PRODUCTION OF SUNFLOWER MEAL PROTEIN HYDROLYSATE BY SEQUENTIAL HYDROLYSIS WITH ALCALASE AND FLAVOURZYME IMMOBILIZED ON FUNCTIONALIZED SILICA NANOPARTICLES

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ABSTRACT

The objective of this research was to study the production of sunflower meal protein hydrolysate (SMPH) from sunflower meal protein isolate (SMPI) prepared by sedimentation/flotation and isoelectric precipitation of sunflower meal (SM). SM is the main co-product formed during the industrial process of extracting oil from sunflower seed. By fractionation of SM, the crude protein content was increased from approximately 37.4 % in SM to 80.8 % in SMPI and represent a good source of protein for human consumption. The percentages of fiber, soluble sugars, polyphenols, lignocellulose fibers and residual lipids which may reduce the chemical and nutritive value of proteins in the SMPI were reduced to more than 92 % with respect to the SM.
Since functional properties of SMPI can be improved by enzymatic hydrolysis under controlled conditions, the hydrolysis of SMPI was carry out using endo-protease Alcalase covalently immobilized on epoxy modified silica nanoparticles (Alcalase-GFNS) and/or exo-protease Flavourzyme covalently immobilized on cyanuric chloride activated amino modified silica nanoparticles (Flavourzume-CCAFNS).
Results showed that enzymatic hydrolysis of the SMPI should be performed using sequentially Alcalase-GFNS and Flavourzyme-CCAFNS since the achieved hydrolysis yield was greater by 1.5 and 3 times in comparison to the use of only Alcalase-GFNS or Flavourzyme-CCAFNS, respectively, during the same time. Also, it should be mentioned that Flavourzyme-CCAFNS was added in reaction mixture 2 h after the start of hydrolysis with Alcalase-GFNS. With the predigestion with Alcalase-GFNS, the number of N-terminal sites for the exoprotease activity of Flavourzyme-CCAFNS was increased which directly led to the increment of SMPI hydrolysis degree. Finally, it can be concluded that Alcalase-GFNS and Flavourzyme-CCAFNS have great prospects for usage in the hydrolysis of SMPI to produce SMPH with high nutritional value that can be used directly, for example, in the fortification of liquid foods or high energetic beverages.
***Keywords**: sunflower meal protein hydrolysate, silica nanoparticles, protease, immobilization, enzymatic hydrolysis*

INTRODUCTION

The existing problems of food malnutrition coupled with escalating growth of population and high costs of animal based food supplies have forced finding alternative protein sources to enrich traditional formulations (Awan, 2007). As a result, plant proteins became important in human nutrition due to their high essential amino acids content. Plant proteins are obtained by different isolation procedures (such as salting out and isoionic precipitation) depending on the physicochemical properties of proteins (Nehete et al., 2013). Additionally, supplementation of diet with the plant protein isolates could be an alternative for improvement of people protein intake.

In this study, as a source of plant proteins, sunflower meal is used. Sunflower meal is by-product of the oil extraction process from sunflower seed. So far, sunflower meal has been primarly used as livestock feed, especially for ruminants. Sunflower meal protein, fiber and oil contents depends on the oil extraction process (Siebert, 2018). Unlike other oil meals, sunflower meal does not have intrinsic anti-nutritional factors or require heating or special treatment before being used as food for humans or animals. Its amino acid profile is richer in sulfur amino acids, especially methionine than other protein sources, but its lysine content is much lower than that of soybean meal (Ullah et al., 2016). In the future, due to their high nutritional value and favorable functional properties, proteins isolated from sunflower meal could have very important role in modern food design.

Since plant protein isolates potential usage in food industry depends on their functional properties (bulk density, protein solubility, water and oil absorption capacity, emulsifying and etc.) further processing of isolated plant proteins is necessary (Kachrimanidou et al., 2015). For example, solubility of protein isolates could be improved via enzymatic hydrolysis. Sunflower meal protein hydrolisates were generated using proteases such as kerase, endoprotease-Alcalase and exoprotease-Flavourzyme. However, as a result of the sunflower meal protein hydrolysis with kerases bitter peptides was produced. The bitterness was caused by the exposure of hydrophobic amino acid residues. On the other hand, during usage of Alcalase and Flavourzyme, this problem has not been observed.

