ACID HYDROLYSIS OF CASEIN

M. G. Kurbanova* and S. M. Maslennikova

Kemerovo State Institute of Agriculture,
ul. Markovtseva 5, Kemerovo, 650056 Russia,
*e-mail: kurbanova-mg@mail.ru

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Abstract: Protein hydrolysates have a high biological and nutritional value and are widely used in various sectors of the food, medical, and pharmaceutical industries. This article deals with the chemical hydrolysis of the milk protein casein in the presence of hydrochloric or sulfuric acid and reports the hydrolysis parameters minimizing the loss of amino acids. In casein hydrolysis, peptide bonds of protein molecules break to form di- and tripeptides and free amino acids, enhancing protein absorption by the body. Inadequate intake of digestible forms of protein leads to disruption of growth processes and impairs the immune resilience of the human body. To avoid the decomposition of labile amino acids, hydrolysis was performed with triply distilled 6 M hydrochloric or sulfuric acid in a vacuum in sealed ampoules for 4, 8, or 24 (±0.05) h at a temperature of 110 ± 5°C and a substrate-to-acid ratio of 1 : 15, 1 : 20, or 1 : 25. The compositions of the casein hydrolysates obtained at various hydrolysis times are presented. For a more detailed evaluation of the properties of the casein hydrolysates, the hydrolysis time effect on the molecular weight distribution of proteins and peptides has been investigated. The problem of obtaining protein hydrolysates with the desired composition and properties remains topical.

Keywords: acid hydrolysis, casein, protein, degree of hydrolysis, peptides, amino acids, hydrolysates

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INTRODUCTION

Protein hydrolysates are the products of the hydrolytic decomposition of proteins. They consist mainly of separate amino acids, their sodium salts, and polypeptide residues. During hydrolysis, the peptide bonds in protein molecules break to yield di- and tripeptides and free amino acids, thus increasing protein assimilation in the living organism. Mixtures of various peptides are more rapidly and more completely absorbed in the digestive tract than proteins themselves. In addition, protein hydrolysates may contain various physiologically active peptides necessary for regulation of a number of essential functions of the living organism. Note that peptides contained in hydrolysates can possibly exert a favorable effect on the absorption of some essential micronutrients. Protein hydrolysis is used to produce preparations for the following applications: blood substitution and parenteral nutrition in medicine; protein deficiency compensation, resistance enhancement, and improvement of youngsters' development in veterinary; sources of amino acids and peptides for bacterial and culture growth media in biotechnology. Casein hydrolysates contain peptides that are capable to form stable coordination compounds (chelates) with calcium ions and to considerably raise the absorbability of the latter [2–4]. Again there are data indicating that phosphopeptides of β-casein, like κ-casein glycomacropeptide and peptides of whey proteins, markedly increase the bioavailability of iron and can be considered as favorable factors in anemia prophylaxis.

In view of this, milk protein hydrolysates are widely used in the food, medical, pharmaceutical, and fragrance industries as rich sources of nonmacromolecular nitrogen compounds, amino acids, and proteins.

The chemical methods employed in the hydrolysis of milk proteins are facile and do not require use of uncommon or expensive enzymes, but they need severe processing conditions. The production of protein hydrolysates by acid hydrolysis is carried out above 100°C at pH 1–2 using inorganic (hydrochloric, sulfuric, orthophosphoric) acids. The rate of release and destruction of individual amino acids depends mainly on the nature of the protein.

The peptide bonds (H–N–C=O) forming the polymer chain of a protein molecule undergo hydrolysis in the presence of an acid or alkali. This causes polymer chain scission and can finally lead to the constituent amino acids. The peptide bonds occurring in α-helices or β-structures are more resistant to hydrolysis and various chemical treatments than the same bonds in single chains [1]. Investigating the chemical hydrolysis of casein, we revealed considerable structural changes in protein subunits. In addition, spectroscopic data indicate that oxyacids, dicarboxylic acids, and other compounds undergo decomposition and racemization as well, though to a lesser extent. Some authors reported that terminal structural deformations take place in nonmacromolecular peptides, which are biologically active compounds, to make them unrecognizable by cell receptors [4, 5, 7]. However, the following advantages of acid hydrolysis are noted in some reports: protein breakdown proceeds to a sufficient extent, and any bacterial contamination of the hydrolysate (including by metabolism products) is ruled out.

Here, we report a promising method of the acid hydrolysis of casein and characterize the resulting hydrolysates.

