Inhibitory effect of enzymatic browning products on trypsin activity

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Efecto inhibitorio de los productos del pardeamiento enzimático sobre la actividad de la tripsina

Efecte inhibitori dels productes d'embruniment enzimàtic sobre l'activitat de la tripsina

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RESUMEN

El objetivo de este trabajo fue evaluar si las melaninas sintetizadas a partir de polifenol oxidasa de *Agaricus bisporus* y uno de sus principales sustratos (L-tirosina) actúan como inhibidores de la enzima pancreática tripsina y, por tanto, pueden tener un efecto tóxico en la digestión proteica. Se encontró que estos polímeros disminuyen la afinidad aparente entre la enzima y su sustrato (Nα-benzoyl-L-Arginine ethyl ester, BAEE). Además, la velocidad máxima de reacción (r_{max}) disminuye con el incremento de la concentración de melanina (hasta un 32,6% añadiendo 0,07 mg·mL^{-1}). Se puede concluir que la presencia de melaninas inhibe la actividad de la tripsina de forma mixta. La constante alpha (α) resultó ser 2,95.

Palabras clave: pardeamiento enzimático; inhibición enzimática, digestión, tripsina, melaninas.

SUMMARY

The aim of this piece of work was to assay if the melanins synthesized from *Agaricus bisporus* polyphenol oxidase and one of its main substrates (L-tyrosine) act as trypsin inhibitors, and therefore may have a toxic effect on protein digestion. It was found that these polymers decrease apparent affinity between trypsin and its substrate (Nα-benzoyl-L-arginine ethyl ester, BAEE). In addition, the maximum reaction rate (r_{max}) decreases with the increase of melanin concentration (up to 32.6% adding 0.07 mg·mL^{-1}). It can be concluded that the presence of melanins from L-tyrosine inhibits trypsin activity in a mixed way. Alpha (α) constant was found to be 2.95.

Keywords: enzymatic browning; enzyme inhibition; digestion; trypsin; melanins.

RESUM

L'objectiu d'aquest treball fou avaluar si les melanines sintetitzades a partir de polifenol oxidasa d'*Agaricus bisporus* i un dels seus principals substrats (L-tirosina) actuen com a inhibidors de l'enzim pancreàtic tripsina i, per tant, poden tenir un efecte tòxic en la digestió proteica. Es va trobar que aquests polímers disminueixen l'afinitat aparent entre l'enzim i el seu substrat (Nα-benzoyl-L-arginine ethyl ester, BAEE). A més a més, la velocitat màxima de reacció (r_{max}) disminueix amb l'increment de la concentració de melanina (fins a un 32,6% afegint-ne 0,07 mg·mL^{-1}). Es pot concloure que la presència de melanines inhibeix l'activitat de la tripsina de manera mixta. La constant alpha (α) resultà 2,95.

Paraules clau: embruniment enzimàtic; inhibició enzimàtica; digestió; tripsina; melanines.

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1.INTRODUCTION

Enzymatic browning products are a heterogeneous group of polymers formed by polyphenol oxidase action in vegetable tissues containing phenolic or polyphenolic molecules, which have been considered up to the present substances that produce deterioration in many foods, especially in mushrooms, fruit juices and other fruit derivatives, decreasing its market value. Nevertheless, recent discoveries on beneficial properties on health, such as anti-oxidative, anti-inflammatory, immune and anti-tumour properties, have done that not only their elimination should be reconsidered, but also their addition could be proposed in order to take advantage of these properties (Falguera *et al*., 2010a).

Polyphenol oxidase (E.C. 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen with various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity). The later polymerization of these compounds leads to the formation of the enzymatic browning final products, called melanins (De Faria *et al*., 2007; Falguera *et al*., 2010a).

