Technical Paper

PREPARATION AND CHARACTERIZATION OF CHICKEN EGG WHITE HYDROLYSATE

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Abstract

Albumen of freshly laid chicken eggs was thermally treated at various pH values in order to find optimal conditions for subsequent enzymatic hydrolysis with Alcalase (Subtilisin Carlsberg). Alkalisation to pH 10 followed by 30 minutes heating between 75 °C and 80 °C resulted in irreversible denaturation of Alcalase inhibitors and formation of soluble aggregates of denatured proteins. Denatured albumen was most effectively hydrolysed at 60 °C and pH 8.5. Hydrolysis rate between pH 7 and pH 9 was relatively high and therefore hydrolysis without pH regulation was performed. Bitter peptides, which were formed as a result of Alcalase hydrolysis, were degraded with Flavourzyme, a mixture of fungal exopeptidases and endopeptidases. Degree of hydrolysis over 30 and high yield of peptides soluble over a wide pH range were obtained by combination of peptidases. Hydrolysate, which contained substantial amount of dipeptides and tripeptides was only moderately bitter and had an overall acceptable flavour.

Key words: egg white, hydrolysate, hydrolysis, Flavourzyme, Alcalase, peptides, inhibitor

Introduction

Chicken egg white (CEW) is a ≈ 11% aqueous solution of proteins, containing less than 1% of carbohydrates, ≈ 0,5% of minerals and is practically devoid of lipids. Proteins of the chicken egg white (CEWP) also referred as albumen have a balanced content of essential amino acids and therefore an excellent nutritional value.[1] Not only high nutritional value, but also unique functional properties, as gelling and foaming[2] contributes to the extensive use of albumen as ingredient in various processed foods.

Major drawback of albumen use in human nutrition is relatively high antigenicity.[3] Up to few percents of babies can be hypersensitive to egg white,[4] which is despite its optimal amino acid content therefore scarcely used in the nutrition of infants. The antigenic epitope of ovomucoid, a major CEW allergen, is partially destroyed upon prolonged boiling[5] and antigenicity is further reduced by enzymatic hydrolysis.[6]

Extensive hydrolysis in generally reduces the antigenicity of food proteins and result in new functional properties as high solubility over a wide pH range and low viscosity of highly concentrated solutions. Due to the property of dipeptides and

tripeptides, which are absorbed directly through the brush border membrane,[7] extensively hydrolysed proteins can be used as high quality protein supplement for the nutrition of individuals with impaired digestive function or when high demand for intake of proteins is present.

Enzymatic hydrolysis of proteins is unfortunately associated with bitterness of short hydrophobic peptides, which are formed during the process. The bitterness is dependent on the protein hydrolysed, degree of hydrolysis and on the type of the enzyme used. Endopeptidases with broad substrate specificity as Alcalase (subtilisin Carlsberg), which are frequently used for enzymatic hydrolysis of food proteins yield certain amount of bitter peptides. Bitterness of such hydrolysates can be substantially reduced by introduction of exopeptidases at the late stages of the process.[8]

The aim of present work has been to (I) optimise the process of CEWP denaturation prior to enzymatic hydrolysis, (II) find optimal conditions for hydrolysis and (III) prepare hydrolysate with high yield of soluble peptides and acceptable flavour.

Materials and methods

Materials

Chicken egg white of freshly laid eggs was separated from the yolk and gently stirred without foam formation to provide homogeneous mixture. Alcalase 2.4L (2.4 AU/g) and Flavourzyme 1000 (1000 LAPU/g) were a kind gift of Novo Nordisk (Bagsvaerd, Denmark). All other chemicals were of analytical grade.

Optimisation of substrate preparation

Homogeneous mixture of CEWP was adjusted to appropriate pH using 4 M HCl or 4 M NaOH, followed by dilution with distilled water to 5.5% protein concentration. Each sample with adjusted pH was heated for 30 minutes on 75 °C or in boiling water. Samples were then cooled to the room temperature and homogenised with Potter–Elvehjem glass homogeniser with a Teflon pestle. Cooled samples were diluted with distilled water to 2% protein concentration and pH of all samples was adjusted to 8.