OBJECTS AND METHODS OF STUDY

The substrate was edible casein containing 85 wt % protein. In order to avoid the decomposition of the resulting labile amino acids, acid hydrolysis was performed using triply distilled 6 M hydrochloric or sulfuric acid in a vacuum in sealed ampoules at 110 ± 5°C for (4–24) ± 0.05 h. After the experiment time was over and the hydrolysis process was complete, the ampoules with the resulting casein hydrolysates were cooled and unsealed and their contents were transferred into a small conical or round-bottom flask. Hydrochloric or sulfuric acid was evaporated to dryness using a rotary evaporator at 40–65°C. For more complete removal of hydrochloric (sulfuric) acid from the hydrolysates, 1.5 mL of water was introduced into the flask and evaporation was repeated. This operation was done two times.

Total nitrogen was quantified on a RAPID N ELEMENTAR protein analyzer according to European standards. The total protein content was calculated by multiplying the total nitrogen content by a conversion factor of 6.38.

Amino nitrogen was determined spectrophotometrically using 2,4,6-trinitrobenzenesulfonic acid (TNBS). This method is based on spectrophotometric determination of the chromophores resulting from primary amine--TNBS reactions. The degree of hydrolysis was determined as the ratio of amino nitrogen to total nitrogen. The molecular weight distribution of proteins and peptides in the hydrolysates was estimated by Laemmli's protein electrophoresis method.

RESULTS AND DISCUSSION

Some experiments were intended to find rational casein hydrolysis conditions that would maximize amino acid survival in the hydrolysate. As distinct from some globular proteins, caseins are readily decomposable by chemicals because, even in their native state, they are in a low-ordered conformation that is like a disordered structure of denaturated globular proteins [3]. This is explained by the very low proportion of α-helices and by the low structural organization of the main casein components. This fact is due to the high proline content of these proteins (8.5 to 16%), which apparently deforms the helix into a disordered ball [1, 6].

The compositions of the casein hydrolysates obtained by treating casein with 6 M hydrochloric acid are presented in Table 1.

The data listed in Table 1 demonstrate that, at a substrate-to-acid ratio of 1 : 25 and a temperature of 110 ± 5°C, the hydrolysis process is directed in the right way and proceeds to a sufficient extent. The degree of hydrolysis is 32.75 ± 2.29% in 4.00 ± 0.05 h and 65.50 ± 4.59 % in 8.00 ± 0.05 h and reaches its maximum value of 96.20 ± 6.73% in 24.00 ± 0.05 h. As the substrate-to-acid ratio is decreased to 1 : 15 and 1 : 20, the degree of hydrolysis in 24.00 ± 0.05 h falls to 82.69 ± 5.79 and 92.99 ± 6.50%, respectively. This is possibly due to the polypeptide chain being insufficiently strongly attacked by the hydrochloric acid solution. It was also discovered that ammonia accumulates as the hydrolysis time is extended. For example, as the hydrolysis time is lengthened from 4.00 to 24.00 h at substrate-to-acid ratios of 1 : 15, 1 : 20, and 1 : 25, the ammonia weight fraction increases from 0.015 to 0.095%, from 0.034 to 0.156%, and from 0.085 to 0.200%, respectively.

The same trend is observed for the weight fraction of .amino nitrogen. This is obviously due to the increase in the number of cleaved amide bonds in separate amino acids. For the sake of comparison, we carried out casein hydrolysis with 6 M sulfuric acid under the same conditions. The results of these experiments are presented in Table 2.

The data listed in Table 2 suggest that, as the hydrolysis time is extended, amino nitrogen and ammonia accumulate and the degree of hydrolysis increases. The intensity of the process increases with an increasing volume of the acid. For example, the degree of hydrolysis at a substrate-to-acid ratio of 1 : 15 and a hydrolysis time of 24.00 ± 0.05 h is 74.42 ± 5.20%, while the degree of hydrolysis at substrate : acid = 1 : 25 and a hydrolysis time of 24.00 ± 0.05 h is 88.52 ± 6.19%.

In the sample with substrate : acid = 1 : 15, the weight fraction of ammonia increases by a factor of 1.78; as the substrate-to-acid ratio is increased to 1 : 20 and 1 : 25, the ammonia weight fraction grows by a factor of 2.81 and 3.49, respectively. This finding is not in conflict with data obtained by other researchers [3–5, 7]. Thus, we have ascertained that casein hydrolysis proceeds more rapidly and more efficiently under the action of 6 M hydrochloric acid.

