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Characterization of commercially available peptidases in respect of the production of protein hydrolysates with defined compositions using a three-step methodology

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ABSTRACT

Enzymatic hydrolysis of food proteins is an effective way to implement a desired functionality into protein containing foods. Since these functionalities are highly dependent on the degree of hydrolysis (DH) of the hydrolysates, the enzymatic hydrolysis process must be carefully controlled. Hydrolysates with both free amino acids and long-chain peptides are usually not desirable which leads to strict requirements for the commercial peptidases used. In the study presented, we characterized ten commercially available peptidase preparations in respect of the production of protein hydrolysates with defined compositions. Therefore, we utilized a three-step methodology to investigate the composition of the commercial peptidase preparations and to characterize the obtained protein hydrolysates applying a HPLC-based system for automated small-scale enzymatic reaction and analysis. With the presented methodology we identified two suitable preparations for the production of hydrolysates with high DH and high amounts of free amino acids, and six suitable preparations for the production of hydrolysates with low DH and negligible release of amino acids.

1. Introduction

The enzymatic modification of food proteins is one way to increase their value by enhancing a desired functionality. One option to achieve the latter is the hydrolysis of the proteins. By this means, for example, technological functionalities, such as emulsification and foam stabilization, can be improved [1,2]. Additionally, angiotensin-I converting enzyme (ACE; EC 3.4.15.1) inhibitory and radical scavenging activity can be enhanced in protein hydrolysates [1]. The usage of protein hydrolysates as a flavoring ingredient is also of industrial interest [3]. The enzymatic hydrolysis of proteins is mainly performed in batch processes [4] with defined process parameters. Each of the functionalities desired is highly dependent on the degree of hydrolysis (DH) and the peptidase preparation utilized [1,2,5–8]. Therefore, the hydrolysis process must be carefully controlled. In general, the emulsifying and foam-stabilizing capabilities of protein hydrolysates are enhanced at lower DH [1,2,9–11]. By contrast, the ACE inhibitory and radical scavenging activity is improved with increased DH [1,6]. Furthermore, a high DH is preferred for flavoring hydrolysates, since free amino acids and smaller peptides contribute to the formation of the flavors desired [3].

Due to the different dependencies of the functional property, the production of hydrolysates with both free amino acids and long-chain peptides is usually not desirable. The choice of the peptidases and the knowledge about them are crucial elements for the production of defined hydrolysates with certain functionality. The type of peptidase and the combination of exopeptidases and endopeptidases in the peptidase preparation mainly influences the DH and, therefore, the peptide/amino acid composition of the resulting hydrolysates. The complexity of the commercial peptidase preparation Flavourzyme, for example, was recently demonstrated [12].

Abbreviations: ACE, angiotensin-I converting enzyme; Bis–tris-propane, 1,3-bis(tris(hydroxylmethyl)methylamino)propane; DH, degree of hydrolysis; h, concentration of free amino groups; h_{tot}, maximum concentration of free amino groups at complete hydrolysis; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; M^*, average amino acid molecular mass of lupine protein; OPA, *ortho*-phthalaldehyde; SDS-Page, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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Eight key enzymes were identified in Flavourzyme and the significant impact of six peptidases on a wheat gluten hydrolysis was shown [13].

In the present study, we characterized ten commercially available peptidases using a three-step methodology. By this means, the composition and the desired property of the hydrolysate can be increased, because the mechanisms of action of the chosen industrial peptidases are better understood. First we investigated the composition of the commercial peptidase preparations by mass spectroscopic analysis. Next, classical batch hydrolyses of a model food protein (lupine) were conducted and, last, the release of free amino acids during the protein hydrolysis was monitored using a HPLC-based system for automated small-scale enzymatic reaction and analysis. Thereby, the overall exopeptidase activity of the preparations was quantified and the hydrolysates were characterized in respect of their amino acid composition. With this study, we revealed the potential of the peptidases tested for the production of defined hydrolysates. Furthermore, the HPLC-based system described enables the automated enzymatic hydrolysis, sampling and subsequent derivatization in mL-scale and is a versatile modification of the methods for the automated precolumn derivatization using *ortho*-phthalaldehyde (OPA) described already [14–17].

2. Materials and methods

2.1. Materials and chemicals

Lupine protein isolate (Prolupin, Prolupin GmbH, Grimmen, Germany) was a gift from Nestlé Product Technology Centre (Singen, Germany). All chemicals were of analytical grade and were obtained from Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Sigma–Aldrich (Taufkirchen, Germany).

