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A Comparison of Silk Fibroin Hydrolysates by Hydrochlonic Acis and Proteolytic Enzymes

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Enzymatic hydrolysis of different forms of silk fibroin (soluble, gel and insoluble forms) by industrial and commercial enzyme preparations to obtain aqueous and powdered silk fibroin in relatively mild conditions was investigated. A mono-enzymatic hydrolysate systems were tested for hydrolysis of water-soluble form of fibroin as most productive form of protein substrate. Insoluble forms of substrate usually were hydrolyzed less effective. In some cases from soluble fibroin substrate gel was formed during hydrolysis process. This hindered intermixing and decreased rates of hydrolysis. Insoluble sediments were formed in enzymatic hydrolysates in other cases. These sediments and also sediment after chemical hydrolysis were purified and tested on amino acids content for comparison. Sediments formation in these conditions are considered as pure tyrosine isolation method. Obtained hydrolysates were characterized by gel-chromatography analysis and other standard biochemical methods. Possibility of application of enzymatic hydrolysis for preparation of silk fibroin hydrolysates is discussed.

Key words : Silk fibroin, Proteinases, Hydrolysates, Peptides, Amino acids, Food and cosmetics additives

Introduction

In recent years the more and more number of investigations are devoted to non-clothing directions of application of silk containing materials for obtaining of wide range of new materials with outstanding properties (Demura *et al.*, 1992; Gotoh *et al.*, 1998; Kaplan, 1998; Motta and Freddi, 1998; Nakayama, 1989). Production of water soluble and powdered forms of silk protein by chemical hydrolysis is a one of the trends of such application (Chen *et al.*, 1991; Lu *et al.*, 1996). These forms of silk fibroin can find application in food industry, medical food, in cosmetics (Nakayama, 1989). Although at the present chemical method is relatively cheap method of obtaining of silk hydrolysate it has numbers of shortages limiting its wide application. In improved method of obtaining of fibroin acid hydrolysates (Madyarov *et al.*, 1999) which also uses high temperatures and pressure even at short time treatment there are losses of part of amino acids. Using of industrial protease with different specificity of action in mild conditions, the enzymatic production of hydrolysates with total saving of amino acids composition of hydrolysing proteins in hydrolysates are the most perspective way with regard to this. Enzymatic hydrolysis today is considered yet as expensive one and is used for manufacture of the high quality food and medical protein's hydrolysates. But with increasing of production of industrial proteases, hydrolysis of pure protein substrates for production of their hydrolysates from year to year will becomes more and more economically acquitted.

There are a few works devoted to enzymatic hydrolysis of silk proteins (Chen *et al.*, 1996; Chen *et al.*, 1996; Kim *et al.*, 1997; Kovalenko *et al.*, 1987; Lee *et al.*, 1976a, 1976b; Lee *et al.*, 1975; Madyarov, 1995). In early publications (Lee *et al.*, 1976a, 1976b; Lee *et al.*, 1975) hydrolysis by pronase P the fibroins of cocoons obtained

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in different conditions of silkworms feeding by artificial diets as well as of cocoons of different silkworms breed varieties was used as testing probe for estimation of silk fibers quality. Later a method for hydrolysis of industrial protein wastes (including silk wastes) by alkali protease *B. subtuilis* entrapped into carboned aluminum Θ-oxide was developed (Kovalenko *et al.*, 1987). High efficiency of application of enzymatic hydrolysis for utilization of industrial waste waters and protein wastes on the example of immobilized protease was described in this patent. In recent work (Madyarov, 1995) problems and perspectives application of proteins and protein hydrolysates as additives to artificial diets and cultural mediums as well as questions of wastes utilization in sericulture and silk processing are discussed.

Transition of silk fibroin as a result of its partial proteolysis by α-chymotrypsin from water solution to crystalline fraction was revealed and studied in the work (Kim *et al.*, 1997). Interesting results have been obtained in the work (Chen *et al.*, 1996). Among free amino acids preferentially tyrosine was revealed in fibroin hydrolysate obtained by action of proteolytic enzyme actinase. Authors of this work supposed that actinase acts on peptides bonds of both sides of tyrosine localization in amino acid sequence of fibroin molecule that providing more higher degree of fibroin hydrolysis. Optimal conditions for preparation of preferentially tyrosine composed hydrolysate of fibroin by aktinase were found in the next work (Chen *et al.*, 1996).

