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Food Chemistry 100 (2007) 616–622
Food Chemistry
www.elsevier.com/locate/foodchem

Screening of D-amino acid oxidase inhibitor by a new multi-assay method

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Received 20 June 2005; received in revised form 30 September 2005; accepted 30 September 2005

Abstract

This paper describes an investigation of the inhibition of a D-amino acid oxidase (DAAO) activity in several kinds of food additives and fruit. To screen for an inhibitor of DAAO, the method employed checked for two kinds of indicators, namely, pyruvic acid (indicator-1) and hydrogen peroxide (indicator-2), both of which are formed by digestion by DAAO of D-alanine (the standard D-amino acid used in this study). For measurement of pyruvic acid, a reliable and authenticated method was employed: the presence of pyruvic acid was determined by converting it into a chromophore derivative by tagging with 2,4-dinitro phenylhydrazine (DNP), followed by measurement of absorbance at 445 nm. The pyruvic acid thus determined is referred to as "(colorimetric) indicator-1". To measure hydrogen peroxide, a highly sensitive fluorogenic substrate, 3-(*p*-hydroxyphenyl)propionic acid (HPPA), was employed in the present study. Hydrogen peroxide was detected by measuring fluorescence intensity (Ex. = 320 nm, Em. = 405 nm) of 2,2′-dihydroxybiphenyL-5,5′-dipropionic acid (oxidize-HPPA), which is produced by horseradish peroxidase (HRP)-catalyzed oxidation of HPPA with hydrogen peroxide. The hydrogen peroxide thus determined is referred to as "(flourometric) indicator-2" After optimizing the multi-assay procedures, the inhibition of DAAO activity against D-alanine (used as the standard D-amino acid) in 11 kinds of food additives and five kinds of fruit was evaluated, first in terms of indicator-1. Then, two substrates, potassium sorbate and apple juice, that were screened by indicator-1, were further evaluated by indicator-2 to conform the inhibition of DAAO activity. This is the first demonstration of the inhibition of DAAO activity by potassium sorbate, which is widely used as a synthetic preservative in food and apple juice.

Keywords: D-Amino acid; D-Amino acid oxidase; Horseradish peroxidase; Inhibitor; Food additive(s); 3-(*p*-Hydroxyphenyl)propionic acid; 2,4-Dinitrophenylhydrazine

1. Introduction

It has generally been believed that no D-amino acid (DAA) of any kind existed in the bodies of mammals. Recently, however, several kinds of DAAs were, indeed, found to be present in mammalian bodies (Dunlop, Neidle, McHale, Dunlop, & Lajtha, 1986; Hashimoto et al., 1992). By contrast, the flavoenzyme D-amino acid oxidase (DAAO, EC 1.4.3.3), which is associated with conversion of DAAs to their corresponding α-keto acid, was found by Krebs (1935). Since then, DAAO has become recognized as being widely present in mammals, and is characterized as a peroxisomal enzyme and a prototypical member of the glutathione reductase family of flavoproteins. DAAO (from yeast) is a stable homodimer with a flavin adenine dinucleotide (FAD) molecule tightly bound to each 40 kDa subunit (Pilone, 2000). Although the biological roles of neither DAAO nor of DAAs, themselves, have yet been completely clarified, our understanding of their physiological importance is gradually increasing. For example, a recent study suggested that the brain-expressed genes for G72 and DAAO exert an influence on susceptibility to schizophrenia (Schumacher et al., 2004). Hasegawa, Matsukawa, Shinohara, Konno, and Hashimoto (2004) studied the metabolic pathway of DAAs by using

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doi:10.1016/j.foodchem.2005.09.076