Hereby, potential usage of Alcalase and Flavourzyme immobilized on silica nanoparticles in hydrolysis of sunflower meal protein isolate was investigated. The main goal was generation of extensive sunflower meal protein hydrolisate.

Amongst numerous inorganic nanomaterials silica nanoparticles are selected to be used for the immobilization of proteases, particularly due to their very favorable features such as high specific surface area, thermal and mechanical stability and biocompatibility. Nevertheless, silica nanoparticles have potential of forming only weak hydrogen and electrostatic enzyme-carrier interactions which makes them unsuitable for widespread usage. The easiest way to overcome this problem is incorporation of reactive organic moieties onto silica nanoparticles surface and enabling the establishment of preferred stable covalent bonds with enzymes. So, in this study, surface of silica nanoparticles was treated with aminoalkyltriethoxysilane (APTMS) in order to introduce amino groups, with subsequent activation by cyanuric chloride (CC) to introduce functionalities that can easily form covalent bonds with amino and thiol groups on enzyme surface. In addition, silica nanoparticles were treated with epoxyalkyltriethoxysilane (GOPTMS) in order to introduce epoxy groups which also allow formation of covalent bonds with enzyme. Immobilization of Alcalase and Flavourzyme on both obtained supports (cyanuric chloride activated amino modified silica nanoparticles (CCAFNS) and epoxy activated silica nanoparticles (GOPTMS)) was examined. In terms of activity (IU/g), the obtained results showed that CCAFNS nanoparticles were the best support for immobilization of Flavourzyme, while it turned out that the best support for Alcalase immobilization were GOPTMS nanoparticles.

MATERIAL AND METHODS

Defatted sunflower meal, provided by Bankom (Belgrade, Serbia) was used as protein source. For hydrolysis two commercial enzymes preparations used were: Alcalase® and Flavourzyme® (Novozyme®, Bagsvaerd, Denmark). Alcalase is an endoprotease from *Bacillus licheniformis,* with Subtilisin Carlsberg as the major enzyme component, while Flavourzyme is mixture of exoproteases and endoproteases obtained from *Aspergillus oryzae*. As supports for immobilization of Alcalase and Flavourzyme hydrophilic fumed silica nanoparticles AEROSIL® 380 (7 nm nonporous particles which have specific surface area of 380 ± 30 m^2 g^{-1}, tamped density of 50 g l^{-1} and pH 3.7-4.7) were used.

Preparation of sunflower meal protein isolate (SMPI)

SMPI was obtained by sedimentation/flotation and isoelectric precipitation of sunflower meal (SM) (Figure 1). The fractionation of sunflower meal is carried out in distilled water,

whereupon three fractions are separated: lignocellulosic (LCF), liquid fractions (LF) and protein fraction (PF). Then the LCF fraction is mechanically separated. The separation of the LF and PF was done by decanting. Afterwards, the separated PF was suspended in distilled water and then treated with concentrated NaOH (30%) until a pH of 10.5 was established. At this pH, all proteins are in dissolved form, which means that the proteins were transferred from the PF into the formed liquid phase. The liquid phase is separated from the PF by decantation. The liquid-phase suspended proteins from the acid precipitate are separated from the solution by adjusting its pH to the isoelectric point of the SMPI (pI 4.3). The newly developed precipitate represents the protein isolate of the sunflower meal. By fractionation of SM, a protein isolate with 1.98% dry matter and a pH of 4.3 was obtained. Ultimately, the neutralization of the protein isolate was performed with 0.1 M phosphate buffer pH 7.5 in a 1:1 ratio. The obtained sunflower meal protein isolate (SMPI) had a pH of 7.0 after neutralization and a dry matter content of 1 %.