Effect of different proteases on hydrolysis of fibroin substrates performed by different methods have been studied in this work. Screening of hydrolysing effect of industrial and commercial enzymes preparations on fibroin substrate was carried out. The most effectively acting enzymes were selected for more detail investigation in mono- and bi-enzymatic systems. Investigation of some properties of enzyme hydrolysates in comparison with chemical hydrolysates was carried out.

Materials and Methods

Materials

Fibroin was prepared from cocoon shells by boiling in solution of 0.03% of sodium carbonate and 0.05% of marceillous soap (weight/weight of cocoon shells; water : cocoon shells ratio was 1 : 50) during 30 min. This degumming process was repeated and fibered fibroin was twice washed by boiling with distilled water. Fibroin material was dried up to constant weight at room temperature and than in thermostable (60-70°C) dryer. The following commercial chemicals were used in the work: Hydrochloric acid, sodium hydroxide and glycine (Juncei Chem. Co., Japan); calcium chloride and calcium nitrate (Yakuri Pure Chem. Co., Osaka, Japan) and other extra pure and analytical grade mineral chemicals and organic solvents. Distilled and deionized water were used. The following biochemicals were used: Albumin (bovine, frac.V), hemoglobin, casein, pepsin A (porcine), trypsin, type II-S, α–chymotrypsin, type II and IS, (bovine pancrease), bacterial protease, type XIV from *streptomyces griseus*, cytochrom c (bovine heart), vitamin B_{12}, N-benzoyl-L-arginine ethyl ester (BAEE) and N-benzoyl-L-tyrosine ethyl ester (BTEE) from Sigma Chemicals (St. Louis, MO), L-Tyrosine from Fluka AG (Switzerland). Industrial enzymes preparations alcalase 2.5 L, neutrase 0.5 L, flavouzyme, protamex, ceremix 2XL and sumizyme LP were a generous gift from Novo Nordisk (Korean branch). Chromatography media for gel-filtration G-10, G-15 Sephadex and prepacked column PD-10 (Pharmacia Biotech., Sweden). Spectra/pore molecular porous membrane tubing (Spectrum, Medical Industries, USA) were used for dialysis. Microfiltration systems (MFS-25 Disposable syringe filter unit) with pore size-0.45 μm were used for preparation of samples for Gel-chromatography.

Preparation of fibroin solutions

The regenerated silk fibroin solution was prepared by dissolving of degummed fibers with a 5 M $CaCl_2$ solution at 98°C for 1 hour at stirring. The salt was completely removed by dialysis against distilled water for 3 days at room temperature. Another method of fibroin dissolution by the mixture of $CaCl_2$, H_20 and ethanol in ratio 1 : 8 : 2 was also used. In this case the process was carried out for 30 min at temperature 70°C. Dialysis against distilled water during 3 days was used to remove salt and alcohol. The final concentration of silk fibroin was adjusted by slowly drying the solution in air under stream from fan or using Rotary evaporator (Buchi Rotavapor-R-114). Obtained fibroin solutions were used as substrate for enzymatic hydrolysis. The solutions obtained in such a manner and also the gels formed from these solutions when standing during 3 weeks in refrigerator were dried by Tray Freeze Dryer (Il Sin Engineering Co., Korea). This way was used to obtain molecular silk fibroin powder (SFP).

Hydrolysates preparation

Chemical hydrolysis Small portions of fibered fibroin was immersed part by part to solution of hydrolyzing agent-deluted acid in final ratio 25 : 1. Container with components of hydrolysis was set up to autoclave, heated to requisite temperature and enduranced necessary period.

The hydrolysate obtained in this process was cooled at room temperature, insoluble part was filtrated, solution was neutralized by 2 N solution of alkaline at intensive stirring and cooling up to neutral reaction (pH 7.0, Backman digital model 134 pH meter).