the stable isotope tracer technique of D-[(2)H(7)]leucine. Their results indicated that DAAO was indispensable for metabolizing some kinds of exogenous DAAs, such as D-alanine, for nutritional purposes in the mammalian body. Konno et al. (1993) reported that the urine of mutant ddY/DAO-mice lacking DAAO contained 13 times more D-alanine than that of normal ddY/DAO+ mice. They explained that D-alanine is a component of bacterial cell walls, and, the possibility that the urinary D-alanine came from intestinal bacteria was examined. In ddY/DAO- mice, which were born germ-free and reared in a germ-free environment, the level of urinary D-alanine was found to be lower than to that of normal mice. Oguri, Kumazaki, Kitou, Nonoyama, and Tooda (1999) demonstrated that exogenous DAAs are as easily absorbed as L-amino acids at small intestinal sites using a sodium ion-dependent transport system on the brush border membrane of the intestinal cells. Given this background, we believe that one of the physiological functions of DAAO, present in the mammalian body, is to be a "scavenger" of exogenous DAAs, thereby regulating the level of endogenous DAAs in the body. We speculate that, should some inhibitors of DAAO be present in food, a homeostatic imbalance in the natural level of DAAs might be caused. Actually, it has been reported that some kinds of DAAs are toxic (Friedman & Gumbmann, 1984; Ganote, Peterson, & Carone, 1974). Although some inhibitors have been found (Miyano et al., 1991; Moreno, Montes, Catalan, & Galan, 1996; Nohara, Suzuki, Kinoshita, & Watanabe, 2002; Tanaka, Bamji, & Yagi, 1978), detailed investigations regarding their presence in food have not yet been reported.

DAAO activity or inhibition of DAAO activity is sometimes determined by measuring amounts of hydrogen peroxide formed by enzymatic degradation of DAAs by DAAO. If a reductant, such as an antioxidant, exists in a subject being screened for DAAO inhibition activity, the apparent amount of hydrogen peroxide present will be less than the actual value because the hydrogen peroxide would be reduced. Therefore, the indicated DAAO activity, as determined from the amount of hydrogen peroxide alone, is inaccurate. Moreover, there is a method of measuring of alpha-keto acids which is another reaction product from DAA/DAAO reaction. However, measurement of this alpha-keto acid might also lead to errors. As it is already known that alpha-keto acids or pyruvic acid, synthesized by enzymatic degradation of amino acids (Morata, Gomez-Cordoves, Colomo, & Suarez, 2003), is widely distributed in fermented foods such as wine, cheese and Japanese *Sake*. When the sample is relatively rich in alpha-keto acids, it is sometimes difficult to read the difference between blank and sample absorbance values. Additionally, it is known that DAAO is inactivated by some alpha-keto acids, such as pyruvic acid or amino acids (Moreno et al., 1996).

Those factors lead to unreliable information. To prevent this mistake from occurring, the determination of DAAO inhibition activity should be done by double-checking for two kinds of indicators, namely pyruvic acid (indicator-1) and hydrogen peroxide (indicator-2). For this reason, we modified the standard assay methods for the determination of pyruvic acid (D'Aniello et al., 1993) and hydrogen peroxide (Zaitsu & Ohkura, 1980) to meet the requirements of the present study. First, the inhibition of DAAO activity against D-alanine was screened by means of indicator-1 in eleven kinds of food additives and five kinds of fruits. Next, the substrates that were screened as inhibitors were further evaluated with indicator-2. Finally, to confirm the inhibition activity by potassium sorbate in this first finding, kinetic studies of DAAO activity were done and the results compared with sodium benzoate, is a well-known inhibitor of DAAO.

2. Materials and methods

2.1. Chemicals

DAAO (from hog kidney) and HRP (from horseradish root) were obtained form Boehringer–Mannheim GmbH (Germany) and Oriental Yeast Co., Ltd. (Tokyo, Japan), respectively. D-Alanine, HPPA and the other reagents were purchased from Wako Pure Chem. Co. (Tokyo, Japan), and, were of the highest purity or HPLC grade commercially available. They were used without further purification. All aqueous solutions were prepared using water purified with a Milli-Q purification system (Millipore, Milford, MA. USA).

2.2. Apparatus

Absorbance and fluorescence intensity were measured with a Hitachi U-2000 spectrometer (Tokyo, Japan) and a Hitachi F-3010 fluorescence spectrometer in a 3 ml volume quartz cell, respectively.

2.3. Sample preparation

Fresh fruit was squeezed and its juice was diluted twice with 0.2 M Tris–HCl buffer (pH 8.3), and filtered with a 0.45 μm diameter disposable syringe filter unit (DISMIC-13cp; Advantec Toyo; Tokyo, Japan). The pH of the sample solution was adjusted to around pH 8 with either 1 M hydrogen chloride or 1 M sodium hydroxide, as required. The filtrate was placed in a refrigerator until just before using. The food additive sample solution was prepared by dissolving with 0.2 M Tris–HCl buffer (pH 8.3) to make 100 μM.