Surface modification of silica nanoparticles

Cyanuric chloride activated amino modified silica nanoparticles (CCAFNS) was prepared as follows: 300 mg of fumed silica nanoparticles (FNS) was stirred with 1.18 ml APTMS in 36 ml of anhydrous toluene for 48 h at 25°C under inert atmosphere. The obtained amino modified silica nanoparticles (AFNS) was filtrated, rinsed with fresh toluene and sonicated for 5 min. For further modification 300 mg of AFNS was incubated with 1.5 g of CC dissolved in 70 ml of acetone and with addition of a few drops of triethylamine. The mixture was stirred for 2 h at 0°C. Epoxy modified silica nanoparticles (GFNS) was prepared as follows: 300 mg of FNS was stirred with 0.540 ml of GOPTMS in 36 ml of anhydrous toluene for 1 h at 25°C under inert atmosphere. Then, the obtained epoxy modified silica nanoparticles (GFNS) were filtrated, rinsed, twice, with fresh toluene and sonicated for 5 min. Afterwards, CCAFNS and GFNS were filtrated, washed with acetone twice and dried in a vacuum oven at 40°C for 24 h

Enzyme immobilization procedure

The immobilization of protease from *A. oryzae* (Alcalase) and protease from *B. licheniformis* (Flavourzyme) was carried out on GFNS and CCAFNS, respectively. Regardless of which support or protease was used, the same treatment was applied. 2 mg of support (GFNS or CCAFNS) was incubated with 1 ml of protease solutions (Alcalase or Flavourzyme) concentration of 0.05 mg ml^{-1} prepared in 10 mM acetate buffer pH 7.5. The samples were incubated at 25 °C and continuously stirred within an orbital shaker at a speed of 150 rpm. After 3 h, the samples were taken out and centrifuged at 13000 rpm for 2 min. The obtained immobilized protease preparations were washed with 1 ml of immobilization buffer and then used for hydrolysis of sunflower meal protein isolate.

Enzymatic hydrolysis of SMPI

The obtained SMPI (pH 7.0) is hydrolyzed was with Alcalase immobilized on the GFNS nanoparticles and Flavourzyme immobilized on CCAFNS nanoparticles by individual or sequential treatment. For the individual hydrolysis in reaction mixture 20 ml of the SMPI and 30 mg of Alcalase immobilized on GFNS or 35 mg Flavourzyme immobilized on CCAFNS were added. The reaction was carried out at 50 °C for 8 h. The sequential hydrolysis was carried out as follows: an initial hydrolysis using Alcalase immobilized on GFNS alone for 2 h, and a second one by adding Flavourzyme immobilized on CCAFNS for 6 h. During the time (1, 2, 3, 4, 6 and 8 h), samples were taken from all reaction mixtures. Hydrolysis was stopped by heat treatment at 90 °C for 10 minutes. Subsequently, the samples were analyzed by a Ninhydrin reaction to determine degree of hydrolysis by these immobilized enzymes.

RESULTS AND DISCUSSION

Generally, plant protein isolates represent an optimal material for the generation of new types of foods and are a good substrate for the production of enzymatic protein hydrolysates.

Protein isolates have high protein content, while the content of soluble sugars, lipids, phenols or fiber is very low. During production of protein hydrolisates, soluble sugars and lipids are undesirable, because they may react with proteins by Maillard reaction and as result a deficit in essential amino acids such as lysine, tryptophan and methionine could be formed. Furthermore, proteases could be absorbed on the fibers, which may lead to their activity decrease. Also, the present polyphenols may be converted into brown polymers which affects the external appearance of the product.
In this study, for the generation of the protein isolate, sunflower meal (SM) was fractionated by a sedimentation/flotation and isoelectric precipitation as described in section Material and methods (Figure 1).

