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Analysis of Bovine Serum Albumine, Caseine and Tryptone Proteins Hydrolysis by 1H NMR Spectroscopy.

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ABSTRACT

An adequate estimation of a proteinaceous substrate decomposition is one of the problems, frequently appearing during the protein hydrolysate technology. A combination of high-resolution ^{1}H NMR spectroscopy data with other technics could be a good solution of this problem. In present paper we applied high-resolution ^{1}H NMR spectroscopy method to characterize the degree of protein hydrolysis of the Bovine Serum Albumin, Caseine and Tryptone proteins and propose to use it in a combination with other methods to describe quality of the proteinaceous substrates. Due to the fact that the methods of evaluation of the protein hydrolysis degree are not universal so the combination of methods should be used for precise the description of proteinaceous substrate degradation. Based on the results from one dimensional ^{1}H NMR spectra the hydrolysis of the protein molecules was analyzed by changes in the chemical shifts, multiplicities and line widths in ^{1}H NMR spectra. It was shown that ^{1}H NMR spectroscopy is an effective instrument for analyzing the protein hydrolysis reaction in solution.
Keywords: bovine serum albumine, caseine, tryptone, hydrolysis, proteins, NMR

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INTRODUCTION

Proteins are natural macromolecular polymers constructed from amino acid residues linked by peptide (amide) bonds. Each protein is characterized by a specific amino acid sequence. During the hydrolysis due to influence of high temperatures, pressures, aggressive acids, alkali or activity of proteolytic enzymes the peptide bonds can be hydrolyzed [1-3] and break down proteins to a short peptide chains [4-6] and individual amino acids [7, 8]. The more hydrolysis Efficacy is higher – the more individual amino acids appears in a resulting "cleaner" hydrolyzate. Protein hydrolysates recovered from proteins which containing in a waste of food industry can be recycled to produce food additives or used for animal feeding as proteinaceous additives and applied as fertilizers in farming. Higher purification aminoacid hydrolysates can be used for parenteral (inrovenous) application to improve protein absorption by patient; hydrolysates are also used in a cosmetics industry [9].

Adequate estimation of a proteinaceous substrate decomposition is one of the problems, frequently appearing during the design of a hydrolysate technology for obtaing the protein. One of the parameter for the process efficacy is a grade (depth) of hydrolysis, which can be determined by variety of methods. Ratio of amine nitrogen fraction to a total mass of nitrogen fraction in a hydrolysate is the most frequently used estimation method for protein hydrolysis efficacy [9]. One of the most popular method is hydrolysis efficacy of proteins as a fraction of non-protein nitrogen to a total mass of nitrogen or amount of soluble proteinaceous compounds. Another direct method, reflecting degree of the protein degradation, is based on optical density decrease due to formation of a low molecular weight peptides, which remain soluble in 10% solution of trichloracetic acid whereas proteinaceous compounds with molecular mass more than 2 kDa are precepitating under these conditions. Ratio of amino-acids fraction to amine nitrogen fraction can be determined for precise the description of the hydrolysis. Gel filtration method also can be used for estimation of the size distribution of degrading proteins [10, 11].

Since the methods of the protein hydrolysis degree evaluation are not universal so the combination of methods should be used for precise the description of proteinaceous substrate degradation. In present paper we applied ^{1}H NMR spectroscopy to characterize a degree of protein hydrolysis of Bovine Serum Albumin (BSA), Caseine and Tryptone proteins and propose to use this method in a combination with other ones to describe the quality of proteinaceous substrates.

EXPERIMENTAL

Materials

To study the process of hydrolysis three well-known proteinaceous compounds were chosen: bovine serum albumine (BSA), Caseine and Tryptone (Sigma-Aldrich, USA). To carry out acidic hydrolysis proteinaceuos compounds were dissolved in 0,9%NaCl to reach concentration 1g per Liter and then mixed in ratio 1:1 with concentrated HCl solution (37%, Sigma-Tech., USA). Mixture was autoclaved (Sarmat LLC, Russian Federation) for the 3 hours under the pressure of 2 atmospheres and temperature 120°C.