Optimisation of enzymatic hydrolysis

Enzymatic hydrolysis was normally carried out at 2% protein concentration, 55 °C and pH 8 with 24 AU of Alcalase per kg of CEWP (E/S = 1:100) in pH-stat (718 Stat

TITRINO, Methrom) with continuous stirring. When temperature of the hydrolysis (50 °C, 55 °C and 60 °C), pH (7, 7.5, 8, 8.5 and 9) and E/S ratio (12 AU/kg, 24 AU/kg, 48 AU/kg and 120 AU/kg) were optimised, all other parameters were those as normally used.

Hydrolysis with drifting pH

The pH of homogeneous mixture of CEWP was adjusted to 10. Sample was diluted to 5.5% protein concentration and divided into three equal parts. Samples were transferred into Erlenmeyer flasks and heated at 80 °C for 30 minutes in thermostated shaker (90 rpm). Samples were then cooled to 60 °C and 24 AU of Alcalse per kg of proteins was added into each probe. Hydrolysis was allowing to proceed for 1 hour, than another 24 AU of Alcalase per kg of proteins was added into sample 1, 10 LAPU of Flavourzyme per g of proteins (E:S = 1:100) was added into sample 2, whereas no extra enzyme was added into sample 3. Enzymatic reaction was stopped after 9 hours of total hydrolysis time by raising the temperature to 85 °C for 15 minutes. Samples were cooled to the room temperature and centrifuged for 15 minutes at 10000 g, in order to separate soluble peptides from undigested slurry. Supernatant was collected and freeze-dried.

Determination of Degree of Hydrolysis

Degree of hydrolysis (DH) is a measure for the proportion of peptide bonds in protein substrate that are cleaved by hydrolysis. DH values for the experiments performed in pH-stat were calculated from consumption of base, mass of the substrate and degree of protonation of N-terminal amino group at the conditions of hydrolysis.[9] When pH was not held constant during hydrolysis, DH values were determined spectrophotometrically with 2,4,6 trinitrobenzensulfonic acid,[10] using leucine as standard.

Amino acid analysis

Samples were hydrolysed prior to the amino acid analysis for 24 and 72 hours in 6 M HCl at 110 °C in an inert atmosphere. Analysis was performed on Amino Acid analyser Biosystem 421 A (Applied Biosystems). Amino acid composition obtained from 24-hour hydrolysis was corrected with the results of 72-hour hydrolysis for valine

and isoleucine due to incomplete hydrolysis of peptides containing these two amino acids within 24 hours.

Senzorical evaluation

Bitterness of CEWH was quantitatively evaluated by three individuals. Freeze-dried hydrolysates were dissolved in distilled water at a 30 mg/mL peptide concentration. Bitterness of hydrolysates was compared to a range of caffeine concentrations and was expressed as an isointensity concentration.

Determination of protein and peptide concentration

Protein and peptide concentration was determined with Kjeldahl analysis of nitrogen, multiplying the % N by factor 6.68. Concentration was alternatively determined spectrophotometrically, comparing the absorbance at 280 nm of CEWH to the absorbance of CEWP with known concentration.

Size exclusion chromatography

TSKgel G2000SWXL chromatography column attached to the LDC/Milton Roy, HPLC system was used for the estimation of molecular weight of peptides and proteins. Chromatography column, 7.8 mm in diameter and 300 mm in length, with 5 μm particle size and 125 Å pore size, was supplied from TosoHaas Bioscience. HPLC system consisted of constaMetric 3000 isocratic pump, spectroMonitor 3100 UV detector and injection valve (model 7125 Rheodyne) with 100 μl sample loop. 0.1 M sodium phosphate pH 7, containing 0.3 M NaCl was used as elution buffer at a flow rate 1 L/min. Peptides were detected measuring the absorbance at 215 nm.