2.2. Commercial peptidase preparations

The commercial enzyme preparations were a gift from Nestlé Product Technology Center (Singen, Germany). The commercial enzymes tested were Alcalase 2.4 L (Novozymes, Bagsværd, Denmark), Bioprase SP-20FG (Nagase ChemteX, Kyoto, Japan), Collupulin 200 L (DSM, Delft, Netherlands), Corolase 2TS (AB Enzymes, Darmstadt, Germany), Flavourzyme 1000 L (Novozymes, Bagsværd, Denmark), Maxazyme NNP DS (DSM, Delft, Netherlands), Promod 439 L (Biocatalysts Ltd., Nantgarw, Wales), Proteinase T (DuPont, Aarhus, Denmark), Protease AN "Amano" SD-K (Amano Enzyme Inc., Nagoya, Japan) and Protin SD-AY10 (Amano Enzyme Inc., Nagoya, Japan). The preparations are hereinafter referred to as Alcalase, Bioprase, Collupulin, Corolase, Flavourzyme, Maxazyme, Promod, Proteinase T, Protease AN, and Protin, respectively.

2.3. Determination of amino groups with ortho-phthalaldehyde

Primary amino groups were determined after derivatization with OPA, according to the method of Nielsen et al. [18] with some modifications. A volume of 25 μL of the sample was transferred into a microtiter plate and 175 μL OPA reagent was added. For the OPA reagent, 11 mM OPA, 20 mM Dithiothreitol and 11.25% (v/v) methanol were dissolved in 120 mM sodium tetraborate decahydrate (adjusted to pH 9.8 with NaOH). The plate was incubated at 37 °C for 1 min and the absorbance was measured at 340 nm using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). The calibration was performed using L-serine as a reference.

2.4. Degree of hydrolysis

The DH was calculated with Eq. (1) according to Adler-Nissen [19], with modifications [20].

$$\mathrm{DH} = \frac{h}{h_{\mathrm{tot}}} \times 100[\%] \quad (1)$$

where h is the concentration of free amino groups [mol/L; see above] and h_{tot} is the maximum concentration of free amino groups at complete hydrolysis [mol/L] calculated according to Eq. (2).

$$h_{\mathrm{tot}} = \frac{c_{\mathrm{Protein}}}{M^{*} - M_{\mathrm{H_2O}}}[\mathrm{mol/L}] \quad (2)$$

where c_{Protein} is the concentration of protein which is hydrolyzed (g/L) and M^{*} is the average molecular mass of the amino acids in lupine protein (138 g/mol). This lupine-specific average molecular mass was calculated by considering the lupine protein amino acid composition [21] and the amount of nitrogen/amino groups of the amino acids. The molecular mass of water ($M_{\mathrm{H_2O}}$ = 18 g/mol) was subtracted due to the addition of water during the hydrolysis of a peptide bond.

2.5. Determination of the proteolytic activity

The enzyme activity of the commercial enzyme preparations was tested using lupine protein as a substrate. Therefore, 150 μL of lupine protein isolate (2 g/L) and 100 μL of the corresponding buffer (150 mM) were incubated at 37 °C for 5 min. The reaction was started with the addition of 50 μL diluted enzyme solution, was stopped by the addition of trichloroacetic acid (TCA, 1.5 M, 50 μL) and was centrifuged (20,000 × *g*, 4 °C, 5 min) afterwards. The supernatant (25 μL) was transferred into a microtiter plate and the OPA assay was performed (see above). One katal (kat) of enzyme activity was defined as the release of 1 mol L-serine equivalent amino groups per second.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Respective samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5%) according to the method of Laemmli [22]. The protein bands of the SDS gel were located by staining with Coomassie Brilliant Blue R-250 [23]. Every lane was analyzed by mass spectrometric investigation. The protein load was 5 μg per lane and was determined by using the method of Bradford [24] and bovine serum albumin as a reference.

2.7. Mass spectrometric analysis

The Life Science Center at the University of Hohenheim carried out the mass spectrometric investigations. Proteins were in-gel digested (Coomassie stained SDS gel; see above) using trypsin (Roche, Germany), according to Shevchenko et al. [25]. Nano-LC–ESI-MS/MS experiments were performed on an ACQUITY nano-UPLC system (Waters, USA) coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Germany). Tryptic digests were concentrated and desalted on a precolumn (2 cm × 180 μm, Symmetry C18, 5 μm particle size, Waters, USA) and separated on a 25 cm × 75 μm BEH 130C18 reversed phase column (1.7 μm particle size, Waters, USA). Gradient elution was performed from 1% ACN to 40% ACN in 0.1% FA within 30 min. The LTQ-Orbitrap was operated under the control of XCalibur 2.1.0 software. Survey spectra (m/z = 250–1800) were detected in the Orbitrap at a resolution of 60,000 at m/z = 400. Data-dependent tandem mass spectra were generated for the seven most abundant peptide precursors in the linear ion trap. Internal calibration was