodotorula gracilis* and *Trigonopsis variabilis* are used to convert cephalosporin C into 7-aminocephalosporanic acid, the precursor of other semi-synthetic cephalosporins. This review summarizes the recent data on the enzyme localization, physiological role, gene cloning and expression, and the studies on the enzyme structure, stability, catalytic mechanism, and practical applications.

**Key words:** D-amino acid oxidase, cloning, expression, stability, protein engineering, *Trigonopsis variabilis*, *Rhodotorula gracilis*

Flavoproteins include numerous groups of enzymes distinct in structure and function. They catalyze the majority of key biochemical conversions [1, 2] and represent the most widely studied family of proteins. In contrast to dehydrogenases, which catalyze the interconversion of dinucleotides, NAD<sup>+</sup> and NADP<sup>+</sup>, from oxidized form to the reduced one and vice versa, the FAD molecule plays the role of a prosthetic group and its reduction state, oxidized as a rule, is the same in the end of a catalytic act as it was in the beginning. Among the most intensely studied and actively used in practice FAD-containing oxidoreductases, we note glucose oxidase [3], lactate oxidase [4], xanthine oxidase [5], cytochrome P450 reductase [6], and cytochrome *b*<sub>5</sub>-reductase [7], etc.

One of the FAD-dependent conversions that occur in living cells is oxidative deamination of D-amino acids yielding the corresponding  $\alpha$ -keto acids. This reaction is catalyzed by a specific enzyme, D-amino acid oxidase (EC 1.4.3.3, DAAO). Although DAAO was discovered in mammalian tissues in the 1930s [8], the enzyme never attracted serious attention until the 1980s. Intense studies of DAAO in the last 20 years were inspired by the fact that

yeast enzymes, from *Rhodotorula gracilis* and *Trigonopsis variabilis*, can efficiently oxidize cephalosporin C. This is the first step of a two-enzyme process of biocatalytic conversion of the natural antibiotic cephalosporin C into 7-aminocephalosporanic acid (7-ACA), which is the key precursor of many other semi-synthetic cephalosporin antibiotics of different generations [9, 10]. Currently DAAO is also used for the production of  $\alpha$ -ketoacids [11, 12] and unnatural L-amino acids [13] and in biosensors [14–16].

The most studied enzyme among D-amino acid oxidases till the mid 1980s – beginning of 1990s was the enzyme from pig kidney, the first DAAO for which crystal structure was refined by X-ray analysis [17, 18]. The systematic studies of yeast *R. gracilis* DAAO by L. Pollegioni's research group in 1990s in combination with the data on pig kidney enzyme provided the necessary background for DAAO to be a model enzyme for basic mechanistic studies of FAD-dependent oxidases [19, 20].

A recent review on the structure and mechanism of action of DAAO was published four years ago [19]. Some useful information on L- and D-amino acid oxidases can be also found in a recently published book [21]. Recent years are rich in new publications on DAAO studies: novel DAAO have been cloned or found in the genomes of prokaryotes and eukaryotes, novel efficient expression systems for recombinant DAAO in yeast strains and *Escherichia coli* have been developed, important data has been obtained by site-directed mutagenesis, newly

---

**Abbreviations:** DAAO) D-amino acid oxidase; DASPO) D-aspartate oxidase; pkDAAO) pig kidney D-amino acid oxidase; RgDAAO) D-amino acid oxidase from *Rhodotorula gracilis*; TvDAAO) D-amino acid oxidase from *Trigonopsis variabilis*; 7-ACA) 7-aminocephalosporanic acid.

\* To whom correspondence should be addressed.

refined DAAO crystal structures have been added to PDB, and a new highly efficient biocatalyst with immobilized DAAO has been prepared. This review is an attempt to generalize and make systematic all currently available data on DAAO and to include the new aspects of DAAO research that were not included in the previous reviews.

### LOCALIZATION AND BIOLOGICAL ROLE OF DAAO

The enzyme was previously thought to exist in eucaryotic cells only. However, the recently sequenced and annotated bacterial genomes (*Mycobacterium tuberculosis* [22, 23], *Streptomyces avermitilis* [24], *Acinetobacter* sp. IP I-671 [25] and sp. ADP1 [26], etc.) show the presence of bacterial DAAO. Currently the enzyme is known to be present in mollusk, fish, reptile, amphibia, insects, birds, mammals (kidney, lungs, brain) [27], and different microorganisms: alga *Chlorella vulgaris* [28], fungi *Neurospora crassa* [29], *Cephalosporium acremonium* [30], *Fusarium solani* (*Nectria haematococca*) [31], yeast *T. variabilis* [32, 33], *Candida tropicalis* [34], *R. gracilis* (alternate name *Rhodospiridium toruloides*) [35], *Candida boidinii* [36], bacteria *Streptomyces avermitilis* [24, 37], *Arthrobacter protophormiae* [GenBank accession AY306197], *Mycobacterium tuberculosis* [23], *Agrobacterium tumefaciens* C58 [38], etc.

The physiological role of the enzyme is diverse and not fully clarified. In microorganisms, DAAO allows exogenous D-amino acids to be used as growth substrates providing carbon and nitrogen [8, 39]. In addition, the enzyme plays an important role in defending the microorganisms against D-amino acid containing antibiotics.

In eucaryotic cells, DAAO is localized in specific organelles—peroxisomes. Such localization of the enzyme provides the efficient removal of the cell toxicant, hydrogen peroxide, produced in the course of DAAO-catalyzed reaction (detoxification occurs with the involvement of catalase) [40]. A significant amount of DAAO is found in mammalian liver, kidney, and brain cells. We note that some mammals, like pig, possess the enzyme both in liver and kidney, while others, like mice, have the enzyme in kidney only. D'Aniello et al. [41] demonstrated that mammals, including humans, possess a highly specific DAAO, D-aspartate oxidase (DASPO), in addition to the traditional DAAO, which oxidizes a wide spectrum of D-amino acids. The presence of this highly specific enzyme is justified by the fact that the classic DAAO is essentially inactive with D-aspartate. At the same time, the rate of conversion of L-aspartate in its D-isoform in mammalian cells is the highest one among natural L-amino acids. The main role of DAAO and DASPO in mammalian kidney and liver cells is detoxification of

endogenous D-amino acids accumulated in the organism in the course of racemization. An inverse relationship between the D-amino acid content in the cell and the activity of the above mentioned oxidases has been reported [42].

Accumulation of D-amino acids in mammalian cells is one of the characteristics of organism aging. The fastest accumulation of D-amino acids (D-aspartate and D-hydroxyproline especially) is observed for long-lasting tissues, e.g., dentin, teeth enamel, crystalline lens, etc. For instance, the rate of D-aspartate accumulation in lens proteins is up to 0.14% per year [43, 44]. In addition, it has been established that aging results in D-aspartate accumulation in the cerebral white matter in humans [45]. Recent years also revealed the important role of DAAO in maintaining the necessary levels of D-serine in different brain tissues. D-Serine participates in the regulation of N-methyl-D-aspartate receptors (NMDA-receptors) in the form of a free amino acid or a neuroactive peptide [46-49]. (D-Serine is produced in brain by a specific rasemase [50]). There have been some suggestions that the dysfunction of NMDA-receptors resulting from the erroneous expression of the DAAO gene is one of the possible causes of schizophrenia [51-54]. It was also shown that the activity of DAAO in malignant kidney and liver cells is much lower than in the healthy ones [55], which can be used in cancer diagnostics of these organs. Generation of cytotoxic hydrogen peroxide with D-amino acid oxidase was used to make anticancer preparations based on DAAO immobilized in polyethylene glycol [56, 57].

### BASIC PROPERTIES

The most studied enzymes are pig kidney DAAO (pkDAAO) and yeast DAAO from *R. gracilis* (RgDAAO) and *T. variabilis* (TvDAAO). The main properties of these enzymes are summarized in Table 1. The active form of pkDAAO is the monomer, but at high concentrations *in vitro* the formation of oligomeric forms is observed [19]. RgDAAO and TvDAAO function in the cell as homodimers containing two identical subunits with noncovalently bound FAD in each subunit. RgDAAO dimer is rather stable: no dissociation into subunits is observed upon dilution [58]. X-Ray analysis of a closely related enzyme, glycine oxidase from *Bacillus subtilis*, proves its tetrameric structure with three types of intersubunit contacts ("a dimer of two dimers" type) [59]. The literature data on TvDAAO provide evidence for the formation of a tetramer with molecular weight 170 kD and higher aggregates at high enzyme concentrations [33].

Mammalian and microbial enzymes differ significantly in their affinity for FAD. Microbial DAAO bind the prosthetic group in their active center much stronger than mammalian enzymes. The FAD dissociation con-

**Table 1.** General properties DAAO from different sources

Parameter	pkDAAO [19]	RgDAAO [19]	TvDAAO [19, 33]
Absorption maximum, nm			
oxidized state	274, 380, 455	274, 368, 455	272, 360, 455
semiquinone state	—	274, 370, 400, 490	—
reduced state	355, 415	350	—
Fluorescence maximum, nm	530	355, 530	355, 530
Ratio $A_{272}/A_{455}$	9.5	8.2	8.4 [19], 6.5 [28]
$pK_a$ of N(3)-H-bound FAD	9.4	10.6	10.1
Dissociation constant of FAD ( $K_d$ ), M	$2.2 \cdot 10^{-7}$	$2 \cdot 10^{-8}$	—
Benzoate dissociation constant, mM	0.002	0.245	18.8
Sulfite dissociation constant, mM	3.5	0.11	—
Number of amino acids per subunit	347	368	356
Molecular mass of subunit, kD	39.6	40.0	39.3
Isoelectric point (pI)	7.0, 7.2	7.2, 7.4, 7.8	5.1

stant from holo-form of pkDAAO is about  $2.2 \cdot 10^{-7}$  M. In the case of RgDAAO, the dissociation constant  $K_d$  is an order of magnitude smaller ( $2 \cdot 10^{-8}$  M, Table 1). The enzymes from pig kidney and yeast, in addition, differ in their inhibition by benzoate and sulfite (Table 1).

By isoelectrofocusing, TvDAAO was shown to exist as a single isoform, while pkDAAO and RgDAAO are represented in cells by two and three isoforms, respectively (Table 1). The absorbance spectrum of oxidized DAAO is typical for FAD-dependent enzymes: it has two bands in the visible region and one in the UV region. The oxidized form of FAD is fluorescent and exhibits an emission band at 530 nm (Table 1).

### PRIMARY STRUCTURE

As mentioned above, DAAO was not in the focus of intense studies till the mid 1980s – the end of 1990s. This explains why the number of amino acid sequences for this enzyme deposited in the databases in 2000 was less than ten. The search for new DAAOs of microbial origin for biotechnological purposes and the studies of the enzyme role in nerve system regulation in higher mammals resulted in a fourfold increased number of deposited sequences by mid-2004. The sequencing of the genome of pathogenic microorganisms and parasites became an additional source for DAAO sequences. For instance, DAAO and DASPO genes were found in socially important pathogens such as *Mycobacterium leprae* (GenBank Accession AL049571), *Mycobacterium bovis* (GenBank

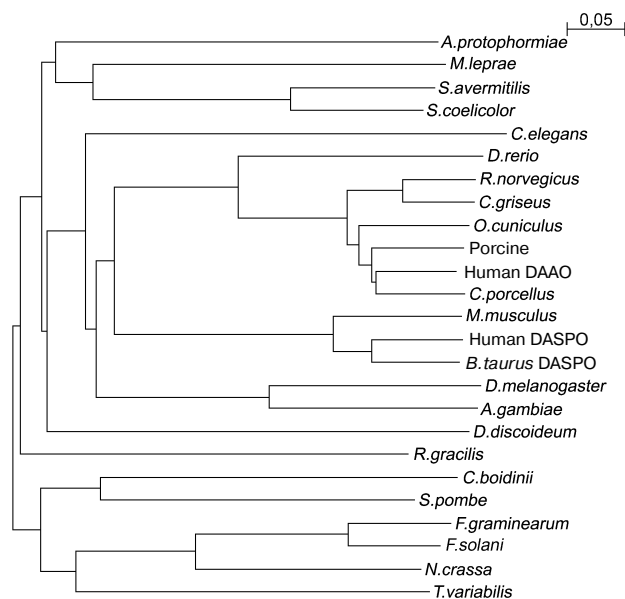
Accession BX248340), *Mycobacterium tuberculosis* [22, 23], *Neisseria meningitides* [60], and *Pseudomonas aeruginosa* [61].