2.4. Measuring colorimetric and fluorometric indicators

Two kinds of indicators for this study were obtained by following standard operation procedures: 5.0 ml of 5 mM D-alanine (dissolved in 0.2 M Tris–HCl buffer, pH 8.3) were mixed with 200 μl of a sample solution (containing

an inhibitor), which was divided into two portions in order to measure both pyruvic acid and hydrogen peroxide, as described next. Using indicator-1, e.g., the presence of pyruvic acid, was determined according to the described method (D'Aniello et al., 1993), but with the following modifications: a 2.0 ml portion of the mixture and 100 μl of DAAO solution (10 U/ml of 0.2 M Tris–HCl buffer, pH 8.2) were mixed and incubated at 37 °C for 30 min. Then, 200 μl of 1 mM 2,4-dinitro phenylhydrazine (DNP) (dissolved in 1 M HCl) were added to the solution and further incubated at 37 °C for 10 min. Subsequently, 800 μl of 1.5 M NaOH were added, mixed, and, after an additional 10 min of incubation time, the absorbance (As) was read at 445 nm against a sample blank consisting of the same assay mixture, but without the D-alanine (using 0.2 M Tris HCl buffer, pH 8.3, instead of D-alanine). A general blank (Ab) was also measured under the same conditions, but with a 0.2 M Tris–HCl buffer (pH 8.2) instead of the sample solution. The colorimetric indicator-1 (Ci) was obtained by the following equation:

$$Ci = As/Ab.$$

Using indicator-2, e.g., the presence of hydrogen peroxide, was obtained as follows. A 100 μl portion of a mixture consisting of 5 μl of DAAO solution (10 U/ml of 0.2 M Tris–HCl buffer, pH 8.3), 100 μl of HRP (10 U/ml of 0.2 M Tris–HCl buffer, pH 8.3), 100 μl of 20 mM HPPA (dissolved in 0.2 M Tris–HCl buffer, pH 8.3), and 2.0 ml of 2 M Tris–HCl buffer (pH 8.3), was incubated at 37 °C for 30 min. Then, fluorescence intensity (Fs) was measured at 405 nm by irradiation a 320 nm against a sample blank consisting of the same assay mixture but without the D-alanine (using 0.2 M Tris HCl buffer, pH 8.3, instead of D-alanine). A general blank (Fb) was measured under the same conditions, but with a 0.2 M Tris–HCl buffer (pH 8.2) instead of the sample solution. The fluorometric indicator-2 (Fi) was obtained by the following equation:

$$Fi = Fs/Fb.$$

Although, in the assay for DAAO, FAD was generally included in the reaction mixture because this co-factor is easily dissociated from the holoenzyme, the present method was performed without FAD.

3. Results and discussion

Many kinds of oxidase are present in mammalian body and they carry out many important biological functions. Examination of their enzymatic activities is usually done by determining the presence of hydrogen peroxide, which is produced by oxidation of a substrate. If some reductant is present in the sample matrix, the amount of hydrogen peroxide detected is much less than the actual amount. Therefore, we re-designed our assay method to study the inhibition of DAAO activity in such a way as to prevent this problem. Our assay scheme was based on measuring two kinds of indicators, namely, pyruvic acid and hydrogen peroxide, as shown in the chemical-reaction pathway in Fig. 1. D-Alanine (as the standard DAA used in this study) is oxidized with DAAO-catalyzed reactions, followed by the production of pyruvic acid (alpha-keto acid), hydrogen peroxide and ammonia. Indicator-1 was obtained by measuring the resulting pyruvic acid after derivatizing with DNP. Indicator-2 was a measurement of fluorescence