NMR spectroscopy

The NMR spectra were acquired on a 500 MHz (^{1}H) Bruker Avance II NMR spectrometer at 20°C. Chemical shifts are given in δ (ppm). For each spectrum, 32 transients were collected using 4 dummy scans with spectral widths of 12 ppm using 1024 complex points. The solvent signal was suppressed using 3-9-19 pulsesequence with gradients. Spectra were processed and analyzed using standard Bruker software (TOPSPIN, version 3.0). Data were Fourier transformed with a sine-bell-squared window function shifted between p/2 and p/4. A polynomial baseline correction was applied to both sides of the residual water signal.

RESULTS AND DISCUSSION

As was mentioned above an adequate estimation of a proteinaceous substrate decomposition is one of the problems, frequently appearing during the protein hydrolysate technology. A combination of high-resolution ^{1}H NMR spectroscopy data with other technics could be a good solution of this problem. In present

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paper we studied a degree of protein hydrolysis of the BSA, Caseine and Tryptone proteins by ^{1}H NMR and propose to use it in combination with other methods to describe quality of proteinaceous substrates. Discovered in 1945 by Felix Bloch and Edward Mills Purcell Nuclear Magnetic Resonance (NMR) become one of the most informative methods to study structure and dynamics of molecules, molecular interaction, chemical reactions and quantitative analysis of the compounds in different state of aggregation of matter.

It was shown that the hydrolysis is improved when additives (proteins for example) are present [12]. But the mechanisms for the enhancement of the enzymatic hydrolysis process through the use of additives are still unclear. NMR spectroscopic techniques applied in a wide range of researches as for native materials such as bone [13] and cartilage[14 which have led to structural and dynamic insights but are normally not sufficiently quantitative. It was shown that ^{1}H NMR spectroscopy is useful tool to monitor the hydrolysis of polysaccharides [15]. This technique is suitable for any soluble carbohydrate that provides at least one separated, discrete resonance between the starting material and its hydrolysed products [15]. In case with protein hydrolysis analysis the amino acid amount can be easily determined in the presence of an internal standard by ^{1}H NMR spectroscopy because amino acids proved to be stable under acidic conditions [16-18].