We performed alignment of the known amino acid sequences of DAAO and DASPO. The most different sequences are presented in Fig. 1 in the order of decreasing homology as compared to TvDAAO. The figure also shows  $\alpha$ -helices and  $\beta$ -sheets regions in RgDAAO and pkDAAO in accordance with the refined crystal structures and in TvDAAO in accordance with computer modeling. DAAO amino acid sequences from different sources (yeast, algae, microscopic fungi, bivalve mollusk, fish, mammals) exhibit low homology score, below 30-40%. The maximum identity with TvDAAO is reported for oxidases from *N. crassa* (40%), *S. pombe* (35%), and *F. solani* fungi (36%). The low homology score between yeast TvDAAO and RgDAAO, which was only 30%, was quite unexpected. The highest homology score between TvDAAO and mammalian oxidases is observed for bovine DASPO (28%) and human DASPO (29%) (Fig. 1).

Amino acid alignment of DAAOs of different origin allows the conservative amino acids residues to be identified, as well as the sequences that form the enzyme active center. For example, the N-terminal sequence of all enzymes, GXGXXG, is characteristic for nucleotide binding domains [62] and points to the presence of the Rossmann fold in the protein structure, which is a specific combination of  $\alpha$ -helices and  $\beta$ -sheets [63]. This structural fold is responsible for the binding of the adenine moiety of  $NAD^+$  and FAD and is conserved in a large number of enzymes.

		<b>β1A</b>	<b>α1</b>	<b>β2A</b>	<b>β1B</b>	<b>α2</b>	<b>α3</b>	
T. variabilis	-----MAKIVVLCAGVACLTALQLLR-KGHEV-----TIVSEFTPGDLS-IGYSPWAGANWLT--FYDGG--KLADYDAVSPILRELARSS--PEAGIRL							85
N. crassa	-----MSITVVVLCAGVSCLTALQLAKQGGNTI-----TVVAKHMPGDYD-PEYSPFAGANVLP--MAP-----EYNRWEGETWPELKLAEFC--PEAGIHF							84
F. solani	-----MSNTIVVLCAGVSCLTALQLSKNKGNTI-----TVVAKHMPGDYD-VEYSPFAGANHSP--MATE-----ESSEWERWTYEFKRLVEEV--PEAGVHF							84
S. pombe	-----MTKENKPRIVVLCAGVCLTTAWILS-DLGLAP-RIVKIAYKTPEDRS-VEYSPWAGANFCS--ISATDD--NALRWKITYHFRFAYLAKTR--PEAGIRF							94
		<b>β3A</b>	<b>α1</b>	<b>β2A</b>	<b>α2</b>	<b>α3</b>	<b>β6B</b>	
R. gracilis	-----MHSQKRVVLCAGVCLSSALILARKGYSVH-----ILARDLPEDVSSQTFASPWAGANWTF--FMTLTDGPRQAKWEESTPKKVVLPVPTG--HAMWL							90
C. boidini	-----MGDQVVVLCAGVCLGLTYTYCLIEYAGCAPAKITIVAEFLPGDQS-TLYTSPWAGGNFSC--ISPADD--TTLAYDKFTLNLLFKIHKLGGAEKPCLDN							93
D. discoideum	-----PKINLLVLCAGVCLGLSTGIIALKSGN-----YKSVISWAKDLPNTTNSKAAALWYF--FLCNPLDLVGKWSAETMOYVKDHIIND--PKSGTIT							86
S. coelicolor	METELDDERDGVVLCAGVCLGLTTAVVLAERGR-----RVLRTREPAERTSVVAGGLWWP--YRIEP-VALAQWALRSIDVYEELAAIRPG--QVGRVM							92
H. sapiens DASPO	-----MDTARIAVLCAGVCLSTAVCISKLVP-----RCSVTIISDKFTPDTSVVAAGMLTP-HTYPTDPIHTQKQWFRETNNHFAIANSAAEGADVHL							91
A. protophormiae	-----MPTAPLRITVLCAGVCLSAHAELAAAGH-----QVTVAVDQELAEVSSVAAAIWFP--YHSEN-SPAADKLLADSLARFEQLSEHP--ETGIDL							86
M. musculus	-----MDTVCIADVLCAGVCLSTAACISQLVP-----GCTVTISDRFTPDTSVVAAGMLTP-HTYADTPVPTQKRCFRETEHLSEIAKSAEADAADVHL							91
D. melanogaster	-----MGCVLVCAGVCLGLTALQLKEFP-----TARVSVIADRFNEDTVSYVAAGLFRFGTSFMGPTQKITQQWMTDAFNWDELRRSKEAPLVCVQ							89
H. sapiens DAAO	-----MRVVVLCAGVCLSTALCIIHERYHVSIL--QPLHIKIVYADRFPLTTSVVAAGLWQP-VLS-DPNNPQEAADSSQOTDYLLSHVSHVPAENLGLFL							91
D. rerio	-----MKVCILCAGVCLSTAQSIYQHFSRV--SPLTIVYDAEFFPTLTSDGAAGLWQP-VLY-DKGIVKTEQWNKEDYLLSFINSFSPSIVKMGIFL							91
		<b>β4A</b>	<b>α1</b>	<b>β5A</b>	<b>α2</b>	<b>α3</b>	<b>α4</b>	<b>β2B</b>
Porcine	-----MRVVVLCAGVCLSTALCIIHERYHVSIL--QPLDVKVYADRFPTTSDVAAGLWQP-VYS-EPSPNPQEAENWQOTFNLYLLSHIGSPNAAMLTPT							91
O. cuniculus	-----MRVVVLCAGVCLSTALCIIHERYHVSIL--QPLDMITVYADRFPLTSDVAAGLWQP-VLS-DPSNPQEAADSSQOTDYLLSHIGSPNAAMLTPT							91
C. elegans	-----MANIPKIAITLCAGVCLSTALCIIHERYHVSIL--QPLDMITVYADRFPLTSDVAAGLWQP-VLS-DPSNPQEAADSSQOTDYLLSHIGSPNAAMLTPT							87
R. norvegicus	-----MRVVVLCAGVCLSTALCIIHERYHVSIL--QPLDMITVYADRFPTTSDVAAGLWQP-VLS-DPSNPQEAADSSQOTDYLLSHIGSPNAAMLTPT							87
M. leprae	-----MASNGQIVVLCAGVCLSTALCIIHERYHVSIL--QPLDMITVYADRFPTTSDVAAGLWQP-VLS-DPSNPQEAADSSQOTDYLLSHIGSPNAAMLTPT							87
		<b>β1C</b>	<b>β1D</b>	<b>α4</b>	<b>β1E</b>	<b>β2C</b>	<b>α5</b>	<b>α6</b>
T. variabilis	INQRSHVLKRDLPK-LEGAMS-AICQRNPWFKNVDSFEIIEDR--SRIVHDDVAYLVEFAS-VCIHTGVYLNLMSCQLSLGATVVKRVNHLKDANLLHSSGSR							186
N. crassa	QKAVLYRRAQDEAGFAGPLSDGLFVRNPWKDLPV--DYVDLP--ASEVPEGMSSASSFTS-VCINTAIYLFVLVGGQCARGVVFKRAVKHSDAAKLSHGTRGR							185
F. solani	QKSRIQRNVDTEKAQRSGFPDALSKEPWFKNMFE--DFREQH--PSEVPIGYSGCCFTS-VCINTAIYLFVLVGGQCKNGVIVKRAINDSEAKLSHAGTK							187
S. pombe	ADLRELWEYEPKHD--KIRSWNTYVR--DFKVIIP--EKDLPGEICVGHKAT-FLINAPHYLNMYKLLEAGVEFEKKELSHKEKTEVEETPEAS-							182
		<b>β6B</b>	<b>α4</b>	<b>β8B</b>	<b>β7B</b>	<b>α6</b>	<b>β1A</b>	<b>α7</b>
R. gracilis	KGTRRFAGNEDGLL--HWYKIDTP--NYRPLP--SSECPG-AIGVYDT-LSVHAPKYCCVLAELQKLGATFERRTVISEQAFDGA--							172
C. boidini	KPSTVEYDFYDGE--KVNSLKQYKL--DFKVIIP--KSELPEGVYEGISYTT-WNFNCVPLQMANFLNKRNVITIRKHLTHSSQ-AYLTVTNK-							180
D. discoideum	KKNEYIFRRPHED--PEWKPYIKSFRRAKD--ELPDGYDGYDAIDDDGFMDDMYMDLVQDFKSLGIIIEQRHVDLDRRAFPVH--							169
S. coelicolor	LEGV-LGETGLDEVDG--WAAARLPGLRAASAA--EYAG--TGLWARL-PLIDMSTHLEVRERLAAAGGTVEVDRVLDIAAAD--							168
H. sapiens DASPO	VSQWQIFQSPTEEV--PFWADVGLFRKLTPE-ELKKFP-QYVFGQAFIT-LKCECPAYLWLEKRIKSGSGWTLTRRDDWELHPS--							175
A. protophormiae	RGLNVHDLPGADR--WTRIVAGTEEAESA--DLDPGAHAGVWATV-PIITMSTYLWLRGRVEELGADFAKTVTDIAOLQKG--							166
M. musculus	VSQWQIFRSVPAAEV--PFWADVGLFRKLTPE-ELKKFP-QYVFGQAFIT-LKCECPAYLWLEKRIKSGSGWTLTRRDDWELHPS--							175
D. melanogaster	LSGYISFHTSPSIVRN--HFIEKLLPVYRRATEE-ELRLCNGWKGYSFETT-CLTESRLFLKATKKFLNCGEVVROHNSFEVFPQN--							175
H. sapiens DAAO	ISGYNLFHEAIPD--PSWKDTVLGRKLTPE-ELDMFP-DYGYGWFTS-LILEGKNYLWLTERTLTERGVKFFQKRVSEFEVAREG--							174
D. rerio	QSGYNLCAEATAD--PSFKDTVLGRKLTPE-ELDMFP-DYGYGWFTS-LILEGKNYLWLTERTLTERGVKFFQKRVSEFEVAREG--							174
		<b>β2B</b>	<b>α5</b>	<b>β1B</b>	<b>α6</b>	<b>β6A</b>	<b>α7</b>	
Porcine	VSQYNLFREAVPD--PYWKDMVLGRKLTPE-ELDMFP-DYRYGWFTS-LILEGRKYLWLTERTLTERGVKFFQKRVSEFEVAREG--							174
O. cuniculus	ISGYNLFRKAVPD--PSWKDTVLGRKLTPE-ELDMFP-DYGYGWFTS-LILEGRKYLWLTERTLTERGVKFFQKRVSEFEVAREG--							174
C. elegans	VSQGHQSDNLESLSKQQ--RAYGDIVYVFRFLDRLERLDIPEPSKHCHYTA-YASEGKNYVFLKLNLLKQIEFKQOEVTLSDAVADAG--							176
R. norvegicus	ISGYNLFRDEVPD--PFWKSTVLGRKLTPE-ELDMFP-DYGYGWFTS-LILEGKSYLWLTERTLTERGVKFFQKRVSEFEVAREG--							173
M. leprae	APAVTVGDLPGANANG--LAAKLIPDLRPADLA--DLDPGFGAGFGTM-PMIMPOYLLTLARLAAAGCELETSVRSIAEAAADT--							169
		<b>β3A</b>	<b>β1F</b>	<b>β2D</b>	<b>β2F</b>	<b>β3F</b>	<b>α7</b>	
T. variabilis	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							289
N. crassa	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							290
F. solani	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							290
S. pombe	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							280
		<b>β4A</b>	<b>β2B</b>	<b>β5B</b>	<b>β4B</b>	<b>β3B</b>	<b>α9</b>	<b>α10</b>
R. gracilis	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							270
C. boidini	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							277
D. discoideum	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							261
S. coelicolor	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							265
H. sapiens DASPO	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							268
A. protophormiae	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							264
M. musculus	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							268
D. melanogaster	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							267
H. sapiens DAAO	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							273
D. rerio	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							272
		<b>β3A</b>	<b>α8</b>	<b>β3C</b>	<b>α9</b>	<b>β1C</b>	<b>α10</b>	<b>α11</b>
Porcine	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							273
O. cuniculus	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							273
C. elegans	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							269
R. norvegicus	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							272
M. leprae	PDVIVNLCAGVCLGLGGVEDKKMYPGRGVVLRNLSLFPMASFSSTPEKENEDALYIMTR-FDG-TSIIIGGCQFQNNWSEFPDPSLTHRLSLRDLRFESTLKD-							265
		<b>β4F</b>	<b>β4A</b>	<b>β5A</b>	<b>α8</b>			
T. variabilis	-----GPLDIVRECYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							356
N. crassa	-----GIALDIVRHAYCRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							362
F. solani	-----GVKGLSVIRHAYCRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							361
S. pombe	-----GPEGAEIQCVRPRPRGGARVELD-----VVPGTSPVLVHDYCAAGCAVQYSSYCADEAISYVERALTRPNL-----							348
		<b>α11</b>	<b>β1</b>	<b>β6A</b>	<b>β5A</b>	<b>α12</b>	<b>α13</b>	
R. gracilis	TIEGLEVLRRNHYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							368
C. boidini	-----EPLHIIIRVAGRRPRRHGGPRTEAE-----VCE-EGKLTINHYGASGYCYQAGYGSYEAVALKLVNDQKVK-----							AKL 345
D. discoideum	-----RIEIGGVKYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							NKL 331
S. coelicolor	-----RVLAHLVGRPRRD-AVRERGTLPDG-----RRLVNHYHGGGAGCVTVAGCAQEAARLAS-----							320
H. sapiens DASPO	-----CNIREKYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							SNL 341
A. protophormiae	-----EVLEKHVGRPRRD-AVRERGTLPDG-----RRLVNHYHGGGAGCVTVAGCAQEAARLAS-----							326
M. musculus	-----YDIKEKYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							SKL 341
D. melanogaster	-----EIVREKYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							SKL 335
H. sapiens DAAO	-----RIIEGAEYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							347
D. rerio	-----RIVEDWYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							353
		<b>β4C</b>	<b>β1A</b>	<b>β2A</b>	<b>α12</b>			
Porcine	-----KIVGEYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							SHL 347
O. cuniculus	-----RIVGEYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							SHL 347
C. elegans	-----KIKIWEAHRPRRK-HVIREAQKRTSVG-----GSKDYMVHYHGGGAGCVTVAGCAQEAARLAS-----							334
R. norvegicus	-----RIMGELHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							SHL 346
M. leprae	-----AVIETIYHRRPREGGPRVELE-----KIPGVGVVHN-VCAAGCAVQYSSYCADEAISYVERALTRPNL-----							320