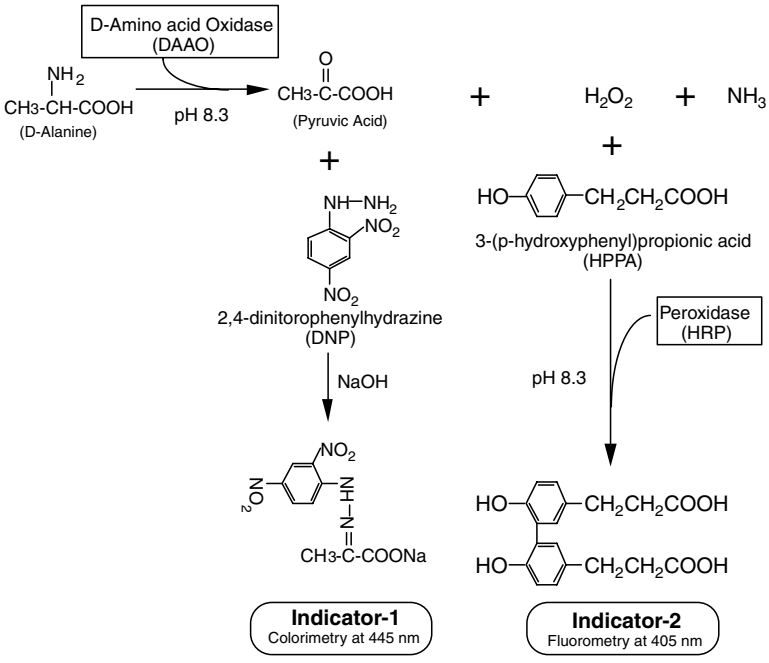

Fig. 1. Scheme showing the principles underlying the assay method inhibition activity of DAAO against D-alanine using two kinds of indicators (colorimetric and fluorometric).

intensity of 2,2′-dihydroxybiphenyL-5,5′-dipropionic acid (oxidize-HPPA), which was delivered by the HRP-catalyzed oxidation of HPPA with hydrogen peroxide. There are various methods by which to measure the amount of hydrogen peroxide, e.g., absorption spectrophotometry (Alexandre, Telegdi, Boutelet, Julien, & Vincent, 1992; Brown & Jenke, 1987; Tietjen & Mancott, 1971), fluorometry, (Keston and Brandt, 1965), luminometry (Ishida, Arakawa, Takada, & Yamaguchi, 1995) and polarography (Borggaard, 1972; Dempsey, O'Leary, & Condon, 1975). In order to achieve high-sensitivity detection of hydrogen peroxide, Zaitsu and Ohkura (1980) found a horseradish HRP-catalyzed reaction to be a suitable fluorogenic substrate HPPA for the fluorometric assay of hydrogen peroxide. In this study, HPPA was chosen as the fluorogenic substrate, and the assay method was optimized as follows.

3.1. Optimization of the method for manifesting the colorimetric indicator (indicator-1)

To obtain the two indicators, optimization of enzymatic digestion of D-alanine with DAAO should be done first. Although the oxidation of D-alanine with DAAO has been reported, the optimal conditions for quantitative determinations have not been described in detail (D'Aniello et al., 1993). Ten units per millilitre of DAAO solution (100 μl) were incubated with D-alanine (5.0 ml), the concentration of which varied over a range from 0.1 to 10 mM at 37 °C for 30 min, in order to determine the condition of complete enzyme reaction between D-alanine and DAAO. The reaction was completed when the D-alanine solution was used at a concentration of 1.0 mM, as shown in Fig. 2(a). To optimize the conditions of the reaction, the effects of the concentration (or number of units) of DAO (unit/ml), reaction time/temperature and concentration of DNP on the absorbance at 445 nm, as the colorimetric parameter, were investigated and those results are shown in Fig. 2(b)–(d), respectively. Consequently, the enzyme reaction was optimized when the concentration of DAO units, reaction time and concentration of DNP were 1 unit/ml, 30 min at 37 °C and 1 mM, respectively.