Results and discussion

Preparation of the albumen for the hydrolysis

Alcalase is a serine proteinase, widely used for hydrolysis of food proteins due to its efficiency and relatively low price. The egg white nevertheless contains a substantial amount of ovomucoid, which is a strong inhibitor of Alcalase.[11] Therefore denaturation temperature and pH of thermal treatment of the albumen prior to the hydrolysis were optimised. The aim of the optimisation was to find the conditions that would lead to

irreversible denaturation of the inhibitor, but would not result in the formation of dense and compact precipitates, which are poor substrates for proteinases. Insoluble agregates or firm gels, which prevent access of the enzyme to the substrate, were formed under all conditions tested, except when the sample was adjusted to pH 10 and heated at 75 °C. Precipitates were formed at the acidic pH and firm gels at the alkaline side.

Samples were after denaturation diluted to 2% protein concentration, homogenised and hydrolysed at pH 8 and 55 °C with Alcalase. Degree of hydrolysis of each sample was followed with NaOH titration (Figure 1).

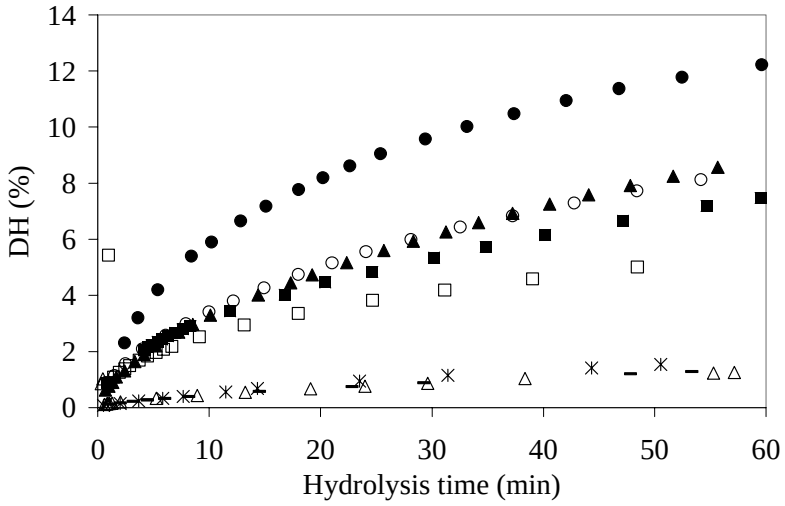

Figure 1. Effect of substrate denaturation on the hydrolysis rate. Chicken egg white albumen was thermally treated at pH 2 and 100 °C (✶), pH 4 and 100 °C (–), pH 6 and 75 °C (Δ), pH 6 and 100 °C (▲), pH 8 and 75 °C (□), pH 8 and 100 °C (■), pH 10 and 75 °C (○), pH 10 and 100 °C (●) and than hydrolyzed with Alcalase 24 AU/kg proteins at 55 °C and pH 8.

Boiling of the samples prior to the hydrolyis and higher pH generally resulted in higher DH values. At pH 2 and pH 4 very low hydrolysis rate was detected, even if samples were boiled prior to the hydrolysis. Major difference between 75 °C and 100 °C was observed at pH 6, when only higher temperature resulted in substantial hydrolysis rate. Low DH values obtained, when CEW was thermally treated at pH 6 or lower can be ascribed to the high reversibility of ovomucoid denaturation at acidic pH.[12] Thermal treatment is associated also with gelling and/or precipitation of CEW proteins. Relatively large particles with low surface/volume ratio that are formed during precipitation at slightly acidic and neutral pH are not optimal substrates.[13] Residual amount of ovomucoid and hampered accessibility of proteinases for substrate therefore accounts for slower hydrolysis rate.

Both, 75 °C and 100 °C were effective at the alkaline pH, hydrolysis was nevertheless faster when higher temperature was applied. Much higher DH values obtained after thermal treatment at basic pH, were probably the result of disulfide exchange and formation of non-native disulfide bonds in ovomucoid,[14] which resulted in irreversible denaturation of the inhibitor.

