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Inactivation of carboxypeptidase A and trypsin by UV–visible light

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ABSTRACT

In the present work, the effect of UV–visible irradiation on the activity of carboxypeptidase A, and trypsin enzymes is shown. The irradiation of the above-mentioned enzymes inhibits their activity, in such a way that sufficiently high irradiation times annul their catalytic action. For carboxypeptidase A a total inactivation after 20 min of irradiation is observed, while trypsin is inactivated completely after 12 min of irradiation. Fitting the data to the Lineweaver–Burk graphs shows that, in the case of CPA enzyme, the inhibition caused by irradiation is similar to that of uncompetitive type. For trypsin, the irradiation acts similarly to a mixed inhibition-type.

Industrial relevance: UV irradiation is a technology used in food treatment, since it has been shown to be effective in the destruction of microorganisms. It can also be applied in the sterilization of enzymatic preparations used in the food industry, but it can have harmful effects, since it can go so far as to inactivate some of the enzymes. In some cases it interests to inactivate enzymes, and it is for it that this treatment type can be effective. In other cases it interests that the enzymes remain active. This way, it will be necessary to avoid that they are exposed to the light.

1. Introduction

The pancreas synthesizes the proteolytic enzymes in their inactive forms (trypsinogen, chymotrypsinogen and pro-carboxypeptidase), which are activated once their secretion has taken place in the intestinal tube. Trypsin catalyzes the protein hydrolysis up to its degradation giving then basic peptides.

Carboxypeptidase acts on these obtained peptides, and may be two types, A or B, depending on whether they act on the aromatic or basic peptides, respectively.

The peptidase activity of carboxypeptidase A (CPA), especially with benzoxyglycil-L-phenylalanine substrate, depends on the pH drawing a flared curve with a maximum at a pH of 7.3 and inflections at pH 6.7 and 7.8. For a Michaelis–Menten kinetics-type, the values of the K_M and k_{cat} constants show a minimum value at pH between 7.5 and 7.8. A constant increase takes place on the acid side and an increase on the alkaline one, reaching a maximum up to pH 8.3, beyond which they start diminishing. The activity of CPA can be inhibited by substances of different nature. The inhibition by substrates is especially evident with N-acyl-dipeptides and similar substrates. CPA can also show a competitive inhibition by product (Hartsuck & Lipscomb, 1971).

Different works report CPA and trypsin inhibition by the presence of melanoidins (Hirano, Miura, & Gomio, 1994; Ibarz, Garza, & Pagán, 2008; Öste, Dahlqvist, Sjöström, Norén, & Miller, 1986).

Generally, the amino acids involved in the action mechanism of the enzymes are identified according to the changes in the enzymatic activity causing the chemical modifications to them. Irradiation on the enzymes was used to identify the function of the amino acids components of the enzyme (Ferrini & Zito, 1963). By means of irradiation, these residues can be modified or destroyed, this being able to cause a change in the structure of the enzyme and its activity. Piras and Vallee (1966a,b) performed a study on the structural changes in the CPA enzyme due to irradiation. These authors showed that irradiation with UV radiation at 253.7 nm increased the esterase activity of the enzyme, and also diminished its peptidase activity. These changes may be due to the fact that irradiation causes an irreversible loss of zinc, this being proportional to the decrease in the enzymatic activities.

UV irradiation is a technology used in food treatment, since it has been shown to be effective in the destruction of microorganisms. It can also be applied in the sterilization of enzymatic preparations used in the food industry, but it can have harmful effects, since it can go so far as to inactivate some of the enzymes.

When a food is exposed to the radiation, a lethal effect takes place on the present microorganisms. It seems that the germicide effect is due to a photochemical reaction in the nucleic acids of the cells (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000). Although, it has also been suggested that the formation of peroxides, free radicals, and the bacteriophage activation for the ultraviolet radiation produce lethal effects by an indirect way (Molins, 2001).