3.2. Optimization of the method for manifesting the fluorometric indicator (indicator-2)

Calculation of (fluorometric) indicator-2, requires carrying out a two-step enzymatic digestion. Using the same conditions of 5 mM D-alanine (100 μl) digestion with

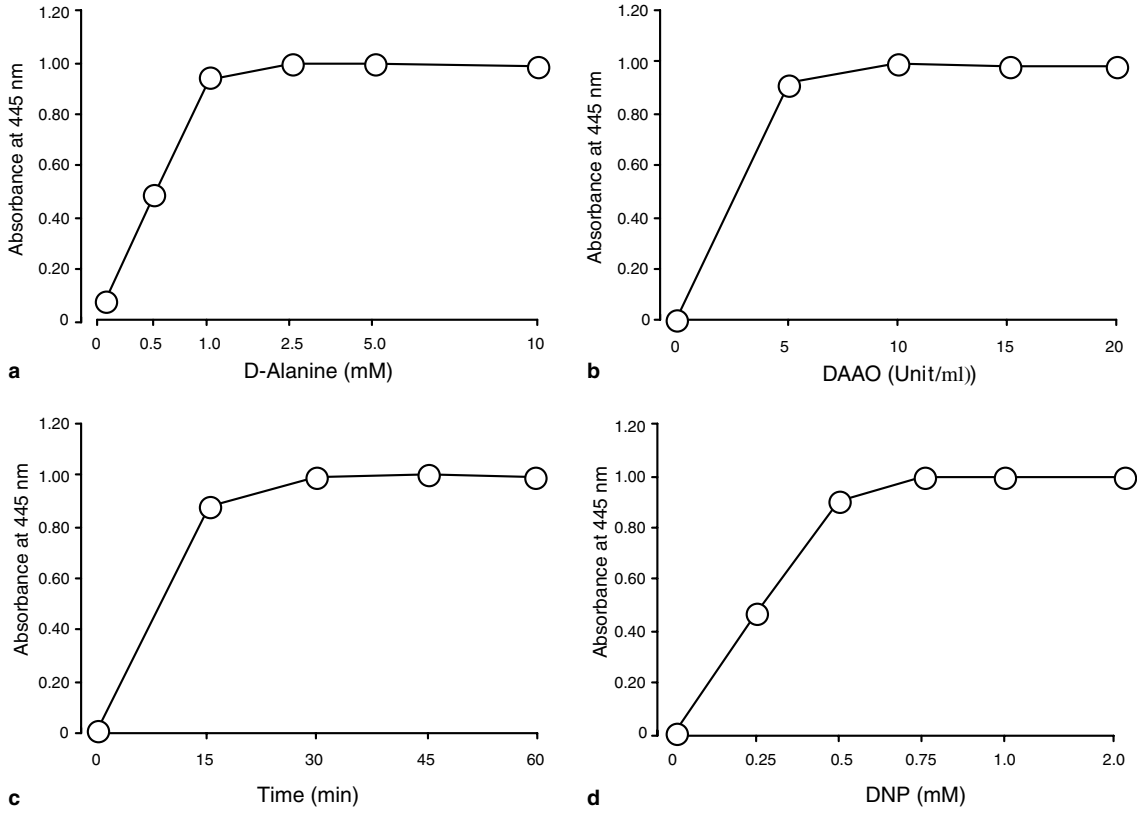

Fig. 2. Optimization of the DAAO reaction with D-alanine and the derivatization reaction of pyruvic acid with DNP for indicator-1. The following conditions were optimized by measuring the absorbance of DNP-pyruvic derivative at 445 nm: (a) the effect of D-alanine concentration (10 U/ml of DAAO, 1 mM DNP, and 30 min at 37 °C for the reaction time); (b) the effect of DAAO concentration (5 mM D-alanine, 1 mM DNP and 30 min at 37 °C for the reaction time); (c) the effect of reaction time of DAAO digestion (5 mM D-alanine, 10 U/ml DAAO, 1 mM DNP and 37 °C for the reaction) and (d) the effect of DNP concentration (5 mM D-alanine, 10 U/ml DAAO, and 30 min at 37 °C for the reaction time).

10 unit/ml DAAO (5 μl) in 2.0 ml of 0.2 M Tris–HCl (pH 8.3) for 30 min at 37 °C, as optimized above, next, the conditions for the HRP-catalyzed oxidation of HPPA with peroxide formed by the former reaction of D-alanine and DAAO were optimized, as shown in Fig. 3. The effect of the concentration (or number of units) of HRP (units/ml), HRP-catalyzed oxidation time at 37 °C and concentration of HPPA on fluorometric intensity of oxidized-HPPA were studied by measuring the fluorescence intensity at a wavelength of 405 nm after irradiating the sample with light at 320 nm, and the results are shown in Fig. 3(a)–(c), respectively. Consequently, the conditions of 10 U/ml of HRP for 20 min at 37 °C and 20 mM HPPA in 0.2 M Tris–HCl buffer (pH 8.3) were chosen. Although the optimum pH of the HRP reaction is known to be around 6, pH 8.3 was chosen for this reaction, because the optimum pH of 8.3 for the DAAO reaction was matched with that of the HRP reaction.