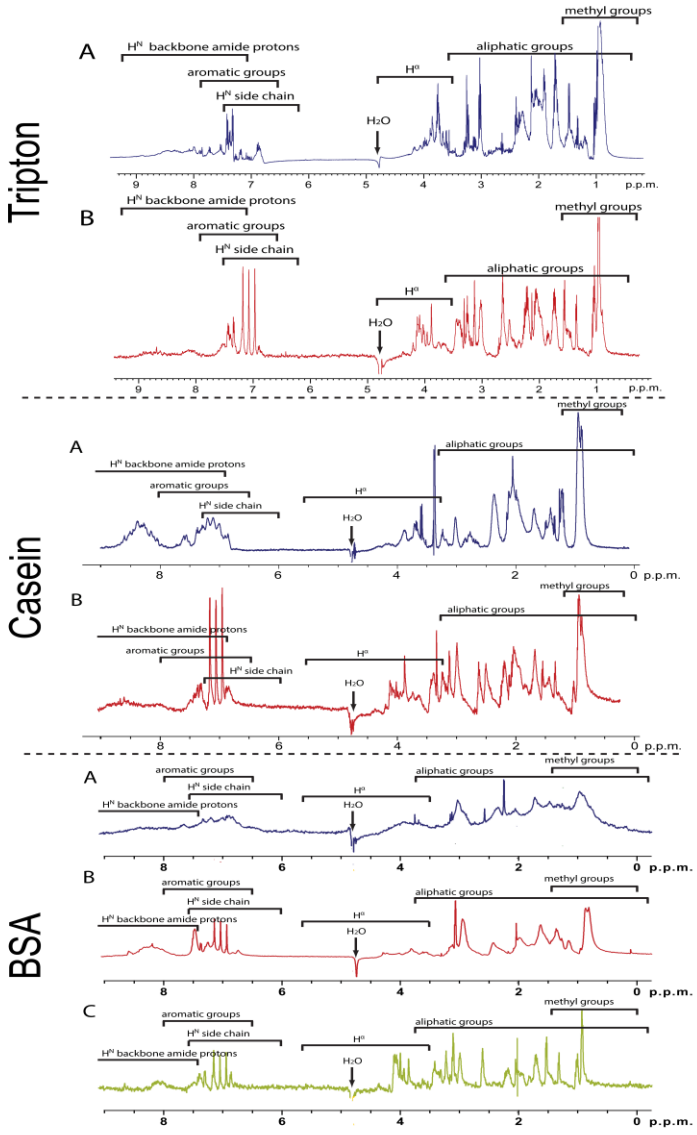

Figure 1: ^{1}H NMR spectra of proteins (Tryptone, casein, bovine serum albumin) in: a) physiological saline solution (90% H_2O + 10% D_2O); b) hydrolyzate in aqueous solution (90% H_2O + 10% D_2O) with concentrated HCl (protein solution and HCl concetration was 1:1); c) hydrolyzate after autoclave in physiological saline solution (90% H_2O + 10% D_2O).

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BSA, Casein and Tryptone were chosen as a model substance for protein hydrolysis due to their well-known proteinaceous compounds and adequate water solubility. BSA is well characterized blood protein with molecular mass 64000 Da. This one -strand protein comprises of 582 amino acid residues [19]. Casein is a mixture of phosphoproteins classified as casein family proteins. Casein has made us interest due to its numerous using in the food, drug, and cosmetic industries [20-22] as well as to its importance as an investigation material for elucidating essential questions regarding the protein chemistry [23, 24]. Proteins of casein family represent 80% of all proteins in cow milk and 20% in human milk. Tryptone is a mixture of peptides varying in size which were formed after hydrolysis of casein by protease tripsine [25-27]. Tryptone is similar to casamino acids, both being digests of casein, but casamino acids can be produced by acid hydrolysis and typically only have free amino acids and few peptide chains. Casamino acids are similar to tryptone, the latter differing by being an incomplete enzymatic hydrolysis with some oligopeptides present, while casamino acids predominantly free amino acids [25].

Commonly the hydrolysis process of proteins characterized by ratio of fraction of amine nitrogen to a fraction of total mass of nitrogen. Due to hydrolysis process the polypeptide chains can break and several short peptide chains appears and so the number of amide protons also increase. The quantity of amide protons are the characteristic of hydrolysis efficiency and it could be measured by calorimetry (by observing the intensity of staining with ninhydrin reagent). Despite the simplicity of this the routine method it is need a quite big sample volume, it is very long in time and requires the expenses of chemical reagents (Ethanol, Calcium Hydroxide, Ethylenediaminetetraacetic acid, Trichloroacetic acid and Ninhydrin), using a variety of equipment (water bath, photoelectrocolorimeter, electric driers). On the other hand it is possible to characterize the degree of protein hydrolysis by changes in the chemical shifts, multiplicities and the line widths in ^{1}H NMR spectra. This approach is relatively fast and requires only a minimum of sample pretreatments. In present paper we recorded ^{1}H NMR spectra for solutions of BSA, Casein and Tryptone in 0,9%NaCl, obtained after acidic hydrolysis of all this proteinaceous compounds (Fig. 1). As during the hydrolysis process the peptide bonds can be hydrolyzed and so break down proteins to a short peptide chains and even to an individual amino acids and based on that fact that ^{1}H NMR chemical shifts [28-30] are very sensitive to changes in chemical structure [31-33] it is possible to use this approach for the monitoring of the hydrolysis process of different peptides [34-36].

