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Inhibition of D-Amino Acid Oxidase Activity by Antipsychotic Drugs Evaluated by a Fluorometric Assay Using D-Kynurenine as Substrate

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A facile fluorometric assay using D-kynurenine as a substrate was utilized for evaluating the inhibition of D-amino acid oxidase (DAAO), which is one of the products of a susceptibility gene for schizophrenia, by commercial antipsychotic drugs, namely, chlorpromazine (CPZ), carbamazepine, sulpiride, quetiapine, and imipramine. CPZ inhibited DAAO ($65.8 \pm 13.2\,\mu$M, $n=3$) as reported previously, and other drugs also inhibited DAAO activity. Among these, quetiapine had the smallest IC_{50} value ($19.5 \pm 2.60\,\mu$M, $n=3$). The proposed assay can be useful for the evaluation or screening of DAAO-inhibitory drugs.

Key words——D-amino acid oxidase; antipsychotic drug; fluorescence; D-kynurenine

INTRODUCTION

It has been considered that endogenous D-serine acts as a co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor,[1] and plays an important role in regulating glutamatergic neurotransmission *via* the NMDA receptor. D-Serine is biosynthesized from L-serine by serine racemase (EC 5.1.1.16)[2,3] in neuronal and/or glial cells, and is decomposed in glial cells by D-amino acid oxidase (DAAO) (EC 1.4.3.3), which is a flavin-adenine dinucleotide (FAD)-containing enzyme.[4–6] With regard to schizophrenia, one of the severe psychiatric diseases, the ''glutamate hypothesis'' has recently been postulated; this hypothesis suggests that the etiology of schizophrenia may be associated with the hypofunction of the NMDA receptor.[7,8] There have been reports stating that DAAO is one of the products of a susceptibility gene for schizophrenia[9] and that the activity of brain DAAO, which degrades D-serine, was found to be increased in the post-mortem brain tissue of patients with schizophrenia.[10] In addition, we previously reported that the serum D-serine concentration was significantly reduced in patients with schizophrenia.[11] In 1998, Tsai *et al*. reported that co-administration of D-serine with atypical antipsychotic drugs improved the symptoms of patients with schizophrenia.[12] Considering these reports together, DAAO inhibition by an exogenous drug could be effective for the treatment of schizophrenia. Therefore, screening or pharmacological studies on compounds that inhibit DAAO activity have come to be performed in the last decade.[13–16]

In our previous study, we originally developed a fluorometric assay for DAAO inhibitors using D-kynurenine (D-KYN) as a substrate.[17] D-KYN is one of the D-amino acids, and is metabolized *in vitro*[17] or *in vivo*[18,19] by DAAO to produce kynurenic acid (KYNA)[20,21] that can emit fluorescence. After the enzymatic reaction of D-KYN with DAAO in the presence of a tested compound, a decrease degree in the fluorescence intensity emitted from KYNA indicates the ability of the tested compounds to inhibit DAAO. Compared with most other assay methods described previously, the proposed assay is facile, because it involves a one-step enzymatic reaction with DAAO. In the present study, some minor modifications were made to further simplify the assay procedure.

Among psychotropic drugs, chlorpromazine (CPZ), which has a planar tricyclic structure, is mainly prescribed to patients with schizophrenia. Although CPZ is a typical antipsychotic drug that blocks the dopamine D_2 receptor, it is known that CPZ has the ability to inhibit DAAO.[22–24] It has been reported that the mechanism by which CPZ can in-

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hibit DAAO activity was through the replacement of the cofactor FAD with CPZ.[23,24] Because most psychotropic drugs generally possess a planar tricyclic structure, other psychotropic drugs may have some DAAO-inhibitory activity. Therefore, we were interested in evaluating the inhibition of DAAO activity by other antipsychotic drugs prescribed to patients with psychiatric diseases.

Thus, in the present study, we tested the ability of commercially available psychotropic drugs to inhibit DAAO activity using the modified fluorometric assay.