It is accepted that processing and storing of foodstuffs may cause a reduction in the quality of the constituents, affecting both its biological value and its digestibility (Öste & Sjödin, 1984). In some cases, protein digestibility may be affected by the formation of non-enzymatic browning (NEB) products, as it has been reported by several authors (Öste & Sjödin, 1984; Öste *et al*., 1986; Hirano *et a*l., 1994; Ibarz *et al.*, 2008; Ibarz *et al*., 2009). Thus, protease inhibitors have been recognized to be significant in protein metabolism and endocrine systems. More than 100 kinds of proteinous and non-proteinous inhibitors have been isolated and identified from various living bodies such as animals, plants and microbes (Hirano *et al*., 1994).

Trypsin (E.C. 3.4.21.4), as pepsin and chymotrypsin, is one of the main digestive proteases. It is synthesized in the pancreas in the inactive form of trypsinogen, and its activity is located in the small intestine where it degrades proteins to polypeptides and amino acids in a medium pH of about 8.0 (Ibarz *et al*., 2009). Concretely, it catalyses the hydrolysis of the peptidic and ester bonds formed by the carboxyl group and the basic amino acids L-lysine and L-arginine.

Trypsin activity is negatively influenced by physical parameters (temperature and pH), by conformational changes (denaturalization), by chemical modifications (substitution of amino acid residues and reduction of disulphite bridges) or by specific interactions with inhibitors. Sometimes, the formation of the catalytically inactive enzyme-inhibitor complex can be useful to understand the formation of the enzyme-substrate complex and the interactions that occur during the catalysis (Schellenberger *et al*., 1994).

Thus, on the one hand, melanins have been reported to be beneficial for health, but on the other hand it has been observed that similar polymers (NEB products) have a toxic effect on proteases activity. The aim of this work has been to assay if enzymatic browning products synthesized by means of *Agaricus bisporus* polyphenol oxidase act as trypsin inhibitors and, in that case, to determine the kind of inhibition and its kinetic parameters.

2. MATERIALS AND METHODS

2.1. Melanin preparation

Agaricus bisporus polyphenol oxidase (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 $U \cdot mL^{-1}$, distributed in aliquots of 1 mL and frozen at -12ºC until use. This solution was stored at 4ºC since 12 hours before experiments started, and then pre-incubated at room temperature (22±2ºC) for 1 hour. L-Tyrosine (Sigma Chemical, St. Louis, MO) was prepared in sodium phosphate buffer (pH 6.0, following the optimal pH determined in a previous piece of work (Falguera *et al*., 2010b)) in a concentration of 4.0 mM. The final enzyme content in the reaction mixture was 10 U/mL.

After 24 h of reaction, melanins were precipitated adjusting the solution pH to 2.0 with HCl. The mixture was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 12 minutes at 12,000 rpm. The supernatant was discarded and the pellet was recovered with distilled water. Melanins were lyophilized and rediluted in dimethyl sulfoxide (DMSO) in a concentration of 0.82 mg/mL.

2.2. Trypsin activity determination

The principle of the enzymatic assayed reaction is the action of bovine trypsin (Sigma Chemical, St. Louis, MO) on Nα-benzoyl-L-arginine ethyl ester (BAEE) in aqueous solution at pH 7.6 and 25 °C, giving Nα-benzoyl-L-arginine (Bz-L-Arg) and ethanol (Bergmeyer *et al*., 1974). This reaction can be followed measuring the solution absorbance at 253 nm with a 1 cm width quartz cell, using an Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) and monitoring the results with the Vision Lite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). A data point was taken every 3 s. Trypsin activity was expressed as the absorbance variation (formation of Bz-L-Arg) for each minute and protein amount of the enzyme (ΔA_{253} s^{-1} mg^{-1}).

2.3. Trypsin inhibition analysis

Experimental series were carried out in a substrate concentration range from 0.2 to 0.6 mM, since higher BAEE concentrations led to inhibition by substrate (Ibarz *et al*., 2009). A trypsin standard solution containing 500 U/mL and three melanin solutions with concentrations of 0.82, 0.41 and 0.205 mg/mL in DMSO were prepared.

