Biocatalysis and Agricultural Biotechnology 4 (2015) 694–702

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

# Synergistic actions of proteolytic enzymes for production of soy protein hydrolysates with antioxidant activities: An approach based on enzymes specificities

Ruann Janser Soares de Castro*, Hélia Harumi Sato

*Department of Food Science, School of Food Engineering, University of Campinas, 80 Rua Monteiro Lobato, Campinas, SP, Brazil*

ARTICLE INFO

*Article history:*
Received 13 May 2015
Received in revised form
22 August 2015
Accepted 23 August 2015
Available online 28 August 2015

*Keywords:*
Proteases
Enzyme specificity
Antioxidant activities
Mixture design

ABSTRACT

The objective of this study was to investigate the enzymatic hydrolysis of soy protein isolate by screening individual and blended commercial protease preparations using a statistical mixture design. Information about the modulation of thermal inactivation of the enzyme by substrate or products of hydrolysis and the determination of synergistic effects between the enzymes on production of soy protein hydrolysates with antioxidant activities were reported. The kinetic parameters for thermal inactivation measured under reactive and non-reactive conditions indicated that product inhibition was not significant on soy protein hydrolysis using the commercial proteases Flavourzyme® 500 L, Alcalase® 2.4 L and YeastMax A. For DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging, the hydrolysates obtained with Flavourzyme® 500 L combined with Alcalase® 2.4 L showed the higher synergistic effect with increases of 10.9% and 13.2% in antioxidant activity as compared to the hydrolysates produced with individual enzymes. The hydrolysates obtained using the ternary mixtures of Flavourzyme® 500 L, Alcalase® 2.4 L and YeastMax A showed the highest power of inhibition of linoleic acid autoxidation. On the other hand, for reducing power assay and total antioxidant activity, the most of all interactions was antagonistic with high antioxidant activity detected for the hydrolysates produced using Flavourzyme® 500 L, individually.


## 1. Introduction

Proteases constitute the most important category of industrial enzymes that catalyze the hydrolysis of proteins to polypeptides and oligopeptides to amino acids (Abraham et al., 2014). These enzymes can be classified according to their biochemical characteristics such as mechanisms of action, catalytic sites or based on the pH for maximum activity. Proteases that cleave peptide bonds within the polypeptide chain are called endopeptidases and those that cleave peptides bonds at N or C termini of polypeptide chains are classified as exopeptidases (López-Otín and Bond, 2008; Hsiao et al., 2014). According to the catalytic sites, these enzymes are classified in six groups: aspartic, cysteine, glutamic, serine and threonine proteases, depending upon the amino acids present in the active site, or as metalloproteases if a metal ion is required for catalytic activity (Li et al., 2013). They may further be classified in acidic, neutral and alkaline proteases depending on the pH at which they show the maximum activity (Ktari et al., 2014). Through structural and functional diversity, proteases carry out a vast array of applications, including food production (e.g. baking and brewing), leather processing, pharmaceutical manufacture, detergent formulations and protein modification (e.g. protein hydrolysis and peptide synthesis) (Anbu, 2013).

Enzymatic hydrolysis disrupts the protein tertiary structure and reduces the molecular weight of the protein, enhancing the interaction of peptides with themselves and with the environment, and consequently altering their functional and biological properties (Liu et al., 2010). Notably, the nature of the protein modification is influenced by several hydrolysis parameters, including the reaction conditions, such as pH, temperature, degree of hydrolysis, and enzyme specificities, and intrinsic characteristics of each protein source, such as amino acids profile (Singh, 2011; Segura-Campos et al., 2012; Fernández and Riera, 2013). The modification of proteins based on enzymatic hydrolysis have broad potential and are likely an innovative tool in food protein processing for optimizing the functional and biological properties of proteins (Hiller and Lorenzen, 2009; Adjonu et al., 2013).

The combined use of proteases with different specificities and mechanisms of action can be applied as a valuable tool to improve the functional and biological properties of protein hydrolysates. Prior knowledge about enzyme characteristics such as purity,

* Corresponding author. Fax: +55 19 35212153.
*E-mail address:* ruannjanser@hotmail.com (R.J.S. de Castro).

http://dx.doi.org/10.1016/j.bcab.2015.08.012
1878-8181/

substrate specificity, specific activity, single or multiple enzymatic activity have been used to obtain products containing multifunctional peptides) or a mixture of different peptides with each contributing to a specific function (Rui et al., 2012; Betancur-Ancona et al., 2014; Li-Chan, 2015).

Statistical methods have been applied for improving the performance, to find the optimum process variables and formulations in different engineering problems (Rao and Baral, 2011). Statistical mixture designs are an interesting class of experimental designs where the components or factors distributed in different proportions are used to verify the interactions between the components of a mixture and maximizing the responses studied using mixture design approach.

The general purpose of mixture design is to make possible estimates, through a contour plots analysis of evaluated responses of a multicomponent system from a limited number of experiments (Anarjan and Tan, 2013). In this experimental design, the total amount of material is held constant because the response depends only on the proportions of the components present, but not on the total amount of the mixture (Rao and Baral, 2011; Anarjan and Tan, 2013). In the simplex centroid design, $2^k-1$ observations are taken, where $k$ is the pure components, $(k/2)$ is the binary mixtures with equal proportions and $(k/3)$ is the ternary mixtures with equal proportions (Scheffe, 1963).