In most of foods the penetration power of irradiation is minimum, being absorbed in the superficial layers, with that the lethal effect of the ultraviolet radiation on the microorganisms only acts in the

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surface (Molins, 2001). Wavelength that the most lethal effect takes place is corresponding to 260 nm, with that the mercury lamps of low pressure (with a maximum of emission to 254 nm) are the most appropriate (Bintsis et al., 2000; Guerrero-Beltrán & Barbosa-Cánovas, 2004).

The main objective of the present work is to observe how the enzymatic activity of CPA and trypsin changes when the solutions containing these enzymes are irradiated using an UV–visible lamp, and to observe which type of inhibition irradiation produces.

2. Materials and methods

2.1. CPA enzymatic activity

The principle of the assayed enzymatic reaction is the action of CPA (EC 3.4.17.1) on an aqueous solution of hippuryl-L-phenylalanine (Hip-Phe) (Sigma Chemical, St. Louis, MO, USA), at a pH of 7.5 and 25 °C, to give hippuric acid and L-phenylalanine (Bergmeyer, 1974), measuring the increase of absorbance that takes place at 254 nm (formation of hippuric acid) (Folk, Piez, Carroll, & Gladner, 1960), using a Philips PV 8720 UV spectrophotometer (Philips Unicam, Cambridge, England). A bovine pancreas solution (Sigma Chemical, St. Louis, MO, USA) containing 19 mg/mL of protein and 65 unit/mg, where 1.0 unit hydrolyses 1.0 μmol min^{-1} of Hip-Phe, is used for the preparation of CPA enzymatic solution. Finally, in the reaction solution the enzymatic protein concentration is 6.3 μg/mL, that is equivalent to 0.41 unit/mL. The absorbance of the samples is due to the appearance of hippuric acid, which possesses a molar absorption coefficient of 360 M^{-1} cm^{-1} at 254 nm (Johnston, 2003), which allows the corresponding molar concentration values of the product formed to be obtained. From the curves of the variation in the concentration with time, it is possible to obtain the initial reaction rate that corresponds to enzymatic activity, expressed as the molar variation of hippuric acid per minute.

2.2. Enzymatic activity of trypsin

The principle of the enzymatic reaction tested is the action of trypsin (EC 3.3.21.4) on an aqueous solution of ethyl Nα-benzoil-L-arginate (BAEE) (Sigma Chemical, St. Louis, MO, USA), at pH 7.6 and 25 °C, to give Nα-benzoil-L-arginine (Bz-L-Arg) and ethanol (Bergmeyer, 1974), the increase of absorbance that takes place at a 253 nm wavelength being measured, using a 1-cm wide quartz cell. Powdered porcine pancreas (Sigma Chemical, St. Louis, MO, USA) containing 16,400 units/mg is used to prepare the trypsin enzymatic solution, where 1 unit hydrolyses 1.0 μmol min^{-1} of BAEE. Finally, in the reaction solution the enzymatic protein content is 2 μg/mL, that is equivalent to 32.8 unit/mL. In this case the value of the molar extinction coefficient of the product formed in the enzymatic reaction is not available, for that reason, the activity of the trypsin is expressed as the absorbance change at 253 nm (formation of Bz-L-Arg) per minute ($\Delta A_{253} \cdot min^{-1}$).

2.3. UV–visible irradiation

The effect of the ultraviolet–visible irradiation on aqueous solutions containing the enzymes CPA and trypsin was studied. The reactor is a methacrylate cell of parallelepiped shape of 45-cm length, 25-cm width and 10-cm depth. The enzymatic solution was placed in the bucket to be irradiated, in such a way that the distance between the surface of the solution and the lamp was 22.5 cm. The radiation emitting system consisted of a Philips PM-12 (Philips, Eindhoven, The Netherlands) of 400 W nominal power high-pressure mercury steam lamp, 4.5 cm in length, centred with respect to the reactor. The lamp emits in a 250 to 750 nm range, and Fig. 1 shows the lamp spectrum of emitted energy (P_λ) at each wavelength (λ) (data provided by the lamp manufacturer). In order to determine the real power reaching

