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Protein enzymatic hydrolysis integrated with ultrafiltration: Thermolysin application in obtaining peptides

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HIGHLIGHTS

- The model for prediction of biopolymer hydrolysis products was verified.
- Reaction kinetics was expressed according to the number of reactive bonds.
- BSA, the protein present in waste whey, was hydrolysed to obtain active peptides.
- The membrane was selected to take off small peptides, including the inhibitors.
- Ultrafiltration process integrated with reactor intensified the hydrolysis process.

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ABSTRACT

In this manuscript, enzymatic proteolysis in order to obtain short peptides was discussed. To hydrolyse serum bovine albumin (BSA) thermolysin (TLN) was used and the molecular mass and concentration of hydrolysates utilising SE-HPLC were identified.

It was found that the substrate pre-incubation at high temperatures (>46 °C) contributed to the increase of hydrolysis rate and final efficiency. At a temperature of 70 °C there is a negative effect, resulting from the aggregation of albumin molecules. It is worthy of notice that the pre-incubation time is significant, up to half an hour.

A strong product inhibition was observed. It resulted in the low values of hydrolysis degree (DH) obtained in classical reactors. Various reaction mechanisms have been tested to elucidate the best kinetic model. The kinetic equation was adopted and its constant values were estimated. A qualitative and quantitative description of the proteolysis products was performed. An ultrafiltration membrane with small pore-size, in order to remove peptides, the enzyme inhibitors, was selected.

Obtained results indicate that the enzymatic process can be significantly intensified in an integrated bioreactor-ultrafiltration environment. In addition, it is clear that process efficiency and permeate composition depend on the proper selection of the dilution rate and the separation properties of the membrane.

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1. Introduction

Protein hydrolysis is a multisubstrate consecutive-parallel reaction, where a given protein is decomposed into a number of oligomers, which are substrates for subsequent polymerisation processes [1]. Theoretically, the products may have significant mass distribution starting from a single mer (amino acid) up to a polymer with only one amino acid cut. The products of partial or final proteolysis are often potentially bioactive peptides, or they present strong tastes and could be applied in the food industry [2,3]. For example, according to the BIOPEP database [4] bovine serum albumin (BSA) may be decomposed to more than 85 biologically active peptides, of which 42 are responsible for generating bitter taste, 17 possess antihypertensive properties, and 15 can be applied in diabetes treatment. In turn, the remaining peptides reveal many other properties, including opioid, immunomodulatory, antithrombotic and antioxidative activities [4]. These are mainly dipeptides, some tripeptides, and one residue consisting of 9 amino acids. Such the high degree of protein hydrolysis requires the application of at least two enzymes: protease initially, and then peptidase with defined specificities (understood as the cleavage site between which amino acids).

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Symbols

A	area under peak in HPLC chromatogram μV min^{-1}
A_v	Avogadro's number –
BSA	bovine serum albumin
C	concentration g L^{-1}, mol L^{-1}
D	dilution rate, (Eq. (9)) h^{-1}
DH	hydrolysis degree –
K_i	inhibition constant g g$_E^{-1}$
k	kinetic constant L g^{-1} h^{-1}
M	molecular weight kDa
N	bonds multiplicity L^{-1}
P1, P1′, P2, P2′	amino acid in the peptide chain
Q	stream L h^{-1}
r	reaction rate g L^{-1} h^{-1}
R	retention coefficient, (Eq. (7))
S1, S1′, S2, S2′	amino acid in substrate-binding pockets
t	time h
V	volume L
λ	wave length nm

Subscripts

E	enzyme
F	given fraction
i	particular reagent
inh	inhibitor(s)
mer	amino acid
per	permeate
R	reactor
Rea	reactive bonds
ret	retentate
t	time
0	initial

It is desirable to use enzymes with well-known substrate specificity and proteins of known amino acid sequence in order to provide predictable hydrolysate composition. One such enzyme is thermolysin (EC 3.4.24.27), an extracellular zinc metalloprotease of molecular weight 34.6 kDa, produced by the gram-positive bacterium *Bacillus thermoproteolyticus* [5].