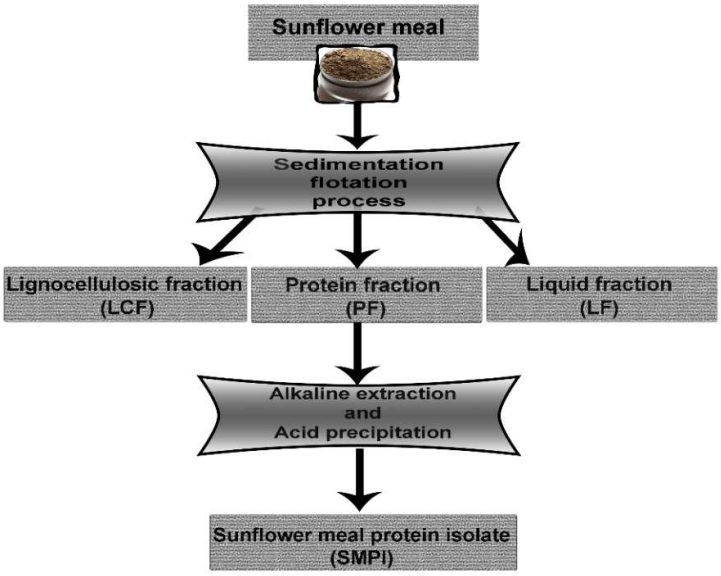

Figure 1. Preparation of sunflower meal protein isolate (SMPI).

As already stated, the procedure starts by dispersing the SM with distilled water and as result homogeneous dispersion is formed. The dispersion was left to settle down by sedimentation resulting in formation of three fractions: lignocellulosic fraction (LCF) made of fiber (floating on surface), protein fraction (PF) enriched in proteins (settled at the bottom) and liquid fraction (LF) containing soluble proteins, sugars and phenols. The PF was separated by decanting, and have reduced content of soluble sugars, lipids and polyphenols by more than 92 % with respect to the SM. The protein content was increased from 37.5 % in the SM to 40 % in the PF (Table 1).

Table 1. The content of crude proteins in fractions obtained by a sedimentation/flotation and isoelectric precipitation of SM.

Sample	**Protein concentration *(%)***	**Moisture *(%)***	**Protein concentration in dry matter *(%)***
Sunflower meal (SM)	34,31	8,45	37,4
Lignocellulosic fraction (LF)	4,5	75,53	18,4
Protein fraction (PF)	4,9	87,73	40
Sunflower meal protein isolate (SMPI)	2,36	97,08	80,8

The next step was the generation of a sunflower meal protein isolate (SMPI) by extraction and precipitation of PF proteins. For the alkaline extraction, a solution of NaOH was used. The extracted proteins were precipitated at the isoelectric point of SM proteins (pH 4.3). The

obtained precipitate is actually the protein isolate (SMPI) which was neutralized with 0.1 M phosphate buffer pH 7.5 and then used in the hydrolysis process. The protein content in the SMPI has increased, with respect to the SM, to content of approximately 80.8 % (Table 1).
SMPI is a very valuable product, and further enhancement of the separation process can be achieved by improving the functional properties of hydrolysis using immobilized Alcalase and Flavourzyme. Alcalase is covalently immobilized on epoxy modified silica nanoparticles (GFNS), while the Flavourzyme is covalently immobilized on cyanuric chloride activated amino modified silica nanoparticles (CCAFNS) (Figure 2). In addition, the hydrolysis of SMPI is carried out by using sequentially the above mentioned immobilized protease preparations (Figure 3).