The reaction mixture contained 3 mL of substrate and 300 mL of a melanin solution (final melanin contents: 0.07, 0.035 and 0.0175 mg/mL). 20 mL of the enzymatic solution (10 U) were added to the different mixtures (final enzyme content: 0.22 mg/mL) and the absorbance at 253 nm evolution was monitored. Blank experiments were carried out in order to prove that there was not any interference of the different substances (mainly melanin and DMSO) with the absorbance at 253 nm.

2.4. Data processing and statistical analysis

From the increase of the absorbance with the time of reaction, it is possible to obtain the maximum reaction rates for each substrate and melanin concentration, which is reached at zero time. With this aim, the monitored variation in the absorbance at 253 nm with reaction time can be fitted to exponential curves:

$$A_\lambda = a - b \cdot \exp(-k \cdot t) \qquad (1)$$

From this expression it is possible to obtain the initial reaction rate, as that is the value of its derivative at the initial time (Ibarz *et al*., 2008):

$$r_0 = \left.\frac{dA_\lambda}{dt}\right]_{t=0} = b \cdot k \qquad (2)$$

From the data of the initial rate of reaction for the different substrate and melanin values used, the Lineweaver–Burk method allows to obtain the Michaelis-Menten (MM) kinetic type parameters, which are the MM constant (K_M) and the maximum reaction rate (r_{max}) (Segel, 1982). The data from this representation was adjusted to a straight line by means of the least squares method.

The fittings to the different kinetic and mathematical expressions were carried out using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The fittings and the estimates were calculated at a 95 % significance level. All shown results are the average of three determinations.

3. RESULTS AND DISCUSSION

In order to study the effect of the melanin synthesized from L-tyrosine on trypsin activity, three different melanin concentrations were assayed on BAEE solutions. It was observed that the initial reaction rate had a great dependence on melanin concentration, being lower as it increased. To characterize and quantify this inhibitory effect these initial reaction rates were transformed following the Lineweaver-Burk method (Figure 1).

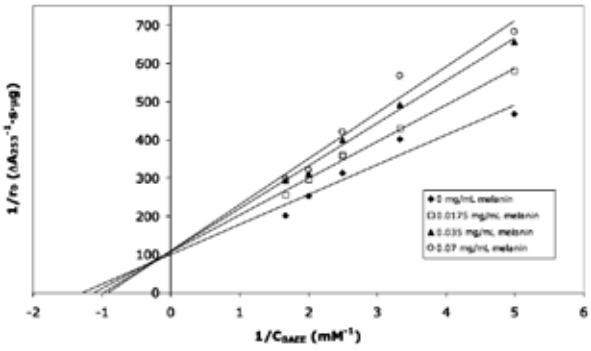

Figure 1. *Lineweaver-Burk representation of samples with different concentrations of melanin from L-tyrosine.*

Table 1. *Kinetic parameters for trypsin inhibition by melanin from L-tyrosine.*

$C_{Melanin}$ (mg/mL)	K_M (mM)	r_{max} (ΔA_{253}·s^{-1}·µg^{-1})	R^2
0	0.86 ± 0.16	0.0108 ± 0.0027	0.9345
0.0175	0.96 ± 0.44	0.00990 ± 0.00039	0.9987
0.035	1.09 ± 0.13	0.00979 ± 0.00095	0.9938
0.07	1.10 ± 0.19	0.0092 ± 0.0015	0.9526

Table 1 shows the values of MM constant and maximum reaction rates for the experimental series carried out with different melanin concentrations. MM constant increases with the melanin concentration in the solution, which indicates that this polymer decreases the apparent affinity between trypsin and its substrate (Nelson & Cox, 2000). In addition, the maximum reaction rate tends to decrease with the increase of melanin content in a linear tendency.

The highest obtained inactivation was 32.6% with a BAEE concentration of 0.6 mM and a melanin content of 0.07 mg·mL^{-1}. By extrapolation of this linear tendency, the necessary melanin concentration to inhibit the enzyme completely would be 0.50 mg·mL^{-1}. However, this deduction could not be empirically proved, since melanin solubility made it impossible to work with concentrations higher than 0.25 mg·mL^{-1}. At higher melanin contents, some of the added polymer remained constantly insoluble.