**Fig. 1.** Comparison of primary structure of DAAOs from different sources. Conserved amino acid residues are marked with asterisks, and catalytically essential residues are indicated with white semi-bold font against black background. Sequences were aligned using Clustal X (version 1.83).



**Fig. 2.** Phylogenetic tree of different DAAO. Full names of organisms (from top to bottom): *Arthrobacter protophormiae* (GenBank accession AY306197), *Mycobacterium leprae* (GenBank accession MLCOSL672), *Streptomyces avermitilis* (GenBank accession AP005027), *Streptomyces coelicolor* (GenBank accession AL939111), *Caenorhabditis elegans* (GenBank accession NM\_075090), *Danio rerio* (GenBank accession BC065882), *Rattus norvegicus* (GenBank accession AB003400), *Cricetulus griseus* (GenBank accession AB016530), *Oryctolagus cuniculus* (GenBank accession D12494), porcine (GenBank accession PIGAAOA), human DAAO (GenBank accession X13227), *Cavia porcellus* (GenBank accession AJ007710), *Mus musculus* (GenBank accession BC027312), human DASPO (GenBank accession NM\_004032), *Bos taurus* (GenBank accession BTDAPO), *Drosophila melanogaster* (GenBank accession AY071416), *Anopheles gambiae* (GenBank accession XM\_315337), *Dictyostelium discoideum* (GenBank accession AC116984), *Rhodotorula gracilis* (GenBank accession RGU60066), *Candida boidinii* (GenBank accession AB042032), *Schizosaccharomyces pombe* (GenBank accession CAB40174), *Fusarium graminearum* (Gibberella zeae PH-1, GenBank accession XM\_389828), *Fusarium solani* (Swiss-Prot accession P24552), *Neurospora crassa* (GenBank accession XM\_326412), *Trigonopsis variabilis* (GenBank accession AY514426).

Clusters of conserved amino acid residues in the 189–202, 299–312, and 326–341 regions (numbered as in TvDAAO) form the catalytic site of the active center including key catalytic residues: Asn192, Arg302, and Tyr326 (see “Kinetic Scheme and Mechanism of Action”). We note that  $\alpha$ C-atom mobility in the Arg302 is significantly restricted because of the adjacent Pro303, while Tyr326, which is responsible for the substrate access to the active center, must have a higher mobility provided by the adjacent Gly327. No high homology is observed for substrate-binding domains in DAAO primary structures. This may reflect the wide variation in the substrate specificity for the enzymes of different origin.

Many DAAOs contain a specific C-terminal sequence (Ser-(Lys/His/Arg)-Leu) (Fig. 1) which is the

first type peroxisomal signal sequence providing enzyme transport to peroxisomes [64]. The existence of three isozymes for RgDAAO reflects the presence of non-processed, half-processed, and fully processed dimeric enzyme with SerLysLeu-peptides cleaved from one or both subunits, respectively [65]. Recombinant RgDAAO expressed from the gene with the deletion of the tripeptide encoding sequence exists as a single isoform [65].

The phylogenetic tree for the DAAO and DASPO enzyme families is presented in Fig. 2. The phylogenetic analysis proves the family evolution diversity and the unusual evolution pathway for this enzyme. For instance, yeast RgDAAO and TvDAAO are much more distant than RgDAAO and mammalian DAAO. The first step of evolution divided the DAAO precursor into two groups: prokaryotes and RgDAAO, and microscopic fungi and yeast including TvDAAO. Later the first group gave rise to invertebrate and vertebrate DAAO.

## QUATERNARY STRUCTURE

Currently, X-ray structural analysis data are available for two enzymes, pkDAAO and RgDAAO. Until 2000, the protein databank had only the structures of free enzyme from pig kidney and its complexes with various inhibitors. The first pkDAAO structures were refined in 1996 by two independent groups, Japanese (PDB1AA8.ENT, free recombinant enzyme with FAD bound, 3.0 Å resolution) [17] and Italian–German (PDB1KIF.ENT, complex with benzoate, 2.6 Å resolution) [18]. Later Miura et al. refined the crystal structure of the recombinant pkDAAO complexed with another competitive inhibitor, *o*-aminobenzoate, with 2.5 Å resolution (PDB1AN9.ENT) [66]. The same year structural data on the reduced DAAO complexed with the reaction product, iminotryptophan (PDB1DDO.ENT), and with 3-methyl-2-oxovalerate (PDB1DAO.ENT) were published [67]. In 1999 the structures of free RgDAAO (PDB1COL.ENT) and its D-alanine (PDB1COP.ENT) and L-lactate complexes (PDB1COK.ENT) with the resolution 1.73, 1.20, and 1.46 Å, respectively, were added to the Protein Data Bank [68], together with the RgDAAO complex with two antranylate molecules (PDB1COIL.ENT, resolution 1.9 Å) [69].

Since 2000, two structures of pkDAAO have appeared in the PDB, the improved 2.5 Å resolution structure of free enzyme (PDB1VE9.ENT) [17] and the structure of its reduced purple intermediate complexed with D-proline (PDB1EV1.ENT) [70].

In December 2002, X-ray analysis data for another amino acid oxidase, *B. subtilis* glycine oxidase, were deposited into PDB: free enzyme (PDB1NG3.ENT) and its complex with acetylglycine (PDB1NG4.ENT) with resolution 2.3 and 2.6 Å, respectively [59]. The enzyme is a tetramer with subunit molecular weight of 42 kD. Mörtl

et al. [71] described the structure of *B. subtilis* glycine oxidase with 1.8 Å resolution, but the structure is not yet available in PDB.

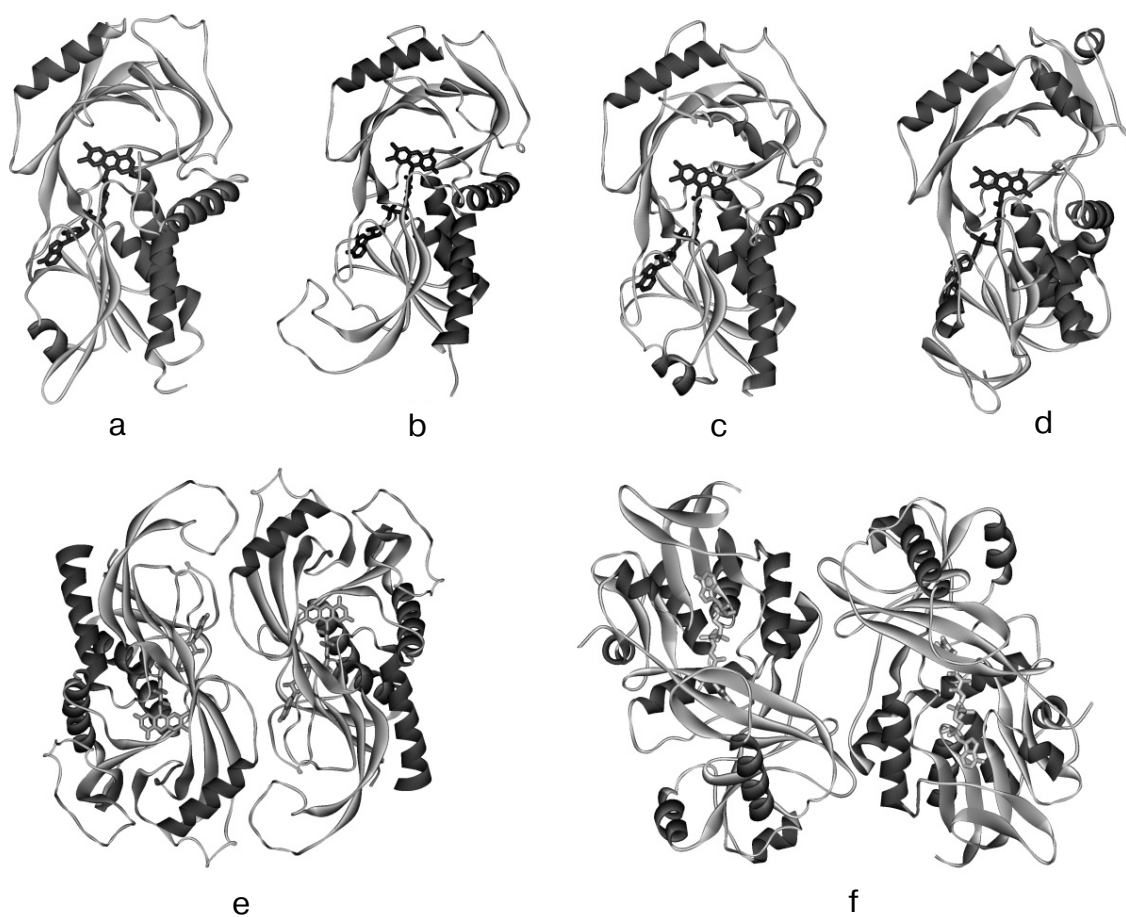
To improve kinetic properties and stability of *T. variabilis* DAAO by means of site-directed mutagenesis in our laboratory, we first performed structural modeling studies for this enzyme in collaboration with Dr. I. V. Uporov using the Insight II program (Accelrys, USA) and the refined structure of RgDAAO complex with D-alanine (PDB1COP.ENT, 1.2 Å resolution) [68] as a template.

