

## New Protein Hydrolysates from Collagen Wastes Used as Peptone for Bacterial Growth

E. Vasileva-Tonkova,<sup>1</sup> M. Nustorova,<sup>2</sup> A. Gushterova<sup>1</sup>

<sup>1</sup>Institute of Microbiology, Bulgarian Academy of Sciences, Academie G. Bonchev Strasse, Building 26, 1113 Sofia, Bulgaria

<sup>2</sup>University of Forestry, 10 Kliment Ochridski Boulevard, 1156 Sofia, Bulgaria

Received: 15 June 2006 / Accepted: 20 July 2006

**Abstract.** A simple and low-cost procedure was developed for the effective processing of native calf skin and blood wastes to produce protein hydrolysates. The method includes extraction of high-molecular-weight protein from the raw material, followed by enzymatic hydrolysis of the extracted residue. The enzymatic hydrolysis was performed by inexpensive commercial subtilisin DY, produced by *Bacillus subtilis* strain DY possessing high specific activity. The contents of protein, nitrogen, ash, and amino acids of the obtained hydrolysates were determined and compared with those of the commonly used commercial casein hydrolysate (Fluka Biochemica, Switzerland). The newly obtained calf skin hydrolysate, called Eladin, was found to be suitable as a low-cost alternative peptone in growth media of different microorganisms, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella dublin*, and *Staphylococcus aureus*. The method allows utilization of waste materials by converting them into valuable protein products that could find widespread application in microbiologic practice.

Peptones are defined as protein hydrolysates that are readily soluble in water and are not precipitable by heat, by alkalis, or by saturation with ammonium sulphate. They are one of the most important constituents of bacterial culture media. Each peptone has its own biologic characteristics, and not one could meet to equal degree the requirements of all microorganisms or cells during cultivation. Different materials from animal and plant sources are used for the production of peptones, most of them valuable and relatively expensive [1–4]. Growth substrate costs often make up the major part of the production costs of microbial cells and bioproducts from the fermentation industry [5]. Therefore, it is promising to search for new, reliable sources of quality peptones that are less expensive than those currently available.

Animal materials, such as skin, wool, bristle, horns, feathers, hoofs, etc., are significant waste byproducts of livestock, meat, and other industries in Bulgaria. Because these wastes contain large amounts of the struc-

tural fibrous proteins keratin and collagen, research has been carried out to develop methods to transform them into useful products. Effective degradation of these materials is difficult because of their stable hard protein structure; thus, industrial utilization of these proteins has remained undeveloped. A promising alternative is to hydrolyse the wastes by acidic or enzymatic hydrolysis to obtain protein hydrolysates that contain valuable peptides and amino acids, which could have many applications [6–11]. The aim of this study was to develop a method to prepare protein hydrolysates from calf skin and blood wastes. The suitability of the obtained hydrolysates as peptones in bacterial growth media was investigated.

### Materials and Methods

**Starting materials.** Calf skin and blood wastes were obtained from a slaughterhouse in the town of Kostinbrod in the region of Sofia, Bulgaria. Calf skin waste was washed with tap water, then air dried and cut into smaller pieces, which were used as a starting material. Crude alkaline proteinase containing subtilisin DY, produced by *Bacillus subtilis* strain DY, was purchased from the Plant for Enzyme Preparations (Botevgrad, Bulgaria). This preparation, named “APB-

Table 1. Some characteristics of calf skin hydrolysate (Eladin), blood hydrolysate, and casein hydrolysate

Properties	Eladin	Blood hydrolysate	Casein hydrolysate
Ash content <sup>a</sup> (%)	4.95	5.10	6.00
Water content <sup>a</sup> (%)	1.90	2.00	4.00
Total protein <sup>a</sup> (%)	53.40	51.40	35.11
Amino nitrogen/Total nitrogen <sup>a</sup>	65.30	68.10	56.40
pH of 2% solution	7.0	7.0	6.7

<sup>a</sup> Values are the averages of two determinations.

72," was standardized at 500 Sigma proteolytic units/g protein (PU/g). Subtilisin DY was purified using a large-scale method [12]. The enzyme product had high specific activity (90 to 100 Committee of Thrombolytic Agents [CTA] U/mg), was odourless, and was free of heavy metals. It was used in the hydrolytic degradation of the waste material.

**Proteolytic activity assay.** The alkaline proteolytic activity was determined by the method of Johnson et al. [13], with modification [12] using casein as a substrate. The enzyme activity was expressed in CTA units. One CTA unit was defined as the amount of enzyme releasing from the substrate 0.1 microequivalents ( $\mu\text{eq}$ ) of tyrosine (Tyr)/min at 37°C. One CTA unit is equal to 0.096 PU per Sigma Chemical Corporation.