MATERIALS AND METHODS

Materials Pig kidney DAAO (pkDAAO) was obtained from Calzyme Laboratories Inc. (San Luis Obispo, CA, USA). D-KYN, KYNA, 3-methylpyrazole-5-carboxylic acid (MPC)[25], 3-methylpyrazole-4-carboxylic acid, Trizma® base (min. 99.9%), bovine serum albumin (BSA), and chlorpromazine hydrochloride were obtained from Sigma (St. Louis, MO, USA). FAD was obtained from Nacalai Tesque (Kyoto, Japan). Zinc sulfate heptahydrate was obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan). (±)-Sulpiride, carbamazepine, and imipramine hydrochloride were from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). Quetiapine fumarate (Seroquel® 25 mg tablets) was obtained from Astellas Pharma Inc. (Tokyo, Japan). Water was purified using a Milli-Q system (Millipore Co. Ltd., Bedford, MA, USA). Dimethylsulfoxide (DMSO) was obtained from Nacalai Chemicals Ltd. (Tokyo, Japan) and Tokyo Chemical Industries Co. Ltd. (Tokyo, Japan), respectively. All other reagents were of reagent grade and were used without further purification. Benz[*e*]MTH-β-carboline[26] was synthesized in our laboratory in the Department of Synthetic Organic Chemistry.

DAAO Assay with D-KYN as a Substrate A DAAO assay was carried out according to the method in our previous paper[17] with a minor modification. We mixed 20 μl of 0.1 mg/ml DAAO in 0.4 M Tris buffer solution (pH 8.3), 30 μl of 200 μM FAD solution, 20 μl of 2.0 mg/ml BSA in H_2O, 390 μl of 0.4 M Tris buffer solution (pH 8.3), and 20 μl of tested drugs or compounds dissolved in DMSO and incubated this at 37°C for 20 min. Next, 7.0 mM D-KYN (20 μl) were added and incubated at 37°C for 60 min. In the case of determination of K_m, varying concentrations (1.4, 2.8, 4.2, 5.6, and 7.0 mM) of D-KYN (20 μl) were added in the presence or absence of the tested drugs, and incubated at 37°C for 60 min. The reaction mixture along with 1500 μl of 0.4 M Tris buffer solution (pH 8.3) and 50 μl of 300 mM zinc sulfate dissolved in H_2O. The final solution was subjected to vortex mixing, and the fluorescence of the solution was measured by a HITACHI F-7000 fluorescence spectrometer (Hitachi Co. Ltd., Tokyo, Japan). The fluorescence intensity of each solution was measured at an excitation wavelength of 250 nm and emission wavelength of 394 nm.

According to the following Eq. (1), the ΔF value was determined and used to calculate kinetic parameters (K_m) by using the Lineweaver-Burk plot:

$$\Delta F = F - F_0 \quad (1)$$

F and F_0 are fluorescence intensities of the sample and blank sample (a sample treated without D-KYN), respectively. For the inhibition curve, the final concentrations of the test compounds were plotted on the X-axis, and the ΔF value in the absence of the inhibitor was set as 100% on the Y-axis. The degree of inhibition for each test compound was expressed as a percentage according to the following Eq. (2):

$$\text{Remaining activity of pkDAAO (\%)} = \Delta F'/\Delta F \times 100 \quad (2)$$

$\Delta F'$ is the ΔF value at each concentration of the inhibitor.

Calculation and Statistical Analysis IC_{50} values of tested drugs were calculated by the following Eq. (3):

$$IC_{50} = 10^{[\log (A/B) \times (50-C)/(D-C) + \log B]} \quad (3)$$

A and B are the higher and lower concentrations near 50% inhibition, respectively, and C and D are the inhibition percentages at B and A, respectively. Statistical analysis of 2 groups was performed using the t test. A p value below 0.05 was considered significantly different.

RESULTS AND DISCUSSION

Modification of the Assay In our previous study, we developed a screening assay method to identify inhibitors of DAAO using D-KYN as substrate. Using the enzymatic assay, production of a fluorescence compound, namely, KYNA, from D-KYN was previously confirmed by fluorescence spectrum, mass spectrometry, and column-switching HPLC.[17] In the present study, Tris buffer (pH 8.3) and zinc sulfate were employed instead of borate buffer (pH 8.3) and zinc acetate, respectively. These

changes in the reagent produced no precipitation in the assay tubes when 300 mM zinc ion was added, thereby eliminating the need for a centrifugation step that removed the precipitate. Furthermore, no deproteinization procedure was performed, and the fluorescence originating from KYNA could be detected in the presence of proteins such as BSA and DAAO. Even though deproteinization was not performed, the obtained excitation and emission spectra were almost identical to those of KYNA, which exhibited 394–398 nm of optimum fluorescence with an excitation wavelength of 250–251 nm (Fig. 1).