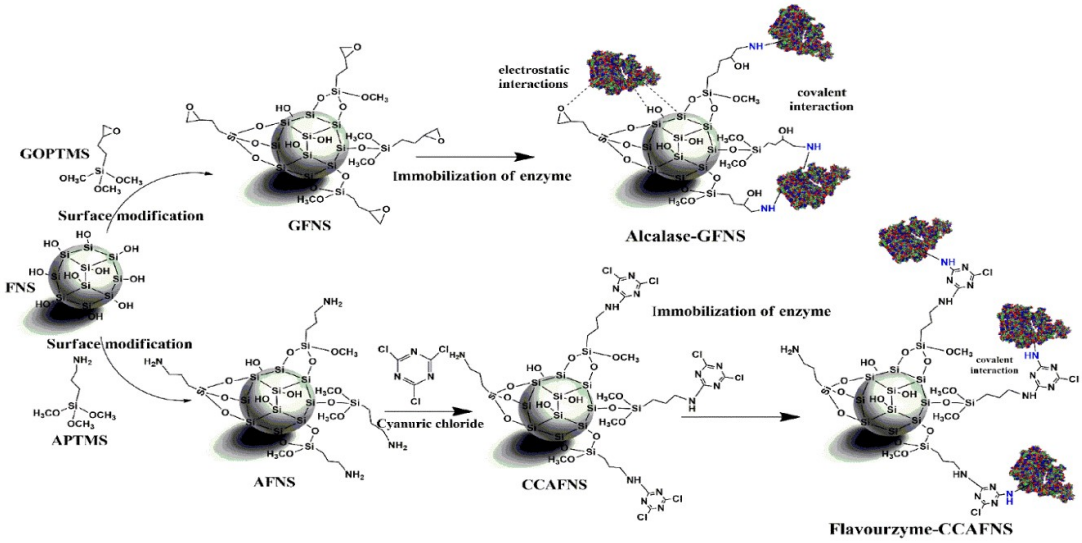

Figure 2. Schematic presentation of silica nanoparticles surface modification and immobilization of proteases on modified silica nanoparticles.

During hydrolysis of SMPI with Alcalase-GFNS, a maximum product yield of 1,539 meq arginine/l of SMPI was achieved after 8 h. It should be emphasized that the kinetic of hydrolysis with Alcalase-GFNS was very fast and 90 % of the total hydrolysis yield was achieved after 1 hour. In the case of a reaction with Flavourzyme-CCAFNS, hydrolysis was slower but with a constant increasment even after 3 h incubation. The maximum hydrolysis yield with Alcalase-GFNS is 1.7 times greater than with Flavourzyme-CCAFNS.
The addition of Flavourzyme-CCAFNS to the reaction mixture after the 2 hours of predigestion with Alcalase-GFNS resulted in a sudden increase in hydrolysis yield by 3 times. This result indicates that the hydrolysis of the SMPI by Alcalase-GFNS increased the number of N-terminal sites for the exopeptidase activity of the Flavourzyme-CCAFNS. The predigestion with Alcalase-GFNS should reduce costs since lower amount of Flavourzyme-CCAFNS is needed to obtain the same degree of hydrolysis. Also results showed that predigestion with Alcalase could be reduced to 1 h, to improve the efficiency of the process.

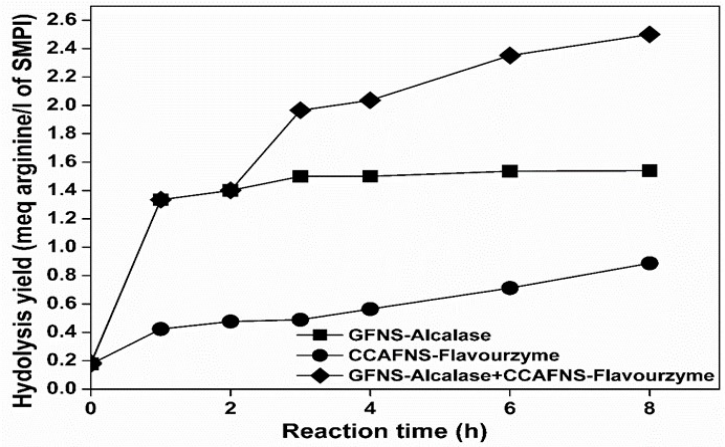

Figure 3. Hydrolysis of SMPI with GFNS-Alcalase, CCAFNS-Flavourzyme and GFNS-Alcalase+CCAFNS-Flavourzyme.

CONCLUSIONS

In conclusion, by combining two immobilized proteases preparations (Alcalase-GFNS and Flavourzyme-CCAFNS) with different catalytic activities and by using high quality protein isolate (sunflower meal protein isolate) as a starting material, an extensive protein hydrolysate (SMPIH) was produced. Obtained SMPIH could be used directly, for example, in the fortification of liquid foods or high energetic beverages, thus spreading the field of application of sunflower proteins.

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