Albumen denatured at pH 10 and 75 °C for 30 minutes was hydrolysed with 12 U, 24 U and 48 U of Alcalase per kg of CEWP at pH 8 for 20 minutes, in order to test whether all inhibitors are denatured under these conditions. Samples hydrolysed with 12 U and 24 U of Alclalase per kg of CEWP, reached 18% and 45% of DH of the sample hydrolysed with 48 U of Alcalase respectively. That is considerably less, even if fourfold and twofold lower concentration of the enzyme is taken into the account, thus giving after normalisation 72% of the reaction rate for 12 U and 90% for 24 U of the sample hydrolysed with 48 U of alcalase per kg of CEWP. As residual amount of inhibitor is present, 24 U or higher concentration of Alcalase per kg of CEWP was used in further experiments.

The length of incubation time was chosen on the basis of the experiments, where samples were adjusted to pH 10 and heated at 75 °C for different periods of time from few minutes to one hour. Subsequent hydrolysis with Alcalase revealed that heating over 30 minutes does not considerably increase the rate of hydrolysis. Gelling tendency and possibility of toxic lysinoalanine formation[15] under prolonged thermal treatment at alkaline pH were also in the favour of shorter incubation time at 75 °C rather than at 100 °C, despite higher hydrolysis rate obtained after boiling of CEW (Figure 1). The actual pH during thermal treatment was lower than 10. As proteins unfold certain ionisable groups are exposed and pH drops from 10 at the beginning of the thermal treatment to 9.2 after 30 minutes.

CEWP remain soluble under these conditions up to 80 °C if less than 5.5% albumen is used. High solubility is a result of repulsive forces due to net negative charge, which keep proteins in the solution[16,17] and moderate protein concentration.[18]

Optimization of the hydrolysis process

Hydrolysis rate is also temperature dependent. A higher temperature speeds up enzymatically catalysed reactions, but also tends to denature the enzyme. The so-called

temperature optimum therefore depends on the time scale of the process. It was previously shown that soybean isolate is most effectively hydrolysed with Alcalase at 50 °C and 55 °C, whereas less peptide bonds are cleaved at 60 °C after 3 hours.[19] We have tested hydrolysis of CEWP at these three temperatures (Figure 2).

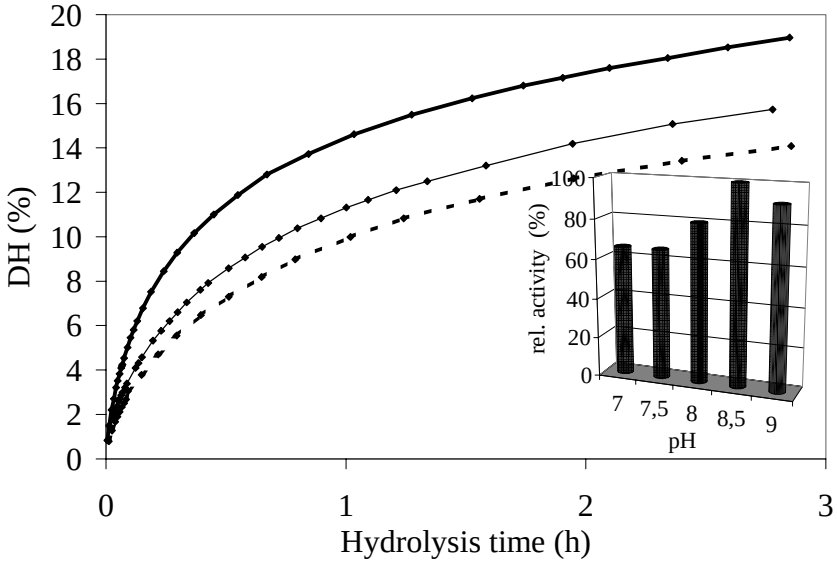

Figure 2. Hydrolysis rate as a function of temperature and pH. Chicken egg white albumen was heated at 75 °C and pH 10 for 30 minutes and than hydrolyzed with Alcalase 24 AU/kg proteins at pH 8 and 50 °C (broken line), 55 °C (thin line) or 60 °C (thick line). Hydrolysis rate at 55 °C as a function of pH is shown as insert in Figure 2.