Figure 3 presents the subunit structures for pkDAAO (a), RgDAAO (b), *B. subtilis* glycine oxidase (d), and the model structure of TvDAAO subunit (c). As seen from Fig. 3, all of the subunits have similar tertiary structures, whereas the quaternary structures for RgDAAO (Fig. 3e) and glycine oxidase (Fig. 3f) significantly differ from each other. The RgDAAO dimer is formed via “head-to-tail” contacts between the subunits (Fig. 3e) [58]. In the case of tetrameric *B. subtilis* glycine oxidase, the strongest contacts are also of “head-to-tail” type, but the subunits

are twisted against each other by 90° as compared to RgDAAO (Fig. 3f) [59].

The active center of all DAAOs and glycine oxidases is a large cavity with the upper wall formed by a β-sheet. The isoalloxazine group of FAD is located in the lower portion of the active center. The upper portion of the active center represents the substrate-binding domain, which binds and orients D-amino acids towards the isoalloxazine group of FAD in the conformation optimal for catalysis. The structure of the upper half of the TvDAAO subunit is notably closer to the structure of RgDAAO substrate-binding domain that may explain the high activity towards cephalosporin C observed for both enzymes as compared to pkDAAO (see below).

The lower half of the subunit represents the FAD-binding domain. All of the enzymes bind FAD in the open conformation (Fig. 3, a-d). In contrast to the catalytic domain, the structure of the coenzyme-binding domain in TvDAAO is very similar to that of pkDAAO, but not RgDAAO. The specific feature of the FAD-binding domain in RgDAAO is the presence of an additional loop



**Fig. 3.** Spatial structures of DAAO subunits from pig kidney (a), yeast *R. gracilis* (b), and subunit of glycine oxidase from *B. subtilis* (d), as well as model structure of subunit from *T. variabilis* (c) and 3D structures of dimeric DAAO from *R. gracilis* (e) and dimeric glycine oxidase from *B. subtilis* (f).

with 14 amino acid residues (Fig. 3b), which is clearly seen in the alignment of the enzyme amino acid sequences (Fig. 1). This loop connects the  $\beta$ F5 and  $\beta$ F6 elements of  $\beta$ -sheet (Fig. 1) and participates in intersubunit interactions in the RgDAAO dimer. It provides the correct orientation and effective interaction between the positively charged Arg305 and Arg314 loop residues of one subunit with the negatively charged Asp269, Glu275, and Glu276 residues of another subunit  $\alpha$ -helices I3' and I3'' [72]. Mutants with partial deletion of the amino acid residues from the above loop have been obtained [58, 72, 73]. The deletion of five residues appeared to be sufficient for RgDAAO to lose the ability to form a dimer. The monomeric form of mutant RgDAAO was less thermally stable than the wild-type enzyme, and the FAD-binding constant was weakened by 5-fold [72, 73]. The data prove the role of the above loop in providing RgDAAO with the dimeric structure *in vivo* and FAD with firm binding in the active center.

## DAAO GENE CLONING AND EXPRESSION

**Cloning.** The list of DAAO genes obtained by direct cloning is not impressive. The enzyme genes from human kidney [41, 74], pig [75, 76], rabbit [77], and mice [78] have been cloned by one and the same research group in 1988-1992. Since that time only four other DAAO genes have been cloned, i.e., rat (1998) [79], guinea-pig (1999) [80], hamster (2003) [81], and carp (2003) [82]. In 2001, the chromosome localization of the human and mouse DAAO genes were determined (12q23-24.1 and 5E3-F chromosomes, respectively) [83].

The first microbial DAAO gene was cloned from the microscopic fungus *F. solani* [31]. RgDAAO and TvDAAO genes were cloned in 1997-1998 simultaneously by different research groups [84-86]. The DAAO gene was also discovered in *Agrobacterium* sp. IP I-671, in the operon responsible for hidantoin utilization [87]. However, no attempts to produce the recombinant enzyme were made. The genes of the *B. subtilis* glycine oxidase [88, 89] and *C. boidinii* DAAO [36] with unusual substrate specificity have been recently cloned. The genes of other DAAOs, i.e., of drosophila, parasites, and pathogens have been discovered from the annotated genomic sequences.

**Expression.** The most popular approach to produce recombinant proteins is their expression in *E. coli* cells. However, in the case of DAAO this approach is connected with numerous problems. First, overexpression of active soluble enzyme will result in oxidation of D-amino acids which are the building blocks of the prokaryotic cell wall, and therefore, in inhibition of cell growth. Second, hydrogen peroxide, the product of DAAO-catalyzed reactions, is a powerful cytotoxic agent. Under normal physiological conditions, hydrogen peroxide is effectively decomposed by catalase, whereas under conditions of

recombinant DAAO overexpression up to 15-30% of the total protein and intensive aeration, the catalase activity will be insufficient to detoxify the hydrogen peroxide produced. Both factors strongly decrease the rate of host strain cell growth and change the cell morphology [90] preventing high cell density in the course of cultivation. For instance, the maximum yield of biomass in the course of recombinant RgDAAO production never exceeded 2 g per liter of medium [91].

All eukaryotic DAAO genes contain introns, and the RgDAAO gene contains an unusually high number of introns (five) [92]. For the purpose of DAAO gene expression in *E. coli*, the introns are deleted from the genes. To produce recombinant DAAO in *E. coli* cells a number of various expression vectors have been constructed, with the enzyme gene under the control of *lac*-, *tac*-, and phage T7 RNA-polymerase promoters [31, 75-82, 88-96]. However, in no case could high yield of either specific or total activity be achieved. Most recombinant DAAO was produced in the form of insoluble inclusion bodies or inactive apoenzyme. Recombinant RgDAAO was totally inactive [91], but its soluble active form could be expressed with an N-terminal leader sequence Met-Ala-Arg-Ile-Arg-Ala [91]. The content of the active enzyme for all DAAO expression cases was no more than 1-3% of the total soluble *E. coli* protein, with the overall yield of no more than 10-30 mg enzyme per liter of medium. Nevertheless, with these yields pkDAAO and RgDAAO were produced in quantities sufficient for protein crystallization [91, 94].

Transport of DAAO into peroxisomes in the case of eukaryotic expression systems allows one to avoid the cytotoxic action of hydrogen peroxide. TvDAAO was expressed in *Saccharomyces cerevisiae*, *Kluyveromyces lactis* [86], and *S. pombe* [97] yeast, but the yield achieved was no higher than for *E. coli* cells [90]. It was probably the result of low-level synthesis of FAD, which is obligatory for holoenzyme production. The lack of FAD for the holoenzyme synthesis was also observed for TvDAAO and RgDAAO expressed in *E. coli* cells [90, 92, 96]. For instance, in the case of TvDAAO, the enzyme activity was increased by 10-fold following the addition of free FAD into the cell-free extract [96].

The best results were achieved for TvDAAO expression in the methylotrophic yeast *Pichia pastoris* under the control of alcohol oxidase 1 promoter [98]. Alcohol oxidase 1 is a FAD-dependent enzyme and its promoter provides the enzyme biosynthesis at the level of 25-30% of the total cell protein [99]. Cultivation of the recombinant strain in the regime of high density cell culture resulted in the enzyme yield of 23,000 activity units (~220 mg) per liter of culture [96]; however, the enzyme activity in the cells was not high, only 473 units per g of dry biomass that corresponds to the recombinant TvDAAO content of ca. 1% of the total cell protein. The cultivation period was 40 h with the productivity of 575 units per liter of medium in 1 h.

Enzyme homologous expression was used in the case of methylophilic yeast *C. boidinii* DAAO [36]. The increase in DAAO gene copies from 1 to 8 in the chromosome resulted in the increase in the enzyme content from 2 to 8% of the total cell protein.

The TvDAAO gene was cloned in this laboratory and the expression vector was constructed to produce the recombinant enzyme [100]. The correct strategy used for the plasmid construction and optimization of cultivation conditions allowed the enzyme yield of 15,000 activity units (or 150 mg protein) per liter of medium with specific activity of ~2000 units per g of raw biomass to be achieved. The content of the active enzyme was 20% of *E. coli* soluble protein. An equivalent amount of the enzyme was synthesized in the form of inclusion bodies. Cultivation was carried out in shaker flasks of 1–5 liter volume for 16 h (process productivity was 935 units per liter of medium in 1 h). Scale-up cultivation in a fermenter, which provides tight control of all process parameters such as aeration, pH, dosage of cultivation medium, will guarantee up to 3–5-fold increase in the enzyme yield.

To conclude this part of the review, we must note that the problem of the development of a highly efficient expression system for the production of commercial DAAO is far from being solved. The process of recombinant protein production for the biotransformation purposes is economically justified if its productivity is no less than 2 g enzyme per liter of medium per day. The currently existing DAAO expression systems are far short (at least by an order of magnitude) with respect to the commercially required productivity.

### SUBSTRATE SPECIFICITY

Analysis of the literature demonstrates the difference in substrate specificity for DAAOs from various sources. Unfortunately, the direct comparison of the data presented by different authors is problematic. There are a number of reasons for this uncertainty. First, the various authors use different assay protocols. The most popular assay is based on hydrogen peroxide determination via the coupled peroxidase assay. Different peroxidase substrates and different reaction conditions (pH, temperature) result in significantly discrepant activity values. In addition, minor traces of catalase in crude extracts have a pronounced effect on the assay results. Moreover, the concentration of oxygen in water under normal atmospheric pressure conditions (0.21 mM at pH 8.0 and 37°C [33]) is much lower than the  $K_m$  value for oxygen (0.72 mM for pkDAAO and TvDAAO [33]).

Table 2 presents the data on activity assay for a number of DAAO obtained in one work using hydrogen peroxide detection with peroxidase and *o*-dianisidine [101] and the data on substrate specificity for *C. boidinii* DAAO [36]. To illustrate the effect of the assay method on activ-

ity values obtained, the TvDAAO assay data with electrochemical detection of oxygen consumption [33] are also presented in Table 2. Table 2 visually justifies the division of all enzymes into two groups based on their activity towards D-alanine: microbial DAAOs from *Fusarium oxysporum*, *Candida parapsilosis*, and *C. boidinii* are highly specific for this amino acid, while other microbial enzymes exhibit maximum activity with D-methionine, D-tryptophan, and D-phenylalanine. The best substrate for pig kidney DAAO is D-proline. The data presented in Table 2 also visualizes the difference in the TvDAAO substrate specificity studied in different works.