This result confirmed the production of KYNA from D-KYN by DAAO. Next, the modified assay was tested by confirming whether DAAO was inhibited by a specific inhibitor such as MPC.[25] As expected, it was observed that MPC inhibited DAAO activity (Fig. 2(a)) and produced an IC_{50} value of 10.2± 2.15 μM (n=3, Table 1). In contrast to MPC, there has been no information on the pharmacological actions of 3-methylpyrazole-4-carboxylic acid, which is the geometric isomer of MPC, as well as the inhibition of DAAO activity. As shown in Fig. 2(b), 3-methylpyrazole-4-carboxylic acid showed very weak inhibition of DAAO activity. The proposed assay could discriminate the difference in the extent of inhibition between MPC and 3-methylpyrazole-4-carboxylic acid, and thus, can be used for evaluating DAAO activity. In total, the number of steps in the procedure was reduced in the modified assay, and therefore, this assay can be applied to high throughput screening in the future.

Inhibition of DAAO by Antipsychotic Drugs

Figure 3 shows the inhibition curves of DAAO activity for representative antipsychotic drugs. In addition to CPZ (Fig. 3(a)), the antipsychotic drugs quetiapine, sulpiride, carbamazepine, and imipramine (Fig. 3(b–e), respectively), which are currently prescribed to patients with schizophrenia, epilepsy, and depression, were tested.

As shown in Fig. 3, these antipsychotic drugs inhibited DAAO activity just as CPZ. Table 1 shows IC_{50} values of antipsychotic drugs that inhibit DAAO activity, as determined by the proposed assay method. The present data on IC_{50} of CPZ (*ca.* 66 μM) was considerably different from that of CPZ (*ca.* 1.9 mM) in the previous report.[17] The reason

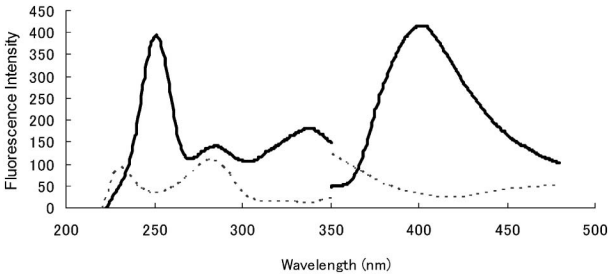

Fig. 1. Excitation and Emission Spectra of the Reaction Product with DAAO (solid line)
The dotted lines are those of samples without D-KYN.

Table 1. IC_{50} Values of Tested Compounds for the Inhibition of DAAO Activity by the Proposed Assay

Tested compounds	IC_{50} (μM) [a]
Chlorpromazine	65.8±13.2
Quetiapine fumarate	19.5±2.60
Sulpiride	85.4±2.18
Carbamazepine	262±53.4
Imipramine	31.1±7.03
3-Methylpyrazole-5-carboxylic acid (MPC)	10.2±2.15
3-Methylpyrazole-4-carboxylic acid	N.D.
Benz[*e*]MTH-β-carboline	72.2±19.1

[a] n=3. N.D.: not determined.

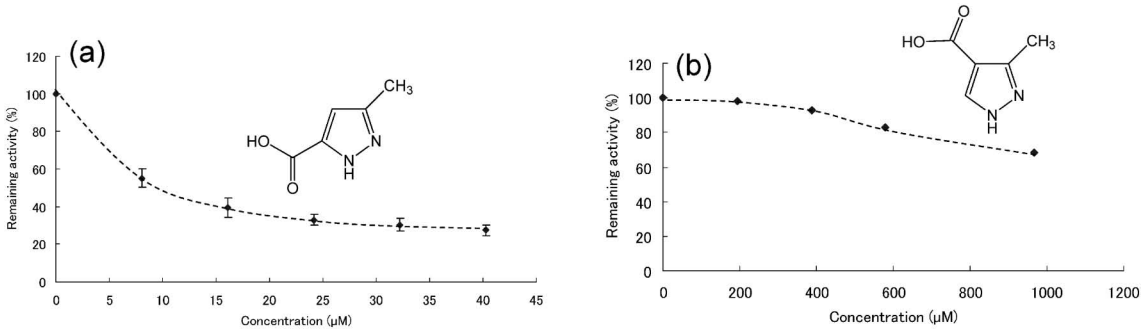

Fig. 2. Dose-dependent Inhibition Curves of pkDAAO by MPC (a) and 3-Methylpyrazole-4-carboxylic Acid (b)