Surprisingly the highest temperature tested, 60 °C, was the most effective even after prolonged hydrolysis. Only 74% and 84% of DH at 60 °C were observed for 50 °C and 55 °C respectively. Preference for higher temperature during the hydrolysis of CEWP with Alcalase can be ascribed to stabilisation of the enzyme by the substrate or more likely to partial denaturation of ovomucoid at 60 °C, which is concomitantly degraded by Alcalase and therefore looses its inhibitory activity. Temperature of 60 °C, which was shown to be optimal for hydrolysis, prevents growth of pathogenic mesophilic microorganisms like salmonela spp.[20] and is therefore appropriate for substrate like CEWP, an optimal media for their growth, especially when proteins are in denatured state.[21] Relatively high temperature nevertheless speeds up chemical modification of amino acids and Maillard reaction of proteins and peptides with carbohydrates of CEW, which could result in lower nutritional value. Therefore higher concentration of Alcalase and Flavourzyme could be used, in order to shorten the hydrolysis.

Alcalase is active at neutral and alkaline pH, nevertheless its pH optimum is dependent on the substrate used. In order to find optimal conditions for the hydrolysis of CEWP, albumen was denatured under standard conditions and than hydrolysed with Alcalase at different pH values. Relative DH after 1 hour of hydrolysis, expressed as a percent of maximal DH obtained, is shown as insert in Figure 2. Alcalase has maximal activity at pH 8.5 similar to haemoglobin and casein,[19] nevertheless drop in activity towards neutral pH is not so pronounced as with haemoglobin, where hydrolysis rate drops to 60% of maximal rate only after shifting the pH from 8.5 to 8. With the CEWP 2/3 of maximal hydrolysis rate is retained down to pH 7.

Extensive hydrolysis of CEWP

We have found that 5.5% protein solution of the albumen can be heated for 30 minutes at 80 °C, without firm gel formation. Therefore denaturation at these conditions followed by hydrolysis at pH 8.5 and 60 °C was used for the prolonged and extensive hydrolysis.

During the hydrolysis free amino acids and peptides are formed and base have to be added in order to maintain alkaline pH. Addition of base results in higher ionic strength and change of taste, which is not desired, if extensively hydrolyzed CEWP would be used as a supplement for human nutrition. As an alternative to the hydrolysis at constant pH, we have performed hydrolysis without pH regulation. Alcalase was added to denatured substrate cooled to 60 °C in order to start the hydrolysis. Sample became very viscous within 10-15 minutes, probably the result of lower pH that promotes formation of the gel.[22] However as hydrolysis proceeded sample liquefied again. After 1 hour substrate was resolubilised and pH dropped to 7.

In order to degrade bitter peptides a mixture of fungal endopeptidases and exopeptidases, active at neutral and slightly alkaline pH, with the commercial name Flavourzyme was added to the albumen partially digested with Alcalase. Introduction of Flavourzyme resulted in higher DH values, compared to the sole Alcalase hydrolysis, as already observed for other complex substrates.[23,24] DH values over 30 were obtained, after 9 hours hydrolysis (Figure 3).

Characterisation of the hydrolysate

Despite relatively high DH values, certain amount of CEWP remained insoluble. In order to determine the yield of soluble peptides, hydrolysate was centrifuged and

amount of nitrogen in supernatant was determined. More than 75% of the initial nitrogen remained soluble after hydrolysis with sequential addition of Alcalase and combination of Alcalase and Flavourzyme (Table 1). All hydrolysates are highly soluble even at slightly acidic pH (results not shown) where solubility is usually the lowest and therefore appropriate as supplement of high quality protein into various beverages.