In order to evaluate the properties of the resulting acid hydrolysates in greater detail, we studied the hydrolysis time effect on the molecular weight distribution of proteins and peptides in the hydrolysates. The results of these experiments are presented in Table 3.

Some experiments demonstrated that the amount of proteins and peptides accumulated is proportional to the hydrolysis time. For example, at a hydrolysis time of 4.00 ± 0.05, 8.00 ± 0.05, and 24.00 ± 0.05 h, the reaction mixture consists mainly of peptides with a molecular weight of >20, 5–20, and <5 kDa, respectively, at any substrate-to-acid ratio.

The same trend is observed in casein hydrolysis with 6 M sulfuric acid (Table 4). For example, at a substrate-to-acid ratio of 1 : 15 and a hydrolysis time of 4.00 ± 0.05 h, the proportion of peptides with a molecular weight of over 20 kDa is 14.24%.

Table 1. Compositions of the casein hydrolysates obtained by treating casein with 6 M hydrochloric acid

Hydrolysis time, h	Weight fraction, %			Degree of hydrolysis, %
	total nitrogen	ammonia	amino nitrogen	
Initial casein sample	13.32 ± 0.93	0	0	0
Substrate : acid = 1 : 15				
4.00 ± 0.05	13.32 ± 0.93	0.015 ± 0.001	0.076 ± 0.005	19.48 ± 1.36
8.00 ± 0.05		0.062 ± 0.004	0.310 ± 0.022	39.30 ± 2.75
24.00 ± 0.05		0.095 ± 0.007	1.976 ± 0.138	82.69 ± 5.79
Substrate : acid = 1 : 20				
4.00 ± 0.05	13.32 ± 0.93	0.034 ± 0.002	0.136 ± 0.010	26.15 ± 1.83
8.00 ± 0.05		0.138 ± 0.010	0.550 ± 0.039	52.38 ± 3.67
24.00 ± 0.05		0.156 ± 0.011	3.126 ± 0.219	92.99 ± 6.50
Substrate : acid = 1 : 25				
4.00 ± 0.05	13.32 ± 0.93	0.085 ± 0.006	0.284 ± 0.020	32.75 ± 2.29
8.00 ± 0.05		0.144 ± 0.024	1.148 ± 0.0080	65.50 ± 4.59
24.00 ± 0.05		0.200 ± 0.070	3.880 ± 0.272	96.20 ± 6.73

Table 2. Compositions of the casein hydrolysates obtained by treating casein with 6 M sulfuric acid

Hydrolysis time, h	Weight fraction, %			Degree of hydrolysis, %
	total nitrogen	ammonia	amino nitrogen	
Initial casein sample	13.32 ± 0.93	0	0	0
Substrate : acid = 1 : 15				
4.00 ± 0.05	13.32 ± 0.93	0.014 ± 0.001	0.068 ± 0.004	17.54 ± 1.23
8.00 ± 0.05		0.056 ± 0.004	0.279 ± 0.02	35.37 ± 2.47
24.00 ± 0.05		0.356 ± 0.025	1.778 ± 0.12	74.42 ± 5.20
Substrate : acid = 1 : 20				
4.00 ± 0.05	13.32 ± 0.93	0.031 ± 0.002	0.122 ± 0.008	23.54 ± 1.64
8.00 ± 0.05		0.124 ± 0.008	0.495 ± 0.034	47.14 ± 3.30
24.00 ± 0.05		0.140 ± 0.01	2.813 ± 0.19	88.19 ± 6.17
Substrate : acid = 1 : 25				
4.00 ± 0.05	13.32 ± 0.93	0.077 ± 0.005	0.256 ± 0.01	29.48 ± 2.06
8.00 ± 0.05		0.099 ± 0.02	1.033 ± 0.07	58.95 ± 4.12
24.00 ± 0.05		0.190 ± 0.006	3.492 ± 0.24	88.52 ± 6.19

Table 3. Molecular weight distribution of the proteins and peptides resulting from casein hydrolysis in 6 M hydrochloric acid