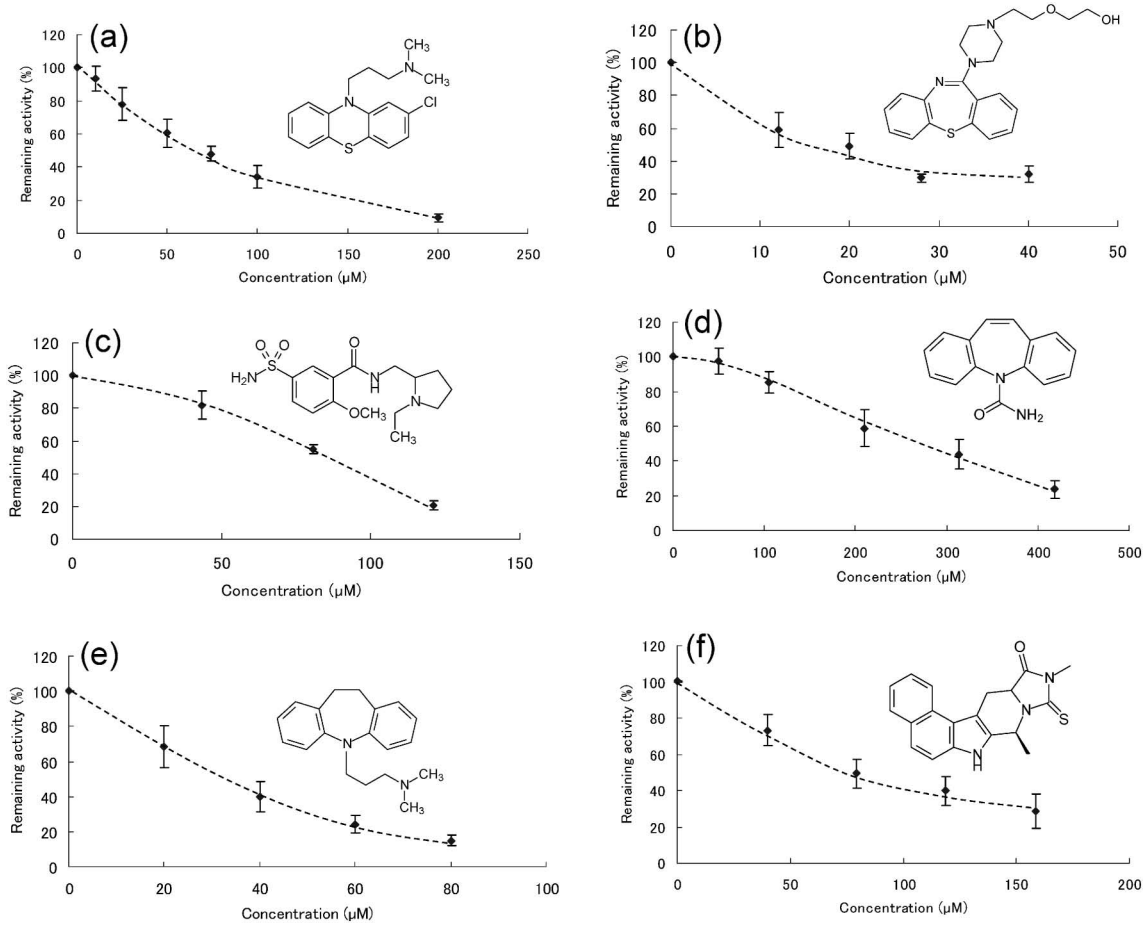

Fig. 3. Dose-dependent Inhibition Curves
pkDAAO by CPZ (a), quetiapine (b), sulpiride (c), carbamazepine (d), imipramine (e), and benz[*e*]MTH-*β*-carboline (f).