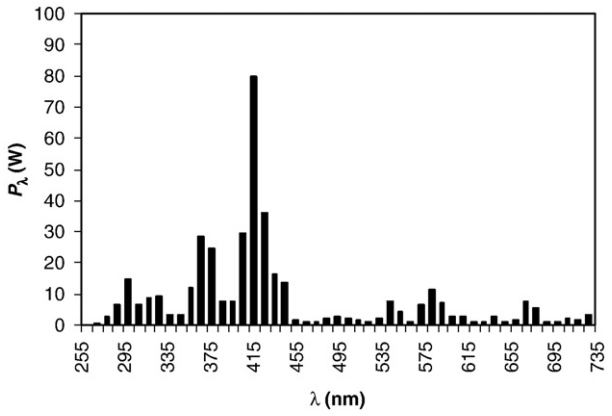

Fig. 1. Emission spectrum for the Philips PM-12 lamp. P_λ is the power of lamp at a wavelength λ.

the surface of the reaction, an actinometry has been performed according to the process described by Esplugas and Vicente (1991), obtaining an incident energy of 3.88×10^{-7} einsteins/s. The radiation equipment (Ibarz, Pagán, Panadés, & Garza, 2005) includes a reactor and the UV–visible lamp, both inside a black box, with the purpose of absorbing all the radiation reaching the reactor, and with an air intake and an extractor to facilitate the circulation of the air and thus avoid an excessive rise in temperature.

The different dissolutions used in the irradiation process were fitted to the optimal pH of the considered enzyme. In case of CPA buffered solutions of TRIS (hydroxymethyl) aminomethane were used, prepared with TRIZMA hydrochloride and NaCl solution (Sigma, Chemical, St Louis, MO, USA), fitting to the pH 7.5 for the solutions of CPA. Experiments for the trypsin solutions were carried out at pH 7.6, which obtained with a phosphate buffered saline–PBS solution (Sigma, Chemical, St. Louis, MO, USA).

In order to study the effect of the substrate concentration on the reaction rate of the two studied enzymes were prepared in solutions of different substrate concentrations. For CPA the substrate concentrations used were 1.0, 0.6 and 0.4 mM, and for trypsin solutions the substrate concentrations were from 0.6 to 2.0 mM. Moreover, working with different substrate concentrations it will enable to obtain Lineweaver–Burk graphs, and therefore it will be possible to assimilate the type of inactivation produced by the irradiation of enzymatic solution samples.

The irradiation process began by igniting the lamp and waiting for 5 min until its emission was constant and stationary. Next, the reactor containing the enzymatic solution was introduced into the lamp box, this moment corresponding to the beginning of the irradiation process. By means of a micropipette, 4 mL of the enzymatic solution were extracted at 4-minute intervals, over a total period of 28 min. Enzymatic activity was measured for each extraction, according to the method described in the previous sections. Enzymatic solutions used were prepared with different substrate concentrations, depending on the irradiated enzyme. For CPA, samples with an enzymatic protein content of 6.3 μg/mL were prepared from an aqueous suspension of CPA of bovine pancreas. In the case of trypsin, its protein enzymatic content was 2 μg/mL and was prepared from powdered porcine pancreas.

As control experiment the buffer solutions without enzyme were irradiated, and it was observed no absorbance variation which indicates that the irradiation process does not produce any effect on the buffer solutions.

All enzymatic solutions were irradiated at 25 ± 0.5 °C. The radiation produces a slight increase of temperature during the inactivation process. This increase of temperature always was less than 2 °C.

Solutions of the two enzymes (CPA and trypsin) were kept at 27 °C during 30 min. No variation in their enzymatic activity was observed.