^{1}H NMR spectra of biomacromolecules (such as proteins) commonly have a lot of overlapped and broadened lines in spectra [37-39], but when hydrolysed proteins are breaking down to peptides or even to single amino acids, which have less molecular mass this can be reflected in ^{1}H NMR spectra as an increasing of signals with more narrow line width and broadening [40]. Since duration of one NMR experiment is approximately 2 minutes, analysis of ^{1}H spectra of NMR can supply with information on current state of hydrolysis in real-time mode (Fig.2).

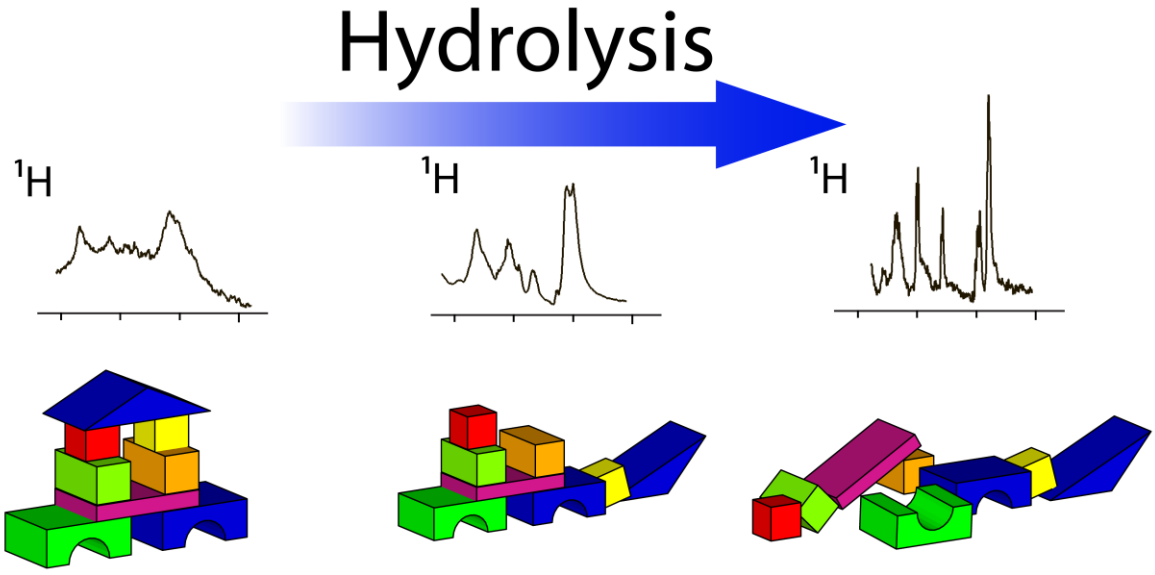

Figure 2:. Scheme of changes in ^{1}H NMR protein spectra during hydrolysis.

We conclude that the suggested NMR technique allows the monitoring of the hydrolysis process of different peptides and despite that this approach does not allow a very detailed compositional analysis, it is relatively fast and requires only a minimum of sample pretreatments. In particular, the used reagents are rather inexpensive and much less expensive than enzymes or biochemicals that are needed in the majority of comparable assays.

CONCLUSIONS

In this work we followed protein hydrolysis using ^{1}H spectroscopy of NMR. Analysis of ^{1}H spectra of NMR of studied proteinaceous compounds (Fig. 1) confirmed sufficient changes in the chemical shifts, multiplicity and lines width of BSA, Caseine and Tryptone proteins, proving the decomposition of the peptide chains to a smaller fragments or even to a single amino acids due the hydrolysis. This is an evidence that it is possible to follow proteinaceous compound hydrolysis in real-time mode using analysis of ^{1}H NMR spectra. Despite its obvious advantages, applications of NMR in protein hydrolysis research are so far very limited because the required spectrometers are expensive but on the other hand this approach does not necessarily need highfield devices and that the required instrument time is only moderate.

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