In this context, the soy protein isolate hydrolysis was investigated by screening individual and blended commercial protease preparations using a statistical mixture design. The study of thermal inactivation modulation of the enzyme by the substrate or products from soy protein isolate hydrolysis using kinetic parameters was reported. The synergistic or antagonistic effects between the different proteolytic enzymes on generation of protein hydrolysates with antioxidant activities were further assessed.

## 2. Material and methods

### 2.1. Reagents

Ammonium thiocyanate, ferrous chloride, linoleic acid, azocasein, trichloroacetic acid (TCA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased in the grade commercially available.

### 2.2. Enzymes

Three commercial preparations of proteolytic enzymes were used in this study. The proteases Flavourzyme® 500 L from *Aspergillus oryzae* and Alcalase® 2.4 L from *Bacillus licheniformis* were purchased from Sigma-Aldrich (Steinheim, Germany). The protease YeastMax A was kindly provided by Prozyn (Sao Paulo, Brazil).

### 2.3. Determination of protease activity

The protease activity was measured using azocasein as a substrate, according to Charney and Tomarelli (1947), with slight modifications. The reaction mixtures containing 0.5 mL 0.5% (w/v) azocasein, pH 7.0, and 0.5 mL of the enzyme solutions were incubated for 40 min at 50 °C. The reaction was stopped by adding 0.5 mL 10% TCA and the test tubes were centrifuged at 17,000xg for 15 min at 25 °C. A 1.0 mL aliquot of the supernatant was neutralized with 1.0 mL 5 M KOH. One unit of enzyme activity (U) was defined as the amount of enzyme required to increase the absorbance at 428 nm by 0.01 under the assay conditions described.

### 2.4. Kinetic parameters for thermal inactivation

The thermal stability of the commercial proteases as a function of the time was evaluated at reactive (50 U $mL^{-1}$ of protease and 100 mg $mL^{-1}$ soy protein isolate solution pH 7.0) e non-reactive (50 U $mL^{-1}$ of protease and 0.2 M phosphate buffer pH 7.0) conditions. For this, the samples were incubated at 50 °C and aliquots were collected at various times for determination of the residual protease activity. The value of the deactivation rate constant ($k_d$) for the proteases, expressed as an exponential decay, was found by plotting ln ($A/A_0$) vs. time using the experimental data as follows:

$$A = A_0 \times e^{-kdt}$$

where $t$ is time, $A_0$ is the initial enzyme activity and $A$ is the enzyme activity at a determined time $t$.

The apparent half-life of the proteases, defined as the time where the residual activity reaches 50%, was estimated as follows:

$$t_{1/2} = ln(0.5)/k_d$$

Decimal reduction time ($D$ value) was defined as the time required for a one-$\log_{10}$ reduction or 90% reduction in the initial enzyme activity at a specific temperature. The $D$ value is related to the first-order deactivation rate constant ($k_d$) and it was calculated as follows:

$$D = 2.303/k_d$$

### 2.5. 2.5. Preparation of protein hydrolysates

The soy protein isolate used as the substrate for enzymatic hydrolysis were kindly provided by Bunge Foods S/A (Gaspar, Brazil). The commercial proteases, Flavourzyme® 500L, Alcalase® 2.4 L and YeastMax A were used for enzymatic hydrolysis. The enzyme concentrations were adjusted to 0 (control) or 50 U $mL^{-1}$ of reaction mixture according to the previously determined protease activity. The soy protein isolate was suspended in phosphate buffer pH 7.0 to a final concentration of 100 mg $mL^{-1}$, and 50 mL aliquots of the mixtures were distributed in 125 mL Erlenmeyer flasks. Hydrolysis was performed at 50 °C and pH 7.0 for 120 min. After hydrolysis, the samples were incubated in a water bath at 100 °C for 20 min for proteases inactivation. The mixtures were centrifuged at 17,000xg at 5 °C for 20 min, and the supernatants containing the peptides were collected and freeze-dried for the determination of their antioxidant activities and TCA soluble protein contents.

### 2.6. Statistical mixture design

The experimental mixture design was used to obtain the optimum mixture compositions of the different proteolytic enzymes for maximum antioxidant activities and to investigate the presence of either synergistic or antagonistic effects in a blend of the components. A three component augmented simplex centroid design was employed in which each component was studied at six levels, namely 0 (0%), 1/6 (16.67%), 1/3 (33%), 1/2 (50%), 2/3 (66.67%) and 1 (100%) (Table 1).

Quadratic or special cubic regression models were fitted for the variations of all the responses studied as a function of significant ($p < 0.05$) interaction effects between the proportions, thereby obtaining acceptable determination coefficients ($R^2 > 0.75$). Equation 1 represents these models as follows:

$$Yi = \sum_{i=1}^{q} \beta iXi + \sum \sum_{i<j}^{q} \beta ij\ XiXj + \sum \sum_{i<j<k}^{q} \sum \beta ijkXiXjXk$$

where '$Y_i$' is the predicted response; '$q$' represents the number of