Five ml of each hydrolysate were used for analysis and remaining parts of hydrolysates were dried in Freeze Dryer to obtain powdered silk fibroin hydrolysates (SFH). To determine degree of fibroin solubilizatrion insoluble remainder after filtration (or centrifugation) was dried in C-DV Vacuum Drying Oven (Chan Shin Sci. Co., Korea) at temperature 50-60°C to constant weight.

Enzymatic hydrolysis Enzyme hydrolysates were prepared by digestion of certain amount of fibroin under different physical conditions (in soluble, gel or solid forms) in water solution of enzyme buffered by appropriate buffer at 30-55°C in water-bath shaker or in thermostatic water bath YB-2 (Yanagimoto MFG Co., LTD, Japan). Reaction process was stopped in dependence of used enzyme and physical state of substrate by adding of definite amount of 0.5 M three-chloracetic acid necessary for protein precipitation or by boiling of reaction media for 15 min. Precipitated part was separated from soluble part by filtration or centrifugation at 15.000 rpm for 10 min on 18-PR-3 Hitachi refrigerated centrifuge. Hydrolysates before examination by gel-chromatography were filtered through 0.45 μm MFS filter unit. Hydrolysis period was varied to attain maximum degree of hydrolysis and not to allow microorganisms propagation. Five ml aliquots of hydrolysates were taken for analysis. To obtain powdered SFH the remaining quantities of hydrolysates were lyophylisated in the freeze dryer. The insoluble fibroin remainder was separated and dried in vacuum oven as in the case of acidic hydrolysis.

Film casting and drying

The definite volume of centrifuged 2-5% fibroin solution was deposited to a polystyrene Petry dish and dried at 25 -30°C and 60-70% humidity for 3 days. Dried film was separeted from Petry dish and crushed to powder. The samples obtained by this method were used in solubility test.

Silk fibroin and its hydrolysates characterization

Spectral characterization Determination of UV-spectra and optical density of fibroin solution and hydrolysates at fixed wavelengths was performed using Perkin Elmer Lamda 10 UV-VIS spectrophotometer or Unicam UV-2-100 spectrophotometer.

Gel filtration of soluble silk fibroin and its hydrolysates Glass column were filled by Sephadex G-10 (0.9× 38) cm. The column was equalibrated by 10 mM phosphate buffered saline (pH 7.4) with 2.7 mM KCl and 138 mM NaCl at flow rate 0.1 ml/min. Calibration of equilibrated columns was performed using bovine serum albumin (MW 67,000) cytochrom c (bovine heart-12,400), vitamin B_{12} (MW-1,355), tyrosine (MW-181) and glycine (MW-75). Prepacked PD-10 columns were used for desalination of hydrolysates. Fractions from columns were collected by Spectrum fraction collector and optical density at 205, 260, 280 and 340 nm was determined by Unicam UV-2 spectrophotometer.

Determination of digestibility (Only for insoluble forms of fibroin) Sanders *et al.* (1973) method with pig pepsin and pancreatic trypsin was used. In a centrifuge tube, 1 g of fibroin material was suspended in 20 ml of 0.1 N HCl and mixed with 50 mg of pepsin in 1 ml of 0.01 N HCl. The mixture was gently shaken at 37°C for 48 hrs, then centrifuged. After removal of the supernatant, the solids were resuspended in 10 ml of water and 10 ml of 0.1 M sodium phosphate buffer, pH 8.0, and treated with 5 mg of trypsin. The mixture was gently shaken at 23°C for 16 hrs. The solids were separated by centrifuging and washed with water (5 × 30 ml), centrifuging and removing supernatant after each washing. In this procedure, centrifuging meant for a period of 5 minutes at 20,000 × g. The solids were finally filtered through a Watman paper filter, air-dried and weighed. The calculation is as follows: Protein digestibility of fibroin (%)=(weight of fibroin before digestion-weight of fibroin after digestion)/(weight of fibroin before digestion) × 100%.

UV-VIS-absorbance test (for soluble samples)

Two methods of absorbance test were used: sun light exposition and FADE -O- meter methods. Two commercial creams were selected as tests background: Johnsons Baby Lotion (Johnson & Johnson) and Vital Care Moisture Cream (Cheil Jedang, Korea).