The key characteristic of TvDAAO is the highest activity towards cephalosporin C among D-amino acid oxidases that justifies the active use of the enzyme in the development of biocatalytic processes of 7-aminocephalosporanic acid (7-ACA) production (see below). TvDAAO exhibits high affinity for amino acids that provide the highest activity values. The  $K_m$  values for D-phenylalanine, D-methionine, cephalosporin C, and D-alanine, determined for TvDAAO at pH 8.0, 37°C, and 0.21 mM oxygen were equal to 0.20, 0.29, 0.83, and 6.5 mM, respectively [33]. The  $K_m$  value for D-alanine ( $7.0 \pm 0.9$  mM) determined for TvDAAO under different conditions (pH 8.5, 25°C, oxygen 21% of saturation) [102] was similar to that previously obtained, while the  $K_m$  value for cephalosporin C showed a 3-fold increase (2.4 mM). The  $K_m$  values for D-alanine in the case of RgDAAO and *C. boidinii* DAAO were determined as 1.0 mM [84, 102] and 4.5 mM [36], respectively.

Concluding this part, we note that the differences in the substrate specificity spectrum for DAAOs from various sources can be used for the selective determination of concentrations of individual D-amino acids in their mixture with an array biosensor based on different DAAOs.

### DAAO STABILITY

**Thermal stability.** The analysis of the residual enzyme activity following incubation of recombinant pkDAAO, RgDAAO, and native TvDAAO for 30 min at different temperatures demonstrated that TvDAAO is superior in its thermal stability [102]. The  $T_m$  values (temperature which gives a 50% decrease in activity after 30 min incubation) for the pkDAAO, RgDAAO, and TvDAAO preparations under study were 39, 44, and 54°C, respectively (pH 8.0). Enzyme thermal stability is significantly affected by pH. The 0.5 unit decrease in pH (to 7.5) results in 8 h half-inactivation period for RgDAAO at 40°C, while TvDAAO loses less than 10% of its activity within the same time period [103]. Thermal stability of glycine oxidase from *B. subtilis* at pH 7.0 is comparable to the stability of TvDAAO at pH 8.0 [88].

Inactivation of monomeric pkDAAO and dimeric RgDAAO and TvDAAO proceeds via different mecha-



**Table 2.** Substrate specificity of D-amino acid oxidases from different species

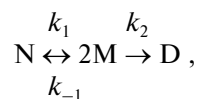
Amino acid	<i>V. luteoalbum</i> [101]	<i>F. oxysporum</i> [101]	<i>C. parapsilosis</i> [101]	<i>C. boidinii</i> [36]	<i>R. gracilis</i> [101]	porcine [101]	<i>T. variabilis</i> [101]	<i>T. variabilis</i> [33]
D-alanine	57	<b>100</b>	<b>100</b>	<b>100</b>	71	40	97	32
D-valine	41	62	15	8	95	28	<b>100</b>	39
D-leucine	42	29	92	7	57	21	32	30-40
D-isoleucine	15	19	n.d.	n.d.	56	35	76	30-40
D-methionine	<b>100</b>	28	n.d.	14	<b>100</b>	75	78	<b>100</b>
D-proline	4	22	41	1.4	57	<b>100</b>	25	2
D-phenylalanine	50	41	19	1.5	79	84	36	80
D-tryptophan	n.d.	14	9	0	56	2	38	<b>100</b>
D-serine	9	18	6	32	41	18	22	2
D-threonine	n.d.	2	n.d.	1.2	10	2	4	30-40
D-tyrosine	n.d.	8	6	0	26	4	17	n.d.
D-cysteine	n.d.	n.d.	n.d.	0	1	n.d.	n.d.	0
D-asparagine	n.d.	3	2	0.18	40	n.d.	65	70
D-glutamine	51	15	5	0	53	1	81	n.d.
D-lysine	n.d.	n.d.	25	0	5	2	17	n.d.
D-arginine	n.d.	7	25	0	2	4	43	30-40
D-histidine	n.d.	14	4	0	58	3	88	88
D-aspartate	7	n.d.	n.d.	0	n.d.	1	4	0
D-glutamate	10	n.d.	54	n.d.	4	n.d.	9	<6
Glycine	2	n.d.	15	n.d.	3	n.d.	n.d.	n.d.
Cephalosporin C	1	1	n.d.	n.d.	2	0.3 [102]	13	25

Note: Activity values are presented in percent of the maximal activity with the best substrate; n.d., no data.

nisms. The rate of inactivation of pkDAAO is independent of enzyme concentration, while a 3-fold decrease in RgDAAO concentration (from 0.15 to 0.05 mg/ml) results in a decrease of the half-inactivation period from 8 h to 45 min [103]. Similar relationships between  $T_m$  value and the enzyme concentration were obtained for RgDAAO thermal inactivation studied by other methods (scanning microcalorimetry, fluorescence, etc.) [73]. Altogether, the data point to the dissociation of RgDAAO into individual subunits as the first step of thermal inactivation of the enzyme. To clarify the role of RgDAAO oligomeric composition on the thermal inactivation mechanism, the enzyme was produced in a monomeric form by means of site-directed mutagenesis [72] (see above). The conversion of RgDAAO into the monomeric form results in its significant destabilization, i.e.,  $T_m$  value drops by 5-6°C, but its value remains constant within the enzyme concentration range studied (0.1-3.5 mg/ml) [73].

In the case of TvDAAO, thermal stability is also dependent on the enzyme concentration [103]. We carried out a detailed study of the inactivation rates for recombinant TvDAAO at different temperatures and dif-

ferent concentrations. At room temperature (20-22°C) and pH 8.0, the enzyme lost activity within several days. In the temperature range 52-60°C, the time course of TvDAAO residual activity changes is fitted to a sum of two exponentials. This proves the minimum two-step inactivation mechanism for this enzyme. The inactivation rate constant for the first step was inversely proportional to the TvDAAO concentration, while the rate constant for the second step was constant for all enzyme concentrations studied. The data were rationalized within a kinetic scheme that includes dimer dissociation into the individual subunits in the first step and then the irreversible denaturation of the subunits:



where N is a native dimer, M is monomer, and D is denatured enzyme.

The values of activation parameters, e.g., enthalpy  $\Delta H^\#$  and entropy  $\Delta S^\#$ , calculated for the second step using

the temperature dependence of  $k_2$ , provide evidence for the enzyme thermal denaturation, i.e., globule unfolding, as the most probable mechanism for this step.

#### The pH dependence of DAAO activity and stability.

D-Amino acids with deprotonated amino group are better DAAO substrates than their positively charged forms [19, 104]. This reflects the fact that proton removal from the amino group increases the rate constant for hydride-ion abstraction from the substrate  $\alpha$ C-atom in the enzyme active center. Therefore, one may suppose that the maximum activity of DAAO should be observed at alkaline pH values providing the uncharged amino group. However, the maximum rate for the RgDAAO reaction with D-alanine is achieved already at pH 8.5 [102, 104], which is much lower than the  $pK$  value for the  $\alpha$ -amino group proton dissociation (9.0–9.5). The explanation of this discrepancy is the change in the microenvironment of the D-alanine amino group upon the substrate binding in the enzyme active center which results in the  $pK$  value drop to 8.0 [104]. The drop in  $pK$  for the D-alanine amino group in the case of TvDAAO is supposedly more pronounced because the enzyme exhibits maximum activity at pH  $\geq 7.5$  [102]. For pkDAAO, the value for the D-alanine amino group drops only by 0.5 pH units upon the substrate binding in the active center [102].

As noted above, a decrease in pH to 7.5 results in a pronounced increase in thermal stability of DAAO. At room temperature (25°C), the enzyme stability is less dependent on pH. pkDAAO, RgDAAO, and TvDAAO are stable in the pH 6.0–8.0 range, the stability of yeast enzyme outside this pH range decreases, especially for RgDAAO, which inactivated more rapidly than TvDAAO. The stability of pkDAAO at pH 9.5 is 2 times higher than at neutral pH [102].

### KINETIC SCHEME AND MECHANISM OF ACTION

Figure 4 presents the general scheme for the reaction catalyzed by DAAO. In the first step, the complex between enzyme and D-amino acid is formed. Hydride-ion transfer from the  $\alpha$ C-atom of the amino acid to the N(5)-atom of the isoalloxazine ring yielding reduced FAD occurs within the binary complex. The reaction proceeds further via two pathways depending on the enzyme origin and amino acid type. In the case of pkDAAO, the reaction with positively charged D-amino acids proceeds along the top pathway because the release of an imino acid is the rate-limiting step of the overall reaction (rate constant  $k_4$ ) and, therefore, FAD is oxidized with dioxygen before the imino acid dissociates from the active center [19]. In the case of RgDAAO, the hydride-ion transfer in the binary enzyme–substrate complex is rate-limiting (rate constant  $k_2$ ), and the overall reaction proceeds via the classic ping-pong mechanism, with FAD reoxidation

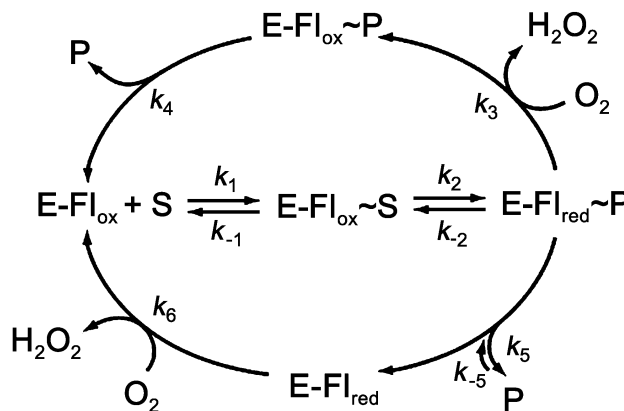


Fig. 4. Kinetic scheme of reaction catalyzed by D-amino acid oxidase.

following the imino acid dissociation from the binary complex enzyme-reduced FAD–product (see  $E-FI_{red}-P$  in Fig. 4). The detailed analysis of the experimental data supporting the above kinetic schemes is presented in [19]. The kinetic studies with *B. subtilis* glycine oxidase demonstrated the identity of its kinetic mechanism to that of mammalian DAAO [105].

Until recently, the mechanism of FAD reduction within the DAAO–D-amino acid complex was unclear. Some authors favored the two-step carbanion mechanism with deprotonation of D-amino acid  $\alpha$ C-atom preceding two-electron transfer to the N(5)-atom of the FAD isoalloxazine ring. Another probable mechanism of FAD reduction is a direct one-step transfer of hydride-ion. The refined crystal structures of pkDAAO and RgDAAO complexes with various D-amino acids, their non-native analogs (such as trifluoroalanine), and inhibitors demonstrates no functional groups, i.e., potential bases or acids in the enzyme active center, that could catalyze the deprotonation step [19, 67–70]. Additional evidence for the mechanism of direct hydride transfer was obtained in studies of the pH dependence of the deuterium primary isotopic effect and solvent kinetic isotopic effect in the reaction of D-alanine and D-asparagine oxidation catalyzed by RgDAAO [104].