Hydrolysis time, h	Relative content, %, at a given molecular weight, kDa			
	>20	10–20	5–10	<5
Substrate : acid = 1 : 15				
4.00 ± 0.05	14.02 ± 0.98	38.50 ± 2.69	26.00 ± 1.82	21.48 ± 1.50
8.00 ± 0.05	6.02 ± 0.42	14.12 ± 0.98	37.06 ± 2.59	42.80 ± 2.99
24.00 ± 0.05	0 ± 0.07	7.02 ± 0.49	8.42 ± 0.59	84.56 ± 5.92
Substrate : acid = 1 : 20				
4.00 ± 0.05	12.72 ± 0.89	26.25 ± 1.84	30.40 ± 2.13	30.63 ± 2.14
8.00 ± 0.05	4.02 ± 0.28	10.12 ± 0.71	33.06 ± 2.31	52.80 ± 3.69
24.00 ± 0.05	0 ± 0.06	2.10 ± 0.15	3.52 ± 0.25	94.38 ± 6.61
Substrate : acid = 1 : 25				
4.00 ± 0.05	10.27 ± 0.72	20.42 ± 1.43	40.80 ± 1.94	28.51 ± 1.36
8.00 ± 0.05	3.73 ± 0.26	7.53 ± 0.53	21.62 ± 1.51	67.12 ± 4.69
24.00±0.05	0 ± 0.06	0.52 ± 0.03	1.87 ± 0.13	97.61 ± 6.83

Table 4. Molecular weight distribution of the proteins and peptides resulting from casein hydrolysis in 6 M sulfuric acid

Hydrolysis time, h	Relative content, %, at a given molecular weight, kDa			
	>20	10–20	5–10	<5
Substrate : acid = 1 : 15				
4.00 ± 0.05	14.24 ± 0.99	39.19 ± 2.74	27.95 ± 1.96	18.62 ± 1.30
8.00 ± 0.05	8.17 ± 0.57	15.06 ± 1.05	38.58 ± 2.70	38.19 ± 2.67
24.00 ± 0.05	0 ± 0.08	7.12 ± 0.50	15.92 ± 1.11	76.96 ± 5.39
Substrate : acid = 1 : 20				
4.00 ± 0.05	12.24 ± 0.86	29.19 ± 2.04	32.95 ± 2.30	25.62 ± 1.79
8.00 ± 0.05	7.17 ± 0.50	12.06 ± 0.84	30.58 ± 2.14	50.19 ± 3.51
24.00 ± 0.05	0 ± 0.08	1.92 ± 0.13	7.12 ± 0.50	90.96 ± 5.39
Substrate : acid = 1 : 25				
4.00 ± 0.05	10.45 ± 0.73	17.20 ± 1.20	41.84 ± 2.93	30.51 ± 2.14
8.00 ± 0.05	3.41 ± 0.23	8.56 ± 0.59	25.91 ± 1.81	62.12 ± 4.35
24.00 ± 0.05	0 ± 0.06	3.92 ± 0.27	5.47 ± 0.38	90.61 ± 6.34

As the hydrolysis time is increased, the amount of peptides with a molecular weight of over 20 kDa decreases to 8.17 ± 0.57%, and disappears entirely in 24.00 ± 0.05 h owing to the attack of the chemical agent on the polypeptide chain and the buildup of nitrogen-containing compounds with a lower molecular weight. As a consequence, the proportion of peptides with a molecular weight of <5 kDa increases to become 18.62 ± 1.30% at a hydrolysis time of 4.00 ± 0.05 h and, as the hydrolysis time is extended to 24.00 ± 0.05 h, their amount increases further by a factor of 4.1 to become 76.96 ± 5.39%. It was observed that, as the concentration of the chemical agent is raised, the degree of hydrolysis increases and this leads to an increase in the proportion of peptides with a molecular weight of <5 kDa. For example, at a hydrolysis time of 24.00 ± 0.05 h, the proportion of peptides with a molecular weight of < 5 kDa increases by a factor of 1.3, specifically from 76.96 ± 5.39 to 90.61 ± 6.3%.

Note also that, as the hydrolysis time is extended, free amino acids build up intensively, and sulfuric acid, a dibasic one, ensures severer hydrolysis conditions than hydrochloric acid. The largest amount of amino acids accumulates in 24 h at a substrate-to-acid ratio of 1 : 25.

CONCLUSIONS

Our experiments demonstrated that the 24-h-long hydrolysis of casein at the optimal substrate-to-acid ratio, which is 1 : 25, affords casein hydrolysates with a high degree of hydrolysis and the maximum amount of amino acids accumulated. It should be taken into account that the proportion of peptides with a molecular weight of <5 kDa is 90.61 ± 6.34% in the hydrolysis with sulfuric acid and 97.61 ± 6.83% in the hydrolysis with hydrochloric acid.

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