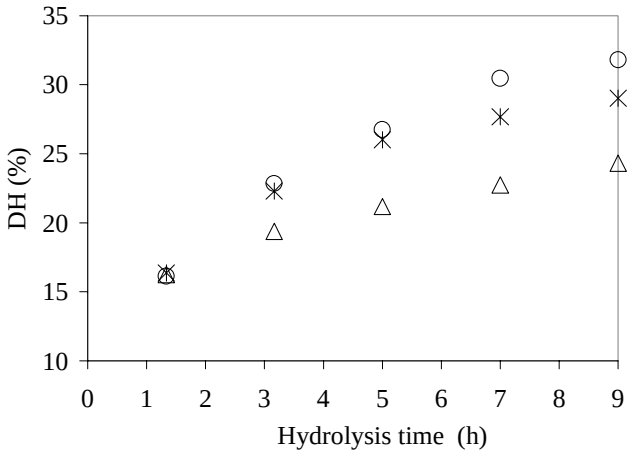

Figure 3. Hydrolysis without pH regulation. Denatured albumen was hydrolyzed with Alcalase 24 AU/kg protein (Δ), 24 AU/kg protein + 24 AU/kg protein added after 1 hour of hydrolysis (✶) and with combination of Alcalase 24 AU/kg protein and Flavourzyme 10 LAPU/g protein, which was added after 1 hour of hydrolysis (○).

Table 1. Characterization of hydrolysate obtained by enzymatic cleavage without pH regulation.

	Alcalase (24 AU/kg)	Alcalase (48 AU/kg)	Alcalase (24 AU/kg) Flavourzyme (10 LAPU/g)
DH (%) after 9 hours of hydrolysis	24	29	32
Soluble proteins and peptides (%)	67	76	77
Solubility in 1 M TCA (%)[a]	90	91	91
Caffeine isointensity bitterness (mM)[b]	8.2 ± 0.6	7.7 ± 0.8	4.2 ± 0.3

[a] Refers to the soluble proteins and peptides obtained after hydrolysis. [b] Means ± standard deviations (n = 3).

Major difference between sole Alcalase hydrolysis and hydrolysis with combination of both enzymes was observed in the bitterness of hydrolysates. Introduction of Flavourzyme yielded the hydrolysate with acceptable flavour and moderate bitterness. CEWH of 30 mg/mL hydrolysed with combination of both enzymes has the same bitterness as 4 mM caffeine. Samples treated only with Alcalase were about twice as bitter (Table 1).

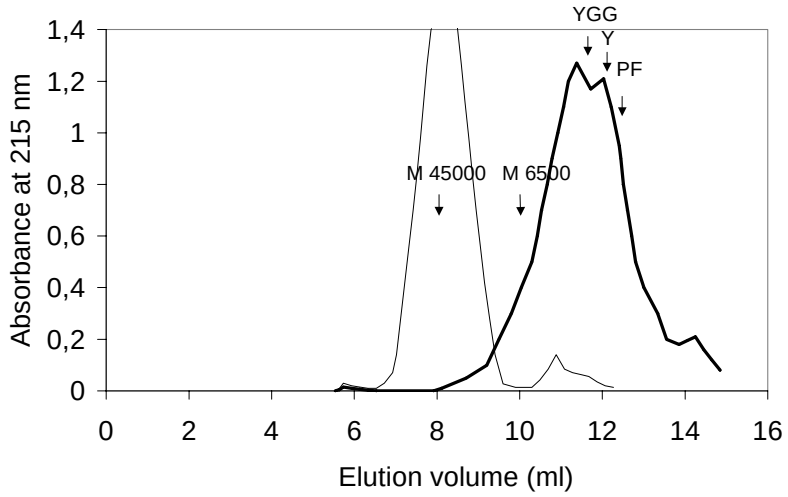

Figure 4. Size exclusion chromatography elution profile of raw chicken egg white albumen (thin line) and hydrolysate obtained by combination of Flavourzyme and Alcalase (thick line). Elution volumes of standards: egg albumin (M = 45 000 Da), aprotinin (M = 6 500 Da), YGG (Tyr-Gly-Gly), PF (Pro-Phe) and Y (Tyr) are indicated.

Elution profile on size exclusion chromatography column of the hydrolysate yielded by combination of both enzymes reveals that practically all proteins and large peptides are hydrolysed to smaller peptides. The elution profile of the hydrolysate and untreated albumen together with the position of elution volumes of certain peptide and protein standards is shown in Figure 4.