To measure the fluorometric indicator, first, a two-step enzyme reaction was carried out, namely, a DAAO-catalyzed oxidation of D-alanine, followed by an HRP-catalyzed oxidation of HPPA with hydrogen peroxide. Then, this two-step enzyme reaction was simplified to a one-step reaction, in which both DAAO and HRP-catalyzed oxidation reactions were carried out simultaneously by mixing of 5 mM of D-alanine (100 μl), 10 U/ml of DAAO (5 μl), 10 U/ml of HRP (100 μl) and 20 mM HPPA (100 μl) in 2.0 ml of 0.2 M Tris–HCl buffer (pH 8.2) at 37 °C for 30 min. Comparing the fluorescence intensities obtained from both the one-step and two-step reactions, by varying the concentration of D-alanine from 0.05 to 1.0 mM, confirmed that the two methods produced similar results. Fig. 3(d) shows the results of the comparison of the one-step and the two-step reactions, as indicated by closed circles and open circles, respectively. As can be seen, the one-step reaction is almost equivalent to the two-step reaction; hence, because of its greater simplicity, the one-step reaction was employed in this study to obtain the fluorescence indicator-2.

3.3. Validation of the methodology

To validate the present methodology, the reproducibilities of both colorimetric and fluorometric indicators were obtained, separately, by measuring the absorbance of DNP-pyruvic acid derivative and the fluorescence intensity of oxidized-HPPA both with and without a 100 μM sodium benzoate solution (as the verified inhibitor of