for the difference is not clear, but in the previous method, the IC_{50} value of CPZ might be imprecise, because some pretreatment steps for the determination were performed as described above. Among the antipsychotic drugs, quetiapine had the smallest IC_{50} values ($19.5 \pm 2.60\,\mu M$). With regard to the manner of inhibition, non-competitive inhibition between D-KYN (substrate) and quetiapine may occur, because no significant difference in K_m values in the presence ($137 \pm 20.5\,\mu M$) or absence ($164 \pm 14.0\,\mu M$) of 250 μM quetiapine was observed. With regard to CPZ, it was reported that DAAO activity was inhibited probably because of the replacement of FAD, which is a co-factor of DAAO, with CPZ that may occur during the enzymatic reaction.[22,23] Similar to CPZ, quetiapine may be able to replace FAD and thus inhibit DAAO activity because it has a tricyclic structure like CPZ. The precise mechanism for the replacement or binding of these drugs to the FAD binding site is still unclear, but structural differences between these drugs may produce the difference in IC_{50} observed in the present study.

In addition, benz[*e*]MTH-*β*-carboline[26] was originally a derivative of L-tryptophan, and in an *in vitro* screening assay, it was found to be an inhibitor of indole 2,3-dioxygenase (IDO),[27,28] which was responsible for cleavage at the 2,3-position of the indole skeleton. It has recently been reported that IDO is in some manner associated with diseases such as Alzheimer's disease[27] and cancer,[28] but the preclinical and clinical trials of benz[*e*]MTH-*β*-carboline have not yet been performed.

As shown in Fig. 3(f), benz[*e*]MTH-*β*-carboline was also found to inhibit DAAO activity. Benz[*e*]MTH-*β*-carboline possesses a planar polycyclic structure, and therefore, inhibited DAAO just like the antipsychotic drugs.

At present, much attention has been focused on the use of DAAO as a target drug for schizophrenia, and the creation of novel drugs that specifically inhibit

DAAO.[13–16,29] There have to date been few information on the DAAO inhibition activity of antipsychotic drugs tested in the present study. The present data suggest that the tested drugs can inhibit DAAO in the brain after they are administered. In order to ascertain this, analytical studies on the concentration of D-serine in biological specimens, including brain specimens, after treatment with these antipsychotic drugs should be performed. If these drugs have an ability to inhibit DAAO *in vivo*, DAAO-inhibitory activity will be an additional efficacy of these drugs in the pharmacotherapy for psychiatric diseases.

In conclusion, it was found that the proposed fluorescence assay in which D-KYN was used as a substrate was useful for evaluating the potential of drugs to inhibit DAAO activity, and will be useful for high throughput screening of DAAO inhibitors in the future.