Amino acid sequence alignment for DAAOs of different origin together with the refined crystal structures for pkDAAO and RgDAAO confirm the catalytic role of just a few amino acid residues in the enzyme active center [19], e.g., Tyr223, Tyr238, and Arg285 (numbering for RgDAAO). Their role in the mechanism of action of pkDAAO and RgDAAO was studied by site-directed mutagenesis [106–110] and the data obtained confirmed that their main function was to correctly orient the D-amino acid against the isoalloxazine ring of FAD via hydrogen bonding with the substrate carboxy group in the enzyme active center [19]. The Ser335 residue and water

molecule participate in hydrogen bonding of the  $\alpha$ -amino group. Detailed information on the DAAO active center organization and the role of individual amino acid residues in the catalysis and FAD and substrates binding can be found in [111].

DAAOs exhibit very poor activity or none towards D-aspartate (Table 2). To oxidize this amino acid, mammals possess a specific enzyme, D-aspartate oxidase. S. Sacchi et al. [112] attempted to determine the reasons for this phenomenon and to construct a mutant RgDAAO with wide spectrum of substrate specificity. The comparison of the amino acid sequence and quaternary structure of RgDAAO with the model structure of beef kidney DASPO demonstrated that the latter enzyme has Arg237 in the position equivalent to Tyr238 in the yeast oxidase. In addition, the Met213 residue is located 3.8 Å from the substrate in the active center of RgDAAO. To effectively bind the  $\beta$ -carboxy group of D-Asp, a single point mutant RgDAAO Met213Arg and a double mutant RgDAAO Met213Arg/Tyr238Arg were prepared. These substitutions provided in the active center additional one and two positive charges. The Met213Arg mutation resulted in 8-fold increase in  $k_{\text{cat}}$  and 10-fold decrease in  $K_m$  in the reaction of D-aspartate oxidation compared to those parameters for the wild-type RgDAAO. The catalytic efficiency ( $k_{\text{cat}}/K_m$  ratio) of the mutant RgDAAO was comparable to that of the native beef kidney DASPO. In the case of the double mutant, Met213Arg/Tyr238Arg, the  $k_{\text{cat}}$

value increased 53-fold, however, the worsened Michaelis constant resulted in 2-fold lower  $k_{\text{cat}}/K_m$  ratio compared to the single-point RgDAAO Met213Arg mutant. The cited work is the first study on engineering of substrate specificity of D-amino acid oxidase and is promising for the development of enzyme mutant forms with higher catalytic activity in the reaction of cephalosporin C oxidation, a process of key biotechnological importance.

## PRACTICAL APPLICATIONS OF DAAO

Interest in DAAO is mainly due to prospects of its use in the production of 7-ACA from the natural antibiotic cephalosporin C in a two-enzyme process. The general scheme of the process is presented in Fig. 5. In contrast to the single enzyme biocatalytic process of 6-aminopenicillanic acid production from penicillin G catalyzed by penicillinase, hydrolysis of cephalosporin C into 7-ACA cannot be achieved as a one-step process since no enzyme with such type of activity has been discovered so far. DAAO catalyzes the first step, i.e., cephalosporin C oxidation into  $\alpha$ -ketoadipyl-7-ACA (Fig. 5). Further non-enzymatic oxidative decarboxylation of  $\alpha$ -ketoadipyl-7-ACA by hydrogen peroxide formed in the course of the oxidase reaction yields 7- $\beta$ -(4-carboxybutanamide)cephalosporanic acid (glutaryl-7-ACA). The latter is hydrolyzed into free 7-ACA using a second enzyme,

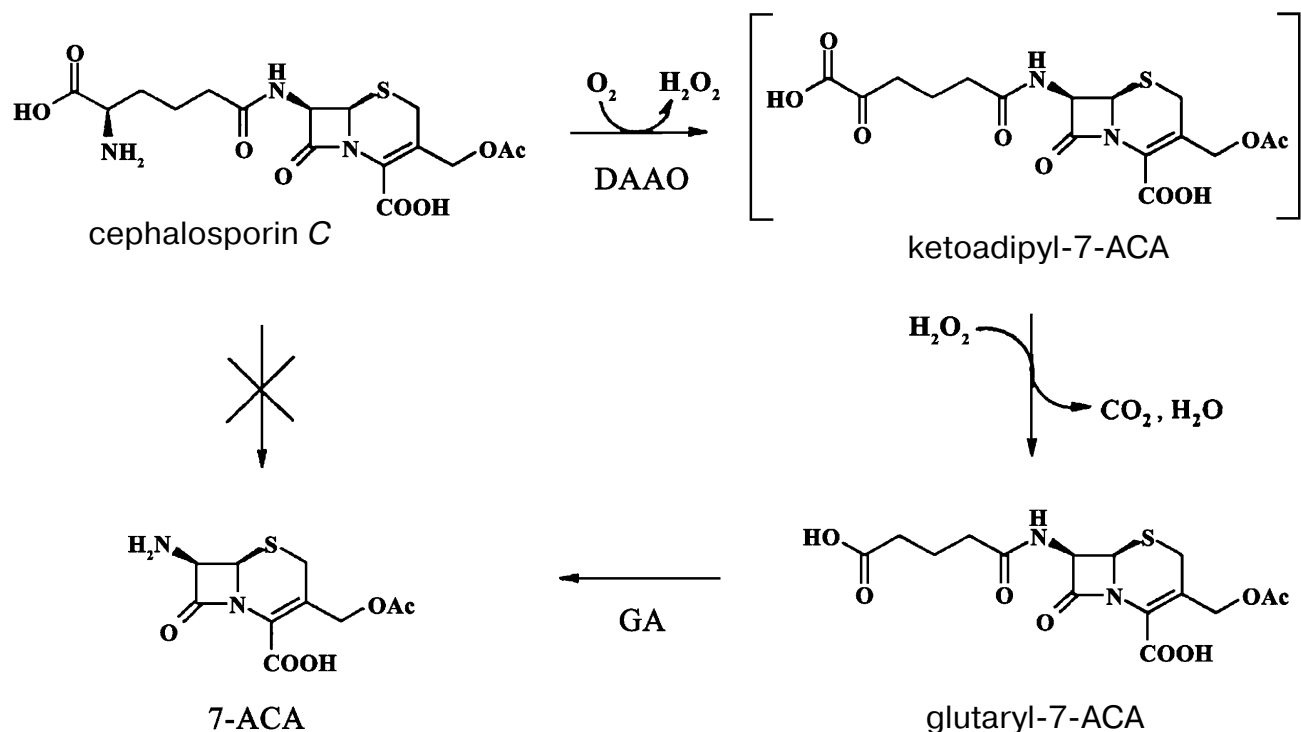


Fig. 5. Production of 7-aminoccephalosporanic acid (7-ACA) from cephalosporin C using two-enzyme system D-amino acid oxidase (DAAO)–glutaryl hydrolase (GA).

glutaryl hydrolase. The process is based on microbial DAAOs due to their high, at least two orders of magnitude higher than that of mammalian enzymes, activity with cephalosporin C [102]. The same two-enzyme system can be used for the production of 7-aminodeacetoxyamino-cephalosporanic acid [113].

The "Derwent" patent database has ~60 national and international patents covering various methods for this process. Originally, free and immobilized cells of *T. variabilis* and *R. gracilis* were used as the biocatalysts for this process [114-116]. However, the serious disadvantage of this approach was in diffusion limitations and the presence of catalase decomposing hydrogen peroxide required for ketoadipyl-7-ACA oxidation. To improve the activity, cells were permeabilized with various compounds [117, 118]. To decrease the catalase activity, either mutant catalase-deficient strains have been constructed or the cells have been preliminary treated with elevated temperature (40°C [117]) or alkali (pH 11 [119]).

The key disadvantage of whole cell catalysts is the low content of the enzyme. Attempts have been made to increase the enzyme yield by optimizing cultivation conditions for the wild-type and mutant strains of *T. variabilis* and *R. gracilis* [120, 121] and recombinant strains of other microorganisms [80]. Nevertheless, 25 years of research did not result in a high activity commercial catalyst based on whole cells.

More successful was the development of an immobilized enzyme biocatalyst [103, 122, 123]. For instance, immobilization of TvDAAO and RgDAAO on activated glyoxyl-agarose resulted in significantly improved thermal stability of the enzymes, and the operational stability of RgDAAO was increased 15,000-fold. To protect the enzymes from hydrogen peroxide-induced inactivation [124], methionine residues susceptible to oxidation causing the loss of enzymatic activity were deleted by means of site-directed mutagenesis [125].

One of the parameters defining the process effectiveness is the concentration of dissolved oxygen. Bubbling with pure oxygen was used to get the maximum level of the oxidant [102]. Recombinant DNA techniques allowed a principally new approach to improve DAAO catalytic effectiveness [126]. By means of genetic engineering, a chimeric protein consisting of protobacterial *Vitreoscilla* hemoglobin (Vhb) and RgDAAO has been constructed. Oxygen binding by hemoglobin in the close vicinity of the RgDAAO active center increased the local oxygen concentration resulting in a 12-fold increase of the catalytic activity of the immobilized chimeric enzyme compared to immobilized RgDAAO. Simultaneously, the affinity for cephalosporin was increased 2-fold and the operational stability was improved 3-fold. The chimeric enzyme Vhb-RgDAAO preserved more than 90% of the original activity after 50 reaction cycles with the cephalosporin C, conversion degree of 99%, and glutaryl-7-ACA purity of 99.77% [126].

The recently achieved success makes the development of a commercial catalyst based on the immobilized recombinant DAAO highly probable within the next few years. The problem with the production of the second enzyme, glutaryl hydrolase, is about to be solved [127]. Taking into account the production volume of cephalosporin antibiotics, the biocatalytic process of 7-ACA and deacetoxy-7-ACA is expected to be the major one among large-scale commercial processes with the use of immobilized enzymes.

High stereospecificity of DAAO towards D-amino acids provides its applications in analytical biotechnology. Enzyme immobilization on the surface of electrodes monitoring either oxygen consumption or hydrogen peroxide production gives rise to reagentless electrochemical biosensors for continuous fermentation control in the course of D-alanine production [128], for the detection of D-amino acids in food [129, 130] and medications [14, 131, 132], etc.

DAAO from yeast and mammals have been already used for the chiral synthesis of various sintons, the starting compounds for the production of chiral medications [133]. The enzymatic synthesis of omapatrylate, an inhibitor of angiotensin converting enzyme, is one of the successful examples [134].

This work was carried out under the federal program "Science and High School Integration" 2002-2006 (grant No. L-0114/999).

## REFERENCES

- Ghisla, S., and Massey, V. (1989) *Eur. J. Biochem.*, **181**, 1-17.
- Palfey, B. A., and Massey, V. (1996) in *Comprehensive Biochemical Catalysis* (Sinnott, M., ed.) Academic Press, N.Y., pp. 83-154.
- Swoboda, B. E. (1969) *Biochim. Biophys. Acta*, **175**, 365-379.
- Choong, Y. S., Shepherd, M. G., and Sullivan, P. A. (1975) *Biochem. J.*, **145**, 37-45.
- Massey, V., and Harris, C. M. (1997) *Biochem. Soc. Trans.*, **25**, 750-755.
- Vermilion, J. L., and Coon, J. M. (1978) *J. Biol. Chem.*, **253**, 8812-8819.
- Stritmatter, P. (1961) *J. Biol. Chem.*, **236**, 2329-2335.
- Krebs, H. A. (1935) *Biochem. J.*, **29**, 1620-1644.
- Conlon, H. D., Baqai, J., Baker, K., Shen, Y. Q., Wong, B. L., Noiles, R., and Rausch, C. W. (1995) *Biotechnol. Bioeng.*, **46**, 510-513.
- Kurochkina, V. B., and Nys, P. S. (2002) *Antibiot. Khimioterap.*, **47**, 29-37.
- Brodelius, P., Nilsson, K., and Mosbach, K. (1981) *Appl. Biochem. Biotechnol.*, **6**, 293-308.
- Beard, T. M., and Turner, N. J. (2002) *Chem. Commun. (Camb.)*, **3**, 246-247.
- Nakajima, N., Conrad, D., Sumi, H., Suzuki, K., Esaki, N., Wandrey, C., and Soda, K. (1990) *J. Ferm. Bioeng.*, **70**, 322-325.