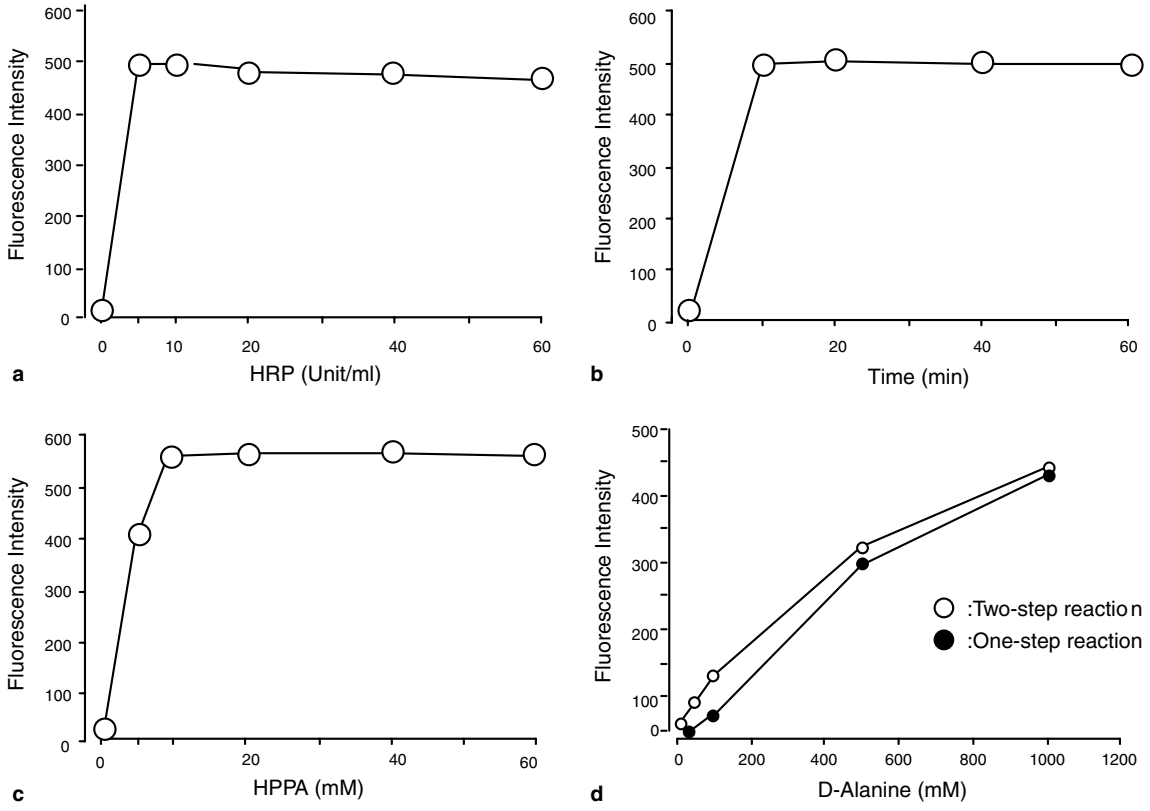

Fig. 3. Optimization of DAAO digestion prior to HRP/HPPA reaction for indicator-2. The following conditions were optimized by measuring the fluorescence intensity (Em. = 405 nm) of the resulting HPPA-pyruvic derivative at 430 nm: (a) the effect of HRP (units/ml) (20 mM HPPA, and 30 min at 37 °C for the reaction time); (b) the effect of HRD reaction time at 37 °C (10 U/ml HRP and 20 mM HPPA); (c) the effect of HPPA concentration (10 U/ml HRP, 20 mM HPPA, and 30 min at 37 °C for the reaction time). (d) Also shown is a comparison of the performance of the one-step reaction vs. the two-step reaction. Both conditions were described in Section 2.

Table 1
Reproducibility test

	1	2	3	4	5	Av.	c.v(%)
Indicator-1*	0.503	0.507	0.513	0.498	0.488	0.5018	1.69
Indicator-2**	0.738	0.776	0.728	0.735	0.701	0.7356	3.27

* Each value represents Ci = As/Ab in the presence of 100 mM sodium benzoate as an inhibitor.
** Each value represents Fi = Fs/Fb in the presence of 100 mM sodium benzoate as an inhibitor. Refer to Section 2 for further details.

DAAO) being added to the 5 mM D-alanine solution. Both indicator-1 and -2, after 5 repetitions, showed 0.502 (1.69% of c.v.) and 0.736 (3.27% of c.v.), respectively (Table 1).

Table 2
DAAO inhibition activity in food

Sample	Indicator-1*
Caffeine	0.84
Potassium phthalate	0.95
Potassium sorbate	0.67
Soduium acetate	0.87
Sodium benzoate	0.50
Sodium citrate	0.92
Sodium dehydroacetate	0.94
Sodium D,L-malate	0.92
Sodium lactate	0.87
Vitamin B2	1.16
Vitamin C	0.84
Apple juice	0.61
Cherry juice	1.00
Grape juice	0.96
Kiwi juice	0.87
Watermelon juice	1.02

* Refer to Section 2.

Table 3
DAAO inhibition activity in food

Sample	Indicator-2*
Potassium sorbate	0.79
Sodium benzoate	0.73
Apple juice	0.13
Vitamin C	0.09

* Refer to Section 2.

3.4. Screening for DAAO inhibitors in food

Inhibition of DAAO activity against various food additives and fruits was investigated by using the present methods. The methods described above were next employed to investigate the inhibition of DAAO activity in samples containing various food or fruit substances. Table 2 shows the results of indicator-1 with eleven kinds of food additives and five kinds of fruits. These results indicated that potassium sorbate (0.67), sodium benzoate (0.50) and apple (0.61) were obviously less than the 1.0 of indicator-1. Then, three samples plus vitamin C (sodium ascorbate, 0.83) were further examined for DAAO inhibition activity by means of indicator-2. Indicator-2 values for the three samples, potassium sorbate, sodium benzoate and apple juice, were shown to be 0.79, 0.73 and 0.13, respectively, as shown in Table 3. Indicator-2 for vitamin C shows 0.09, which is the lowest value in Table 3, Indicator-1, however, is 0.83 in Table 2. We thus concluded that vitamin C has little inhibition potency against D-alanine because vitamin C plays a role of antioxidant against hydrogen peroxide.

Most notably, with regard to apple juice, indicators 1 and 2 were 0.65 and 0.13, respectively. However, just what kind of component in apple juice inhibits DAAO activity against D-alanine has not yet been identified. This result taught us that there are many more inhibitors than we expected.