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REFERENCES

1) Schell M. J., Molliver M. E., Snyder S. H., *Proc. Natl. Acad. Sci. USA*, **92**, 3948–3952 (1995).
2) Wolosker H., Sheth K. N., Takahashi M., Mothet J. P., Brady R. O., Ferris C. D., Snyder S. H., *Proc. Natl. Acad. Sci. USA*, **96**, 721–725 (1999).
3) Wolosker H., Blackshaw S., Snyder S. H., *Proc. Natl. Acad. Sci. USA*, **96**, 13409–13414 (1999).
4) Molla G., Sacchi S., Bernasconi M., Pilone M. S., Fukui K., Pollegioni L., *FEBS Lett.*, **580**, 2358–2364 (2006).
5) Khoronenkova S. V., Tishkov V. I., *Biochemistry (Moscow)*, **73**, 1511–1518 (2008).
6) Kawazoe T., Park H. K., Iwana S., Tsuge H., Fukui K., *Chem. Rec.*, **7**, 305–315 (2007).
7) Paz R. D., Tardito S., Atzori M., Tseng K. Y., *Eur. Neuropsychopharmacol.*, **18**, 773–786 (2008).
8) Lindsley C. W., Shipe W. D., Wolkenberg S. E., Theberge C. R., Williams D. L., Sur C., Kinney G. G., *Curr. Top. Med. Chem.*, **6**, 771–785 (2006).
9) Boks M. P. M., Rietkerk T., van de Beek M. H., Sommer I. E., de Koning T. J., Kahn R. S., *Eur. Neuropsychopharmacol.*, **17**, 567–572 (2007).
10) Madeira C., Freitas M. E., Vargas-Lopes C., Wolosker H., Panizzutti R., *Schizophrenia Res.*, **101**, 76–83 (2008).
11) Hashimoto K., Fukushima T., Shimizu E., Komatsu N., Watanabe H., Shinoda N., Nakazato M., Kumakiri C., Okada S., Hasegawa H., Imai K., Iyo M., *Arch. Gen. Psychiatry*, **60**, 572–576 (2003).
12) Tsai G., Yang P., Chung L. C., Lange N., Coyle J. T., *Biol. Psychiatry*, **44**, 1081–1089 (1998).
13) Smith S. M., Uslaner J. M., Hutson P. H., *Open Med. Chem. J.*, **4**, 3–9 (2010).
14) Ferraris D., Duvall B., Ko Y. S., Thomas A. G., Rojas C., Majer P., Hashimoto K., Tsukamoto T., *J. Med. Chem.*, **51**, 3357–3359 (2008).
15) Duplantier A. J., Becker S. L., Bohanon M. J., Borzilleri K. A., Chrunyk B. A., Downs J. T., Hu L.Y., El-Kattan A., James L. C., Liu S., Lu J., Maklad N., Mansour M. N., Mente S., Piotrowski M. A., Sakya S. M., Sheehan S., Steyn S. J., Strick C. A., Williams V. A., Zhang L., *J. Med. Chem.*, **52**, 3576–3585 (2009).
16) Sparey T., Abeywickrema P., Almond S., Brandon N., Byrne N., Campbell A., Hutson P. H., Jacobson M., Jones B., Munshi S., Pascarella D., Pike A., Prasad G. S., Sachs N., Sakatis M., Sardana V., Venkatraman S., Young M. B., *Bioorg. Med. Chem. Lett.*, **18**, 3386–3391 (2008).
17) Song Z., Ogaya T., Ishii K., Ichiba H., Iizuka H., Fukushima T., *J. Health Sci.*, **56**, 341–346 (2010).
18) Fukushima T., Sone Y., Mitsuhashi S., Tomiya M., Toyo'oka T., *Chirality*, **21**, 468–472 (2009).
19) Ogaya T., Song Z., Ishii K., Fukushima T.,

Neurochem. Res., **35**, 559–563 (2010).
20) Swartz K. J., During M. J., Freese A., Beal M. F., *J. Neurosci.*, **10**, 2965–2973 (1990).
21) Hilmas C., Pereira E. F. R., Alkondon M., Rassoulpour A., Schwarcz R., Albuquerque E. X., *J. Neurosci.*, **21**, 7463–7473 (2001).
22) Yagi K., Nagatsu T., Ozawa T., *Nature.*, **177**, 891–892 (1956).
23) Yagi K., Ozawa T., Nagatsu T., *Biochim. Biophys. Acta*, **43**, 310–317 (1960).
24) Iwana S., Kawazoe T., Park H. K., Tsuchiya K., Ono K., Yorita K., Sakai T., Kusumi T., Fukui K., *J. Enzyme Inhib. Med. Chem.*, **23**, 901–911 (2008).
25) Adage T., Trillat A. C., Quattropani A., Perrin D., Cavarec L., Shaw J., Guerassimenko O., Giachetti C., Greco B., Chumakov I., Halazy S., Roach A., Zaratin P., *Eur. Neuropsychopharmacol.*, **18**, 200–214 (2008).
26) Takikawa O., Akimoto H., Okuno H., Yokoyama Y., Japan Patent 2007–041962 (2007).
27) Takikawa O., *Biochem. Biophys. Res. Commun.*, **338**, 12–19 (2005).
28) Macchiarulo A., Camaioni E., Nuti R., Pellicciari R., *Amino Acids*, **37**, 219–229 (2009).
29) Caldinelli L., Molla G., Bracci L., Lelli B., Pileri S., Cappelletti P., Sacchi S., Pollegioni L., *Protein Sci.*, **19**, 1500–1512 (2010).