Fine homogeneous layer of pure cream (without additives) was supplied on smooth silk fabric-control test. Excesses of cream were removed. In test with hydrolysates additives the cream was mixed with dry powdered SFH, which were easy soluble in the cream composition. This mixture was supplied on silk fabric in the same manner. The obtained samples were exposed under natural sun irradiation (for one week) and 20 hrs under rays of FADE -O- meter, Model CH-20 N (Shimadzu Corp., Japan). Effect of action in both cases were measured according to yellow index by SQ-300 H Colour difference meter (Nippon Den Shoku Ind. Co. LTD., Japan).

Solubility test

Two hundreds mg of samples (SFP or SFH) were dissolved in 5 ml deionized water using Tube Shaker (Chang

Shin Co., Korea). Each sample was shaken three times for 2 min . Than the samples were centrifuged at 6.000 rpm for 10 min. Supernatant was separated for analysis and insoluble part was dried to constant weight in thermostatic vacuum dryer at 50-60°C and weighed.

Protein and amino-acid determination

Two methods of protein determination were used: Lowry method (1951) and biuret method (1986). The technique described in modified Anson method (1975) was used for tyrosine determination. Corresponding calibration curves for determination of fibroin and tyrosine concentration were used.

Results and Discussion

Enzymic method for degumming of silk threads, fabrics and silk wastes is used for silk products manufacturing. Object of the degumming is digestion of sericine remainder associated with silk fibers. Industrial proteases alcalase 2,5 L (at pH 8-9) and esperase 8.0 L (pH 9-10) are recommended to be used for this purpose. The following industrial food grade proteases (Novo Nordisk)-alcalase 2.5 L, neutrase 0.5 L, ceremix, protamex, (*Aspergillius orizae*)-flavourzyme and sumizyme LP (Type XIV), trypsin and α-chimotrypsin from Sigma were used for fibroin enzymatic digestions investigation. All these enzymes are differed by their origin, specifity and specific activities.

Regenerated water-soluble fibroin and solution of caseinate (for comparison) were used as substrate. The reaction was carried out at 50°C, phosphate buffer pH 7.4, 10 min. Standard reaction mixture contained 40 mg of substrate, 50 μkl of enzyme solution and 2.0 ml of buffer. Results of comparative analysis of casein and fibroin hydrolysis are represented in Table 1. As it follows from the results casein for all enzymes (except α-chymotripsin) is better substrate than fibroin, which peculiarity is weak hydrolysis by other digestive enzyme-trypsin.

Comparative gel-chromatographic analysis of fibroin

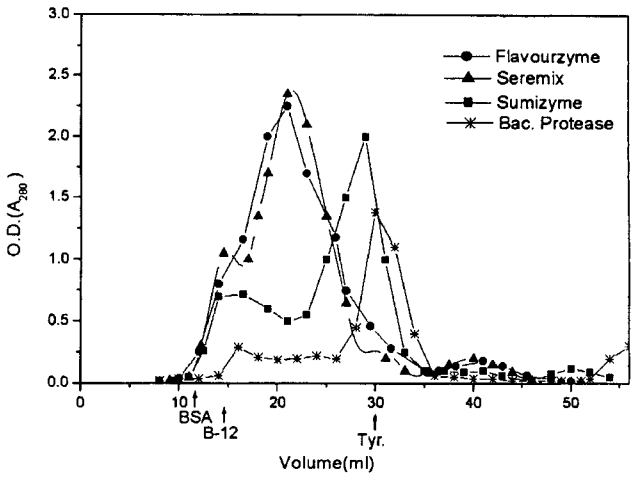

Fig. 1. Gel filteration chromatography of enzymatic silk fibroin hydrolysates on Sephadex G-10.

hydrolysates prepared with help of selected enzyme preparates in mono-enzymatic system (Fig. 1) was further used. It is follows from chromatograms that flavourzyme and seremix produce mainly oligopeptides while sumizyme and bacterial protease could produce free amino acids and dipeptides in big amount. Hydrolysate, produced with help of bacterial protease was chromatographed after separation of fallen sediment. Amino acid analysis of precipitated hydrolysates prepared by bacterial protease showed only one amino acid-tyrosine. The same result was get at analysis of sediment precipitated from 2 N HCl fibroin hydrolysates by traditional method obtained after corresponding purification. These procedures could be used for isolation a pure crystalline tyrosine.