According to the above results, potassium sorbate, like sodium benzoate, also shows inhibition of DAAO. To confirm this inhibition activity with potassium sorbate in this first finding, the kinetic studies of DAAO activity were carried out by means of a reciprocal plot based on the data of

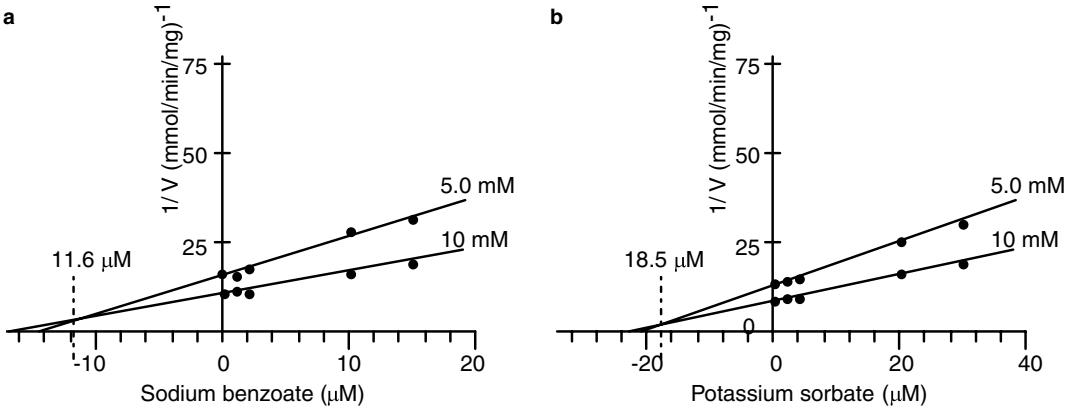

Fig. 4. The reciprocal activity was plotted against the concentration of sodium benzoate (a) and potassium sorbate (b). The DAAO (1 unit) activity was measured by following the decrease in the absorbance of the resulting DNP-pyruvic acid at 445 nm during 15 min at 37 °C according to the method for indicator-1 described in Section 2. D-Alanine, as a substrate, was added as indicated in the figure.

indicator-1 regarding DAAO activity. Competitive inhibition constants (K_i) for sodium benzate and potassium sorbate were 11.6 and 18.5 μM against D-alanine, respectively, as calculated from Fig. 4 by means of the Dixon-plot method (Dixon, 1953). In comparison with the data for sodium benzoate ($K_i = 9$ μM), as reported by Miyano et al. (1991), the present K_i for sodium benzoate was almost the same. In addition, K_i for potassium sorbate was 1.6 times greater than that of sodium benzoate. This result shows that the inhibition activity of potassium sorbate on DAAO is less than that of sodium benzoate, which accorded with our data obtained from Fig. 4.

4. Conclusions

One hypothesis regarding the physiological function of D-amino acid oxidase (DAAO) is that it helps to maintain appropriate levels of D-amino acids (DAAs) in the mammalian body. If, perchance, some inhibitor of DAAO were to exist in food or other substances, ingestion might cause some homeostatic imbalances to occur in the level of DAAs in the body. Although some inhibitors have previously been found, their presence in food has not been investigated in detail. Because there are so many different components in food, any one or more of them might interfere with the attempt to measure real DAAO activity. To overcome this problem, an accurate and convenient method for the screening for DAAO inhibitors was designed on the basis of the determination of the presence of pyruvic acid and hydrogen peroxide, formed by digestion of D-alanine with DAAO. In this paper, we have demonstrated the ability to determine the presence of DAAO inhibitors in various kinds of food or food additives. Although the present experiment was performed in vitro, potassium sorbate, which is allowed for use as a preservative in numerous processed foods, inhibits DAAO at an activity rate of 0.5-fold that of sodium benzoate activity against D-alanine. Furthermore, it was found that apple juice also inhibits DAAO activity for D-alanine. These new findings suggest that a DAAO inhibitor of one sort or another is present in our daily food intake to a significant degree. Based on this finding we recommend that further studies be done to clarify whether or not these kinds of inhibitors effect the homeostatic balance of DAA levels in the mammalian body.

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