Over 90% of peptides are soluble in 15% TCA, which additionally confirms that majority of peptides is composed of less than 4 amino acids, an approximate limit for solubility in TCA.[25] DH values over 30 indicates that hydrolysate contains substantial amount of tripeptides and presumably dipeptides and free amino acids that can be absorbed directly through the brush border membrane[7] and therefore used for the nutrition of individuals with reduced intestinal absorptive area. Thermal treatment followed by extensive hydrolysis drastically reduces the antigenicity of CEWP,[26,27] which is additional advantage of such product.

Chicken egg white proteins have a high nutritional value due to balanced content of essential amino acids. In order to find out whether nutritional value is affected by hydrolysis, amino acid composition of hydrolysate was compared to the composition of untreated albumen. Amino acid composition of both samples was very similar to the average values for CEWP[28] and all amino acids are within (115% - 85%) of declared values. Minor differences still exist between hydrolysate and untreated albumen.

Hydrolysate contains ≈15% less cysteine, phenylalanine and tyrosine. Formation of dehydrolalnine[29] during prolonged heat treatment can result in the lower content of the cysteine in hydrolysate, whereas lower content of phenylalanine and tyrosine can be ascribed to relatively low solubility.[30,31] Tryptophan, an essential amino acid, is similarly to phenylalanine and tyrosine relatively insoluble in water, nevertheless it was destroyed prior to amino acid analysis and its content was not determined by this method. We have estimated its content spectrophotometrically, measuring the absorbance at 280 nm, and comparing protein concentration obtained by absorbance measurement to concentration obtained by Kjeldahl determination of nitrogen. Ideally, if tyrosine and tryptofan, which strongly absorb at 280 nm, are not selectively lost during hydrolysis, mass concentration of peptides determined by two methods should match. Calculated concentration was indeed very similar in both ways and only ≈ 10% lower concentration was determined spectrophotometrically, meaning that only small amount of tryptophan, if any was lost during enzymatic hydrolysis.

Conclusions

Chicken egg white proteins are a source of high quality proteins and can be used for the preparation of hydrolysates with optimal amino acid composition. High content of proteinase inhibitors and gelling tendency are nevertheless the major drawbacks in the process of hydrolysate preparation. We have optimised the process of thermal denaturation as a function of pH in order to find the conditions that would result in irreversible denaturation of protease inhibitors and formation of soluble aggregates. Hydrolysis of denatured albumen with Alcalase was studied at various temperatures and pH values. Flavourzyme, a mixture of fungal exopeptidases and endopeptidases was added to the partially degraded albumen, resulting in degradation of bitter peptides and increase in degree of hydrolysis. High yield of peptides soluble over a wide pH range and overall acceptable flavour were obtained by combination of enzymes.

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Povzetek

Jajčni beljak smo segrevali pri različnih pH-jih z namenom, da ugotovimo najboljše pogoje za encimsko hidrolizo z Alkalazo (Subtilisin Carlsberg). Naalkaljenje na pH 10 in segrevanje pri 75 °C do 80 °C je vodilo do ireverzibilne denaturacije inhibitorjev Alkalaze in tvorbe topnih agregatov denaturiranih proteinov. Optimalni pogoji za hidrolizo tako denaturiranega jajčnega beljaka so bili pri 60 °C in pH 8,5. Ker je bila hitrost hidrolize relativno visoka med pH 7 in 9, smo lahko izvedli tudi hidrolizo brez uravnavanja pH. Grenke peptide, ki so nastali kot rezultat hidrolize z Alkalazo, smo razgradili z mešanico glivnih eksopeptidaz in endopeptidaz pod komercialnim imenom Flavourzyme. S kombinacijo encimov smo uspeli doseči stopnjo hidrolize nad 30, velik izkoristek in dobro topnost peptidov v širokem pH območju. Le zmerno grenak hidrolizat s sprejemljivimi senzoričnimi lastnostmi je vseboval visok delež dipeptidov in tripeptidov.