Results of comparative analysis of enzymatic and acid hydrolysates in chromatographic experiments are summarized on Fig. 2. As it is seen from chromatograms hydrolysate prepared by traditional technology (2 N HCl, 48 hrs at 110°C) is the poorest hydrolysate. There are significantly more amino acids and peptides in acid hydrolysate prepared by improved method with use of 0.5 N HCl, 4 hrs, 130°C (Madyarov *et al.*, 1999). Enzymatic hydroly-

Table 1. Proteinase activities (in relative units) of industrial and commercial enzyme preparations with casein (for comparison) and fibroin as substrates

Enzymes / Substrates	Alcalase 2.5L	Neutrase 0.5L	Ceremix 2XL	Prota-mex	Flavo-urzyme	Sumizyme LP	Bacterial proteinase	Tryp-sin	α-hymo-trypsin
Casein	1.290	0.527	0.400	0.987	0.922	0.965	1.420	0.545	0.040
Fibroin	0.254	0.363	0.358	0.305	0.478	0.356	0.558	0.064	0.361
AU* Fibroin	3.23	4.61	4.56	19.40	30.40	22.60	71.00	8.20	191.30

* AU-Anson Units for fibroin hydrolysis (in μmoles tyrosin/min/g enzyme preparation)

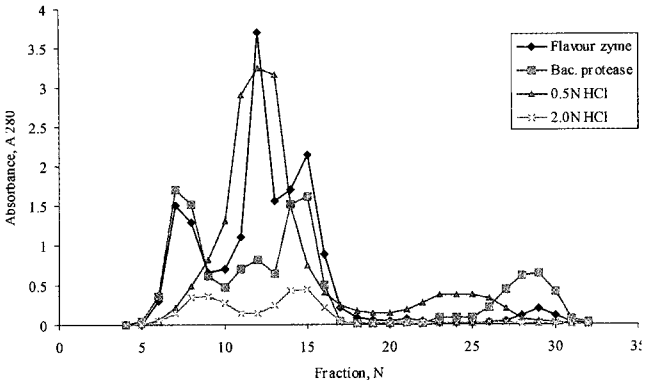

Fig. 2. Results of gel chromatographic analysis of fibroin hydrolysates obtained by enzymatic digestion (Sephadex G-10). Errors indicate BSA, vitamin B-12, tyrosine & glycine standardts elution volumes, consequently.

sates contain both free amino acids and peptides with different molecular weight giving powdered silk materials by freeze-drying.

Solubility data of hydrolysates and fibroin substrates, prepared with use of $CaCl_2$ are represented in Table 2. It is seen that both enzymatic and acid hydrolysates are completely soluble while fibroin substrates to be used for hydrolysis have different solubility. Some enzymatic systems form gel structures from water-soluble fibroin in the process of hydrolysis. Hydrolysis of gel form of fibroin and especially as fibered or other solid forms of fibroin as additional experiments showed (Table 3) by proteinases is hampered.

Table 3 also represents results of different fibroin containing materials analysis for digestibility test by Saunders *et al.* (1973) method with use of pepsin and trypsin-digestive animal and human enzymes. It is follows from these results that some fibroin forms, for example, its residues after acid hydrolysis by 0.5 N HCl (2 hrs) and 1.0 N HCl (4 hrs) by method (Madyarov *et al.*, 1999) are hydrolysed significantly better than degummed pure fibroin. These data extend opportunities of utilization of similar residues (Madyarov, 1985a, 1985b; 1989) simultaneously solving questions of wastelessness of acid hydrolysates production (Madyarov *et al.*, 1999).