14. Van Staden, J. F., Stefan, R. I., and Aboul-Enein, H. Y. (2000) *Fresenius J. Analyt. Chem.*, **367**, 178-180.
15. Dominguez, R., Serra, B., Reviejo, A. J., and Pingarron, J. M. (2001) *Analyt. Biochem.*, **298**, 275-282.
16. Inaba, Y., Mizukami, K., Hamada-Sato, N., Kobayashi, T., Imada, C., and Watanabe, E. (2003) *Biosens. Bioelectron.*, **19**, 423-431.
17. Mizutani, H., Miyahara, I., Hirotsu, K., Nishina, Y., Shiga, K., Setoyama, C., and Miura, R. (1996) *J. Biochem. (Tokyo)*, **120**, 14-17.
18. Mattevi, A., Vanoni, M. A., Todone, F., Rizzi, M., Bolognesi, M., Coda, A., and Curti, B. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 7496-7501.
19. Pilone, M. S. (2000) *Cell. Mol. Life Sci.*, **57**, 1732-1747.
20. Fraaije, M. W., and Mattevi, A. (2000) *Trends Biochem. Sci.*, **25**, 126-132.
21. Curti, B., Ronchi, S., and Pilone, M. S. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Mueller, F., ed.) Vol. 3, CRC Press, Boca Raton, pp. 69-94.
22. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry III, C. E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, S., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, S., Squares, S., Squires, R., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature*, **393**, 537-544.
23. Camus, J. C., Pryor, M. J., Medigue, C., and Cole, S. T. (2002) *Microbiol.*, **148**, 2967-2973.
24. Omura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazawa, H., Osonoe, T., Kikuchi, H., Shiba, T., Sakaki, Y., and Hattori, M. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 12215-12220.
25. Hils, M., Munch, P., Altenbuchner, J., Syltatk, C., and Mattes, R. (2001) *Appl. Microbiol. Biotechnol.*, **57**, 680-688.
26. Barbe, V., Vallenet, D., Fonknechten, N., Kreimeyer, A., Oztas, S., Labarre, L., Cruveiller, S., Robert, C., Duprat, S., Wincker, P., Ornston, L. N., Weissenbach, J., Marliere, P., Cohen, G. N., and Medigue, C. (2004) *Nucleic Acids Res.*, in press.
27. Meister, A. (1965) in *Biochemistry of the Amino Acids*, 2nd Edn., Vol. 1, Academic Press, N. Y., pp. 297-304.
28. Pistorius, E. K., and Voss, H. A. (1977) *Biochim. Biophys. Acta*, **481**, 395-406.
29. Sikora, L., and Marzluf, G. A. (1982) *Mol. Genet. Gen.*, **186**, 33-39.
30. Benz, F., Liersch, M., Nuesch, J., and Treichler, H. J. (1971) *Eur. J. Biochem.*, **20**, 81-88.
31. Isogai, T., Ono, H., Ishitani, Y., Kojo, H., Ueda, Y., and Kohsaka, M. (1990) *J. Biochem. (Tokyo)*, **108**, 1063-1069.
32. Sentheshanmuganathan, S., and Nickerson, W. J. (1962) *J. Gen. Microbiol.*, **27**, 465-471.
33. Schröder, T., and Andreesen, J. R. (1996) *Arch. Microbiol.*, **165**, 41-47.
34. Yoshizawa, M., Ueda, M., Mozaffar, S., and Tanaka, A. (1986) *Agric. Biol. Chem.*, **50**, 2637-2638.
35. Simonetta, M. P., Vanoni, M. A., and Curti, B. (1982) *FEMS Microbiol. Rev.*, **15**, 27-31.
36. Yurimoto, H., Hasegawa, T., Sakai, Y., and Kato, N. (2001) *Biosci. Biotechnol. Biochem.*, **65**, 627-633.
37. Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., Sakaki, Y., Hattori, M., and Omura, S. (2003) *Nat. Biotechnol.*, **21**, 526-531.
38. Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Qurollo, B., Goldman, B. S., Cao, Y., Askenazi, M., Halling, C., Mullin, L., Houmiel, K., Gordon, J., Vaudin, M., Iartchouk, O., Epp, A., Liu, F., Wollam, C., Allinger, M., Doughty, D., Scott, C., Lappas, C., Markelz, B., Flanagan, C., Crowell, C., Gurson, J., Lomo, C., Sear, C., Strub, G., Cielo, C., and Slater, S. (2001) *Science*, **294**, 2323-2328.
39. La Rue, T. A., and Spencer, J. F. T. (1967) *Can. J. Microbiol.*, **13**, 777-788.
40. Momoi, K., Fukui, K., Watanabe, F., and Miyake, Y. (1988) *FEBS Lett.*, **238**, 180-184.
41. D'Aniello, A., D'Onofrio, G., Pischetola, M., D'Aniello, M., Vetere, A., Petrucelli, L., and Fisher, G. H. (1993) *J. Biol. Chem.*, **268**, 26941-26949.
42. Hasegawa, H., Matsukawa, T., Shinohara, Y., Konno, R., and Hashimoto, T. (2004) *Am. J. Physiol. Endocrinol. Metab.*, **287**, 160-165.
43. Ohtani, S., Matsushima, Y., Ohira, H., and Watanabe, A. (1995) *Growth Dev. Aging*, **59**, 55-61.
44. Poinar, H. N., Hoss, M., Bada, J. L., and Paabo, S. (1999) *Science*, **272**, 864-866.
45. Man, E. H., Fisher, G. H., Payan, I. L., Cadilla-Perezrrios, R., Garcia, N. M., Chemburkar, R., Arends, G., and Frey, W. H. (1987) *J. Neurochem.*, **48**, 510-515.
46. Schell, M. J., Molliver, M. E., and Snyder, S. H. (1995) *Neurobiology*, **92**, 3948-3952.
47. Wolosker, H., Shetch, K. M., Takahashi, M., Mothet, J. P., Brady, R. O., Ferris, C. D., and Snyder, S. H. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 721-725.
48. Hamase, K., Inoue, T., Morikawa, A., Konno, R., and Zaitus, K. (2001) *Analyt. Biochem.*, **298**, 253-258.
49. Haradahira, T., Okauchi, T., Maeda, J., Zhang, M. R., Nishikawa, T., Konno, R., Suzuki, K., and Suhara, T. (2003) *Synapse*, **50**, 130-136.
50. Stevens, E. R., Esguerra, M., Kim, P. M., Newman, E. A., Snyder, S. H., Zahs, K. R., and Miller, R. F. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 6789-6794.
51. Cloninger, C. R. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 13365-13367.
52. Chumakov, I., Blumenfeld, M., Guerassimenko, O., Cavarec, L., Palicio, M., Abderrahim, H., Bougueleret, L., Barry, C., Tanaka, H., La Rosa, P., Puech, A., Tahri, N., Cohen-Akenine, A., Delabrosse, S., Lissarrague, S., Picard, F. P., Maurice, K., Essioux, L., Millasseau, P., Grel, P., Debailleul, V., Simon, A. M., Caterina, D., Dufaure, I., Malekzadeh, K., Belova, M., Luan, J. J., Bouillot, M., Sambucy, J. L., Primas, G., Saumier, M., Boubkiri, N., Martin-Saumier, S., Nasroune, M., Peixoto, H., Delaye, A., Pinchot, V., Bastucci, M., Guillou, S., Chevallion, M., Sainz-Fuertes, R., Meguenni, S., Aurich-Costa, J., Cherif, D., Gimalac, A., van Duijn, C., Gauvreau, D., Ouellette, G., Fortier, I., Raelson, J., Sherbatich, T., Riazanskaia, N., Rogaev, E., Raeymaekers, P., Aerssens, J., Konings, F., Luyten, W., Macciardi, F.,