These facts are evidence enough to determine that the presence of melanin from L-tyrosine inhibits trypsin activity in a mixed way (Segel, 1982). Inhibition ways are represented in Figure 2. Since melanins are a heterogeneous group of polymers with different chain lengths, the different fractions are expected to act in different moments of the catalysis, joining both trypsin alone or trypsin-BAEE complex and leading to mixed inhibition kinetics. Ibarz *et al*. (2009) found a similar behavior in the interaction between trypsin and melanoidins, which is also a heterogeneous group of polymers with different chain lengths.

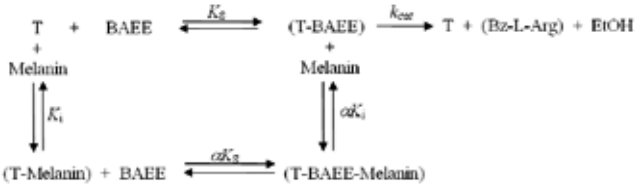

Figure 2. *Mixed enzymatic inhibition mechanism (adapted from Segel, 1982). T: trypsin. BAEE: Nα-benzoyl-L-arginine ethyl ester. Bz-L-Arg: Nα-benzoyl-L-arginine. EtOH: ethanol*

According to Jencks (1969), the electrostatic and apolar contacts dictate enzyme-substrate complementarity, which is necessary for surmounting the activation energy barrier between the ground and transition states. As substrate binding sites are preformed and relatively rigid, the free energy of substrate binding can be converted to catalysis without a large entropic penalty. Therefore, rate acceleration must also depend on the ability of distal portions to stabilize the binding, so the whole protein architecture must play an important role (Perona *et al*., 1995). Moreover, it is known that the differences in substrate specificity between trypsin and chymotrypsin are provided by variations in the distal portions that create a particular electrostatic environment, since the structure of the active site is the same in both enzymes (Stroud, 1974; Hedstrom *et al*., 1992). Then, any molecule present in the reaction medium may have an active effect on these electrostatic and apolar bindings, modifying the local environment that is necessary to create the enzyme-substrate links. Melanin chains with different molecular weight may create bindings with different sites of trypsin molecules, either blocking the active site or modifying these electrostatic forces.

The conclusion that the inhibition is mixed-type can also be stated observing that the intersection of the regression lines in the Lineweaver-Burk plot is found in the second quadrant. Thus, the inhibition constant αK_i (enzyme-substrate-inhibitor complex) must be higher than the inhibition constant K_i (enzyme-inhibitor complex), and then the value of α will be higher than the unit. To obtain the values of these parameters, the slope and the intercept of the Lineweaver-Burk regressions were represented in front of the inhibitor concentration (Segel, 1982). K_i was found to be 0.148 mg·mL^{-1}, while αK_i was 0.438 mg·mL^{-1}. Thus, α

value was 2.95. The fact that α is higher than the unit, but close to it, indicates that, indeed, the inhibition is mixed-type (Copeland, 2000), supporting the evidence observed in Figure 1. Ibarz *et al.* (2009) found an α value of 1.88 in the inhibition of trypsin by melanoidins synthesized from glucose and asparagine.

4. CONCLUSIONS

To sum up, it can be stated that melanins synthesized from L-tyrosine and *Agaricus bisporus* polyphenol oxidase have an inhibitory effect on trypsin activity. The higher melanin concentration was, the more important the inhibition factor was found. The highest obtained inactivation was 32.6%, corresponding to a BAEE concentration of 0.6 mM and a melanin content of 0.07 mg·mL^{-1}. MM constant (K_M) increased with the melanin concentration in the solution, which indicates that this polymer decreases the apparent affinity between trypsin and its substrate. In contrast, the maximum reaction rate (r_{max}) tends to decrease with the increase of melanin content in a lineal tendency. These facts show that this inhibition is mixed-type.

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