Results of investigations on light absorbance tests of standard cosmetics with 10% additives of prepared hydrolysates are shown in Table 4. The lowest photooxydation results (yellow index) were got when used enzy-

Table 2. Solubility and optical characteristics of some powdered samples of hydrolysates and fibroin substrates for enzymatic hydrolysis

Properties \ Sample	Enzyme hydroly-sate (Pro-tamex)	0.5 N HCl, 4 hrs, 130°C	The same, Decolo-rised	$CaCl_2/H_2O$ (regenerated sol.)	$CaCl_2$/ Citric acid (reg. sol.)	$CaCl_2$ Solution refrigerated		
						Gel Sediment	After centrifugation Supernatant***	
Solubility*, %	100%	100 %	100 %	100 %	100 %	52 %	94 %	11 %
Optical density **:								
D_{280}	0.796	0.620	0.615	0.919	0.457	0.229	0.831	0.884
D_{260}	0.445	0.371	0.367	0.564	0.264	0.144	0.482	0.565

* 200 mg of sample and 5 ml of deionized water were taken for dissolution

** Optical density of 40 times dissolved solution

*** Optical density was determined without dissolution

Table 3. Digestibility *in vitro* of fibroin residues, obtained after acid hydrolysis of fibroin in different conditions

Properties \ Samples	Pure fibroin	0.1 N HCl, 2 hrs	0.1 N HCl, 4 hrs	0.2 N HCl, 2 hrs	0.5 N HCl, 2 hrs	0.5 N HCl, 4 hrs	1 N HCl, 4 hrs
Pepsin hydrolysates							
D_{280}	25.26	17.54	19.40	28.25	101.76	27.70	108.60
D_{340}	2.91	1.42	1.74	3.77	6.91	3.38	13.10
[tyrosine], mkg	139.00	89.30	102.50	122.40	427.20	128.00	433.00
Tripsin hydrolysates							
D_{280}	11.90	5.75	5.68	10.70	22.90	9.76	20.06
D_{340}	4.80	0.97	0.97	1.19	2.00	1.70	2.75
Digestibility, %	4.0	4.6	5.0	5.6	22.0	5.2	21.2

Table 4. Results of hydrolysates analysis on light absorbtion tests

Tested base: Jonsons Baby Lotion (JBL)

Type of insolation \ Samples	JBL (control)	+ 10% HCl fibroin Hydrolysate	+10%HCl fibroin decolourized hydrolysate	+ 10% Enzymatic hydrolysate
Sunlight	0.27	9.09	6.03	3.36
Fade-o metre	1.87	11.20	13.19	7.99

Tested base: Vital Care Moisture Cream (VCMC)

Type of insolation \ Samples	VCMC (control)	+ 10% HCl fibroin Hydrolysate	+10%HCl fibroin decolourized hydrolysate	+ 10% enzymatic hydrolysate
Sunlight	1.05	16.16	11.40	8.38
Fade-o metre	3.15	19.51	20.05	16.71

matic hydrolysates in cosmetics both at sun light and at FD-o-meter irradiation. Acidic hydrolysates obtained by improved method [8] even after decolorization in content of used cosmetics were had by both testing methods more higher value of yellow index, then enzymatic hydrolysates.

Thus effect of some industrial and commercial enzyme preparations on silk fibroin hydrolysis for preparation of aqueous and powdered biomaterials was investigated. Utilization of insoluble residues after silk fibroin acid hydrolysis was studied. Some physico-chemical and biochemical properties of acid and enzymatic hydrolysates were investigated.

Major results are as following: Enzymatic hydrolysis was performed in relatively mild conditions: 30-55°C, under normal atmospheric pressure, without chemicals like acids and alkali. Varying temperature and pH, as well as by selection of enzymes, enzymes concentration and time of hydrolysis the same degree of hydrolysis as at acid hydrolysis could be achieved. From isolated of precipitates formed in the process both of acid and enzymatic fibroin hydrolysates manufacturing it is possible to obtain free amino acids.

There is no need to use stage of dialysis and other separating method in processing of final product because of salt and colour content in enzymatic hydrolysates significantly lower than in acid hydrolyses. Colour of final product is mainly determined by colour of used enzyme which is applied in small concentrations.

Most "Novo Nordisc" industrial enzyme preparations are food grade products and that is why enzyme hydrolysates could be used in food industry after sterilization without additional purification. Yield of final product 2-3 fold higher than those obtained by traditional acid hydrolysis method. Content of free amino acids and small peptides in preparations obtained by developed acid and enzymatic methods 7-10 fold higher than in hydrolysates obtained by traditional method.

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