- Sham, P. C., Straub, R. E., Weinberger, D. R., Cohen, N., Cohen, D., Ouelette, G., and Realson, J. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 13675-13680.
53. Harrison, P. J., and Owen, M. J. (2003) *Lancet*, **361**, 417-419.
  54. Schumacher, J., Jamra, R. A., Freudenberg, J., Becker, T., Ohlraun, S., Otte, A. C., Tullius, M., Kovalenko, S., Bogaert, A. V., Maier, W., Rietschel, M., Propping, P., Nothen, M. M., and Cichon, S. (2004) *Mol. Psychiatry*, **9**, 203-207.
  55. Sasamura, T., Matsuda, A., and Kokuba, Y. (2002) *Ann. Clin. Biochem.*, **39**, 595-598.
  56. Fang, J., Sawa, T., Akaike, T., and Maeda, H. (2002) *Cancer Res.*, **62**, 3138-3143.
  57. Fang, J., Sawa, T., Akaike, T., Greish, K., and Maeda, H. (2004) *Int. J. Cancer*, **109**, 1-8.
  58. Piubelli, L., Caldinelli, L., Molla, G., Pilone, M. S., and Pollegioni, L. (2002) *FEBS Lett.*, **526**, 43-48.
  59. Settembre, E. C., Dorrestein, P. C., Park, J. H., Augustine, A. M., Begley, T. P., and Ealick, S. E. (2003) *Biochemistry*, **42**, 2971-2981.
  60. Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C., Nelson, K. E., Eisen, J. A., Ketchum, K. A., Hood, D. W., Peden, J. F., Dodson, R. J., Nelson, W. C., Gwinn, M. L., DeBoy, R., Peterson, J. D., Hickey, E. K., Haft, D. H., Salzberg, S. L., White, O., Fleischmann, R. D., Dougherty, B. A., Mason, T., Ciecko, A., Parksey, D. S., Blair, E., Citterone, H., Clark, E. B., Cotton, M. D., Utterback, T. R., Khouri, H., Qin, H., Vamathevan, J., Gill, J., Scarlato, V., Maignani, V., Pizza, M., Grandi, G., Sun, L., Smith, H. O., Fraser, C. M., Moxon, E. R., Rappuoli, R., and Venter, J. C. (2000) *Science*, **287**, 1809-1815.
  61. He, J., Baldini, R. L., Deziel, E., Saucier, M., Zhang, Q., Liberati, N. T., Lee, D., Urbach, J., Goodman, H. M., and Rahme, L. G. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 2530-2535.
  62. Wierenga, R. K., Drenth, J., and Schulz, G. E. (1983) *J. Mol. Biol.*, **167**, 725-739.
  63. Rossmann, M. G., Liljas, A., Branden, C.-I., and Banaszak, L. J. (1975) in *The Enzymes* (Boyer, P. D., ed.) 3rd Edn., Vol. 11, Academic Press, N. Y., pp. 61-102.
  64. Subramani, S. (1993) *Ann. Rev. Cell Biol.*, **9**, 445-478.
  65. Campaner, S., Pollegioni, L., Ross, B. D., and Pilone, M. S. (1998) *Biochem. J.*, **330**, 615-621.
  66. Miura, R., Setoyama, C., Nishina, Y., Shiga, K., Mizutani, H., Miyahara, I., and Hirotsu, K. (1997) *J. Biochem. (Tokyo)*, **122**, 825-833.
  67. Todone, F., Vanoni, M. A., Mozzarelli, A., Bolognesi, M., Coda, A., Curti, B., and Mattevi, A. (1997) *Biochemistry*, **36**, 5853-5860.
  68. Umhau, S., Pollegioni, L., Molla, G., Diederichs, K., Welte, W., Pilone, M. S., and Ghisla, S. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 12463-12468.
  69. Pollegioni, L., Diederichs, K., Molla, G., Umhau, S., Welte, W., Ghisla, S., and Pilone, M. S. (2002) *J. Mol. Biol.*, **324**, 535-546.
  70. Mizutani, H., Miyahara, I., Hirotsu, K. J., Nishina, Y., Shiga, K., Setoyama, C., and Miura, R. (2000) *J. Biochem. (Tokyo)*, **128**, 73-81.
  71. Mörtl, M., Diederichs, K., Welte, W., Molla, G., Motteran, L., Andriolo, G., Pilone, M. S., and Pollegioni, L. (2004) *J. Biol. Chem.*, **279**, 29718-29727.
  72. Piubelli, L., Molla, G., Caldinelli, L., Pilone, M. S., and Pollegioni, L. (2003) *Prot. Eng.*, **16**, 1063-1069.
  73. Pollegioni, L., Iametti, S., Fessas, D., Caldinelli, L., Piubelli, L., Barbiroli, A., Pilone, M. S., and Bonomi, F. (2003) *Prot. Sci.*, **12**, 1018-1029.
  74. Fukui, K., and Miyake, Y. (1992) *J. Biol. Chem.*, **267**, 18631-18638.
  75. Fukui, K., Momoi, K., Watanabe, F., and Miyake, Y. (1988) *Biochemistry*, **27**, 6693-6697.
  76. Watanabe, F., Fukui, K., Momoi, K., and Miyake, Y. (1989) *Biochem. Biophys. Res. Commun.*, **165**, 1422-1427.
  77. Momoi, K., Fukui, K., Tada, M., and Miyake, Y. (1990) *J. Biochem. (Tokyo)*, **108**, 406-413.
  78. Tada, M., Fukui, K., Momoi, K., and Miyake, Y. (1990) *Gene*, **90**, 293-297.
  79. Konno, R. (1998) *Biochim. Biophys. Acta*, **1395**, 165-170.
  80. Konno, R., Kurabayashi, A., Tsuchiya, M., and Niwa, A. (1999) *DNA Seq.*, **10**, 85-91.
  81. Tsuchiya, M., Kurabayashi, A., and Konno, R. (2003) *Amino Acids*, **24**, 223-226.
  82. Sarower, M. G., Okada, S., and Abe, H. (2003) *Arch. Biochem. Biophys.*, **420**, 121-129.
  83. Konno, R. (2001) *Amino Acids*, **20**, 401-408.
  84. Pollegioni, L., Molla, G., Campaner, S., Martegani, E., and Pilone, M. S. (1997) *J. Biotechnol.*, **58**, 115-123.
  85. Liao, G. J., Lee, Y. J., Lee, Y. H., Chen, L. L., and Chu, W. S. (1998) *Biotechnol. Appl. Biochem.*, **27**, 55-61.
  86. Gonzalez, F. J., Montes, J., Martin, F., Lopez, M. C., Ferminan, E., Catalan, J., Galan, M. A., and Dominguez, A. (1997) *Yeast*, **13**, 1399-2008.
  87. Hils, M., Munch, P., Altenbuchner, J., Syltatk, C., and Mattes, R. (2001) *Appl. Microbiol. Biotechnol.*, **57**, 680-688.
  88. Job, V., Molla, G., Pilone, M. S., and Pollegioni, L. (2002) *Eur. J. Biochem.*, **269**, 1456-1463.
  89. Job, V., Marcone, G. L., Pilone, M. S., and Pollegioni, L. (2002) *J. Biol. Chem.*, **277**, 6985-6993.
  90. Lin, L., Chien, H. R., Wang, W., Hwang, T., Fu, H., and Hsu, W. (2000) *Enz. Microb. Technol.*, **27**, 482-491.
  91. Molla, G., Vegezzi, C., Pilone, M. S., and Pollegioni, L. (1998) *Prot. Exp. Purif.*, **14**, 289-294.
  92. Alonso, J., Barredo, J. L., Díez, B., Mellado, E., Salto, F., García, J. L., and Cortés, E. (1998) *Microbiology*, **144**, 1095-1101.
  93. Pollegioni, L., Fuku, K., and Massey, V. (1994) *J. Biol. Chem.*, **269**, 31666-31673.
  94. Setoyama, C., Miura, R., Nishina, Y., Shiga, K., Mizutani, H., Miyahara, I., and Hirotsu, K. (1996) *J. Biochem. (Tokyo)*, **119**, 1114-1117.
  95. Ciccarelli, E., Massaer, M., Guillaume, J. P., Herzog, A., Loriau, R., Cravador, A., Jacobs, P., and Bollen, A. (1989) *Biochem. Biophys. Res. Commun.*, **161**, 865-872.
  96. Alonso, J., Barredo, J. L., Armisen, P., Díez, B., Salto, F., Guisánd, J. M., García, J. L., and Cortés, E. (1999) *Enz. Microb. Technol.*, **25**, 88-95.
  97. Isogai, A., Kimura, H., Reichert, A., Schorgendorfer, K., Nikaido, K., Tohda, H., Giga-Hama, Y., Mutoh, N., and Kumagai, H. (2002) *Biotechnol. Bioeng.*, **80**, 22-32.
  98. Yu, J., Li, D.-Y., Zhang, Y.-J., Yang, S., Li, R.-B., and Yuan, Z.-Y. (2002) *J. Mol. Catalysis B: Enzymatic*, **18**, 291-297.
  99. Cereghino, J. L., and Cregg, J. M. (2000) *FEMS Microbiol. Rev.*, **24**, 45-66.

100. Davydova, E. E., and Tishkov, V. I. (2002) *Vestnik MGU. Ser. 2. Khimiya*, **43**, 353-355.
101. Gabler, M., Hense, M., and Fischer, L. (2000) *Enz. Microb. Technol.*, **27**, 605-611.
102. Pollegioni, L., Caldinelli, L., Molla, G., Sacchi, S., and Pilone, M. S. (2004) *Biotechnol. Prog.*, **20**, 467-473.
103. Betancor, L., Hidalgo, A., Fernández-Lorente, G., Mateo, C., Rodríguez, V., Fuentes, M., López-Gallego, F., Fernández-Lafuente, R., and Guisan, G. M. (2003) *Biotechnol. Prog.*, **19**, 784-788.
104. Harris, C. M., Pollegioni, L., and Ghisla, S. (2001) *Eur. J. Biochem.*, **268**, 5504-5520.
105. Molla, G., Motteran, L., Job, V., Pilone, M. S., and Pollegioni, L. (2003) *Eur. J. Biochem.*, **270**, 1474-1482.
106. Molla, G., Porrini, D., Job, V., Motteran, L., Vegezzi, C., Campaner, S., Pilone, M. S., and Pollegioni, L. (2000) *J. Biol. Chem.*, **275**, 24715-24721.
107. Pollegioni, L., Porrini, D., Molla, G., and Pilone, M. S. (2000) *Eur. J. Biochem.*, **267**, 6624-6632.
108. Pollegioni, L., Harris, C. M., Molla, G., Pilone, M. S., and Ghisla, S. (2001) *FEBS Lett.*, **507**, 323-326.
109. Nishina, Y., Sato, K., Shi, R., Setoyama, C., Miura, R., and Shiga, K. (2001) *J. Biochem. (Tokyo)*, **130**, 637-647.
110. Setoyama, C., Nishina, Y., Tamaoki, H., Mizutani, H., Miyahara, I., Hirotsu, K., Shiga, K., and Miura, R. (2002) *J. Biochem. (Tokyo)*, **131**, 59-69.
111. Pollegioni, L., Diederichs, K., Molla, G., Umhau, S., Welte, W., Ghisla, S., and Pilone, M. S. (2002) *J. Mol. Biol.*, **324**, 535-546.
112. Sacchi, S., Lorenzi, S., Molla, G., Pilone, M. S., Rossetti, C., and Pollegioni, L. (2002) *J. Biol. Chem.*, **277**, 27510-27516.
113. Velasco, J., Luis, A. J., Angel, M. M., Diez, B., Soler, G., and Barredo, J. L. (2000) *Nat. Biotechnol.*, **18**, 857-861.
114. Vicenzi, J. T., and Hansen, G. J. (1993) *Enz. Microb. Technol.*, **15**, 281-285.
115. Kurillova, L., Gemeiner, P., Vikartovska, A., Mikova, H., Rosenberg, M., and Ilavsky, M. (2000) *J. Microencapsul.*, **17**, 279-296.
116. Becka, S., Skrob, F., Plhachova, K., Kujan, P., Holler, P., and Kyslik, P. (2003) *Biotechnol. Lett.*, **25**, 227-233.
117. Upadhya, R., Nagajyothi, I., and Bhat, S. G. (2000) *Biotechnol. Bioeng.*, **68**, 430-436.
118. Zhu, T. B., Chen, J., Zhang, Y. F., Yang, Y. L., and Jiao, R. S. (2001) *Sheng Wu Gong. Cheng Xue. Bao.*, **17**, 73-77.
119. Vicenzi, J. T. (1995) *Deactivation of Catalase Enzyme Using D-Amino Acid Oxidase Enzyme – by Contacting Enzymes with Aqueous Basic Solutions at pH of 11 to 12*, Patent USA US392438, 22 Feb 1995.
120. Kujan, P., Prell, A., Safar, H., Holler, P., Plhachova, K., and Sobotka, M. (2001) *Folia Microbiol. (Praha)*, **46**, 427-431.
121. Molla, G., Motteran, L., Piubelli, L., Pilone, M. S., and Pollegioni, L. (2003) *Yeast*, **20**, 1061-1069.
122. Mateo, C., Abian, O., Fernandez-Lafuente, R., and Guisan, J. M. (2000) *Biotechnol. Bioeng.*, **68**, 98-105.
123. Dsouza, S. F., and Deshpande, A. (2001) *Appl. Biochem. Biotechnol.*, **95**, 83-92.
124. De la Matta, I., Ramon, F., Obregon, V. V., Castillon, M. P., and Acebal, C. (2000) *Enzyme Microb. Technol.*, **27**, 234-239.
125. Ju, S. S., Lin, L. L., Chien, H. R., and Hsu, W. H. (2000) *FEMS Microbiol. Lett.*, **186**, 215-219.
126. Khang, Y. H., Kim, I. W., Hah, Y. R., Hwangbo, J. H., and Kang, K. K. (2003) *Biotechnol. Bioeng.*, **82**, 480-488.
127. Monti, D., Carrea, G., Riva, S., Baldaro, E., and Frare, G. (2000) *Biotechnol. Bioeng.*, **70**, 239-244.
128. Inaba, Y., Mizukami, K., Hamada-Sato, N., Kobayashi, T., Imada, C., and Watanabe, E. (2003) *Biosens. Bioelectron.*, **19**, 423-431.
129. Voss, K., and Galensa, R. (2000) *Amino Acids*, **18**, 339-352.
130. Dominguez, R., Serra, B., Reviejo, A. J., and Pingarron, J. M. (2001) *Analyt. Biochem.*, **298**, 275-282.
131. Stefan, R. I., van Staden, J. K., and Aboul-Enein, H. Y. (2000) *Biosens. Bioelectron.*, **15**, 1-5.
132. Stefan, R. I., Bokretzion, R. G., van Staden, J. F., and Aboul-Enein, H. Y. (2003) *Biosens. Bioelectron.*, **19**, 261-267.
133. Patel, R. N. (2000) *Adv. Appl. Microbiol.*, **47**, 33-78.
134. Patel, R. N. (2001) *Biomol. Eng.*, **17**, 167-182.