On the surface of thermolysin there are four major substrate-binding pockets: S2, S1, S1′ and S2′ [6]. The main site responsible for specificity is the S1′ pocket, which accepts a wide range of hydrophobic amino acid residues. Thus, thermolysin preferentially hydrolyses peptides and proteins on the N′ site of the peptide bond (P1′ position) formed by leucine (L), phenylalanine (F), isoleucine (I) or valine (V) with lesser efficiency, when the P1′ site is occupied by methionine (M) and alanine (A). Substrates can also be recognised by other pockets less significant for the specificity of thermolysin. Hydrophobic residues are also preferred in the P1, P2, and P2′. This preference according peptide bonds ranks thermolysin as a protease with medium specificity [7]. An advantage of this enzyme is its high activity (especially at elevated temperatures 50–60 °C) and stability [8].

Kinetic description of the proteolysis is complicated. The literature is dominated by descriptions of the overall degree of hydrolysis (DH) [9–12]. A qualitative and quantitative description of the resulting products of proteolysis (i.e. the length of the respective peptides) was only attempted in the studies carried out by Fernandez and Riera [13] in which α-lactoglobulin was analysed. The results of the model simulation were compared to the results based on a simpler model developed by Trusek-Holownia and Noworyta [1]. The Trusek-Holownia and Noworyta model is universal and could be applied for multiple types of biopolymers and enzymes and types of kinetics.

Our present work is focused on hydrolysis of BSA to precursors of bioactive peptides carried out using thermolysin as a biocatalyst. This reaction has not been studied in the literature. BSA can be isolated from easily available whey [14,15] where it is present at relatively high concentrations. BSA is a well-known protein with a defined amino acid sequence [16] thus its hydrolysis can be well controlled.

According to the model applied [1], first a kinetic equation of the considered reaction catalysed by the selected enzyme should be established. Then, kinetic constants have to be experimentally determined.

The proposed model takes into account that during hydrolysis of biopolymers, one molecule of the initial hydrolysed substrate may undergo a number of reactions depending on the number of reactive bonds that contains its molecule. Thus, the number of reactive bonds and their distribution in the chain should be pointed. Based on the thermolysin substrate specificity described above, the reactive bonds in the initial BSA molecule can be identified.

Enzymatic hydrolysis of proteins generates further substrates and final products with different molecular weights that can be fractionated. Le Maux et al. [17] separated the hydrolysates from whey protein hydrolysis using spiral wound NF membrane (200 Da retention coefficient). They used the membrane as a purifying method of reaction mixture and to separate the products (peptides) from unhydrolysed whey protein. [Le Maux et al., 2015]. Uluko et al. [18] used three types of membranes with retention coefficient: 8.0, 3.5 and 0.2 kDa in order to separate angiotensin converting enzyme (ACE) from its inhibitors present in whey protein hydrolysates mixture. The outcome of this work indicates that two steps of membrane filtration can efficiently separate ACE inhibitory.

This process combined with membrane separation could be integrated in one unit – a membrane bioreactor. It provides enzyme immobilisation in the membrane reaction area. The retention of enzyme decreases cost [19], whereas final product is pure and is not contaminated by unreacted substrate. Guadix et al. used membrane reactor with 3.0 kDa membrane. They hydrolysed whey proteins and obtained stable values of conversion degree, permeate flux and the length of hydrolysates with an average peptide chain of approximately 4 amino acids [20]. That leads to producing precursors of bioactive peptides. Other authors [21] used ultrafiltration membrane (8.0 kDa) and thus confirmed that membrane with very small pore size allows to achieve very high DH. Additionally, it was shown [22] for a single substrate that the use of a membrane that retains a considerable amount of substrate enables a significant shortening of the hydraulic residence time in the reactor.

The problem becomes more complicated and interesting in the case of a multi-substrate reaction, where the applied membrane retains particular fractions of substrates (proteins, polypeptides and oligopeptides) differently.

To carry out the mentioned process in a membrane bioreactor it is necessary to select an adequate membrane. The most important parameter is a suitably high retention coefficient (R_i) for the substrate fractions. Finally, the results obtained during the integrated process, proteolysis in a stirred tank reactor connected with

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