**Hydrolysis procedure.** As a preliminary step before enzyme hydrolysis, twofold extraction of collagen from calf skin waste was carried out as follows. The starting material was mixed with tap water (1:2) to obtain a mixture containing 7% to 8% dry matter. The mixture was boiled for 10 minutes, then cooled to 55°C to form a gelatinous protein extract.

The enzyme quantity added to the mixture was 4% (w/w) with respect to dry matter in the starting material. The enzyme hydrolysis was carried out at 50°C to 55°C, and pH was maintained between 8.0 and 8.3 by adding calcium hydroxide. The process lasted approximately 5 to 6 hours, and the end of hydrolysis was determined by a decrease in calcium hydroxide consumption. The obtained turbid suspension was clarified by the method of Tzokov et al. [14], then concentrated in a rotary vacuum evaporator up to 20% to 25% dry matter, autoclaved, filtered, and dried at 50°C to 70°C. The final powder was termed "Eladin." In preparing blood hydrolysate, calf blood waste was used as starting material and subjected to the procedure already described.

**Analytic methods.** The amino nitrogen and the degree of hydrolysis were determined using the trinitrobenzenesulfonic acid method of Adler-Nissen [15]. The total protein content was determined by the method of Bradford [16]. Total nitrogen content was assessed by the Kjeldahl method [17]. Dry-matter concentration and total ash content of the samples were determined using standard analytic methods [18]. The amino-acid analysis was carried out after hydrolysis of the samples in 6N HCl at 105°C in a Biotronic Amino Acid Analyzer LC-6000 (Biotronic, Germany). The amino-acid composition was derived from three separate acid hydrolyses of different time durations 16, 24, and 48 hours.

**Microbiologic test.** The obtained hydrolysate Eladin was checked as sole source of carbon and nitrogen for growth of the strains *Escherichia coli* Ca-58, *Pseudomonas aeruginosa* 2109, *Staphylococcus aureus* 65, and *Salmonella dublin* 1953. Bacterial cultures were obtained from the National Bank of Industrial

Table 2. Amino-acid composition of calf skin hydrolysate (Eladin), blood hydrolysate, and casein hydrolysate used as standard

Amino Acid	Eladin <sup>a</sup>	Blood hydrolysate <sup>a</sup>	Casein hydrolysate <sup>a</sup>
Asp	2.13	11.49	6.35
Thr	2.22	4.06	3.40
Ser	3.34	4.94	4.20
Glu	6.73	8.82	17.40
Pro	9.40	3.69	8.35
Gly	14.29	5.70	1.85
Ala	7.06	8.55	2.55
Cys	0	0	0
Val	3.20	8.29	5.10
Met	1.08	0.92	2.35
Leu	4.72	12.13	7.15
Ile	1.99	1.02	4.25
Tyr	1.65	2.70	1.95
Phe	2.60	5.54	4.05
Lys	3.91	11.20	6.45
His	4.21	7.65	2.70
Arg	6.37	3.78	3.05
Total	74.90	100.48	81.15

<sup>a</sup> Values for amino acids are in number of residues 100 g hydrolysate.

Microorganisms and Cell Cultures (Sofia, Bulgaria). They were maintained between transfers on nutrient agar slants at 5°C.

The test was carried out using the recommendations of the United States Pharmacopeia XXI [19]. The cultivation media (pH 7.2 to 7.4) used were (all dilutions were made in distilled water) (1) hydrolysate (0.1% v/v); (2) hydrolysate (0.1% v/v), NaCl (0.5% w/v), and dextrose (0.5% w/v); (3) hydrolysate (1% v/v); and (4) hydrolysate (1% v/v), NaCl (0.5% w/v), and dextrose (0.5% w/v). Casein hydrolysate (Fluka), which represents milk casein hydrolysed by pancreatin, was used as reference peptone. Each sample (20 ml) was inoculated with 0.4 ml suspension (containing  $1 \times 10^9$  cells  $\text{ml}^{-1}$ ) of the corresponding strain and incubated statically at 37°C in a closed system. Growth was monitored by measuring optical density at 660 nm ( $A_{660 \text{ nm}}$ ) [20]. To compare the changes, all values of optical density were presented as a ratio between  $A_{660 \text{ nm}}$  in determined hours and  $A_{660 \text{ nm}}$  in zero hour.

**Statistical analysis.** Given the small numbers of samples ( $n = 3$ ) and the skewed distribution of results, nonparametric two tailed Mann-Whitney U test was used to determine significance of differences of parameters between different types of peptones ( $P < 0.05$  was considered significant). Statistical analyses were performed using Stawin 5.1 software.

## Results and Discussion

**Preparation of hydrolysates.** In a preliminary step, the starting mixture was heated, and high-molecular-weight protein was extracted. The triple helix of collagen unwound, and the chains separated. When this denatured mass of tangled chains cooled down, it soaked up all of the surrounding water, forming gelatin. Water-insoluble collagen is resistant to most proteases and requires special collagenases for its enzymatic hydrolysis.

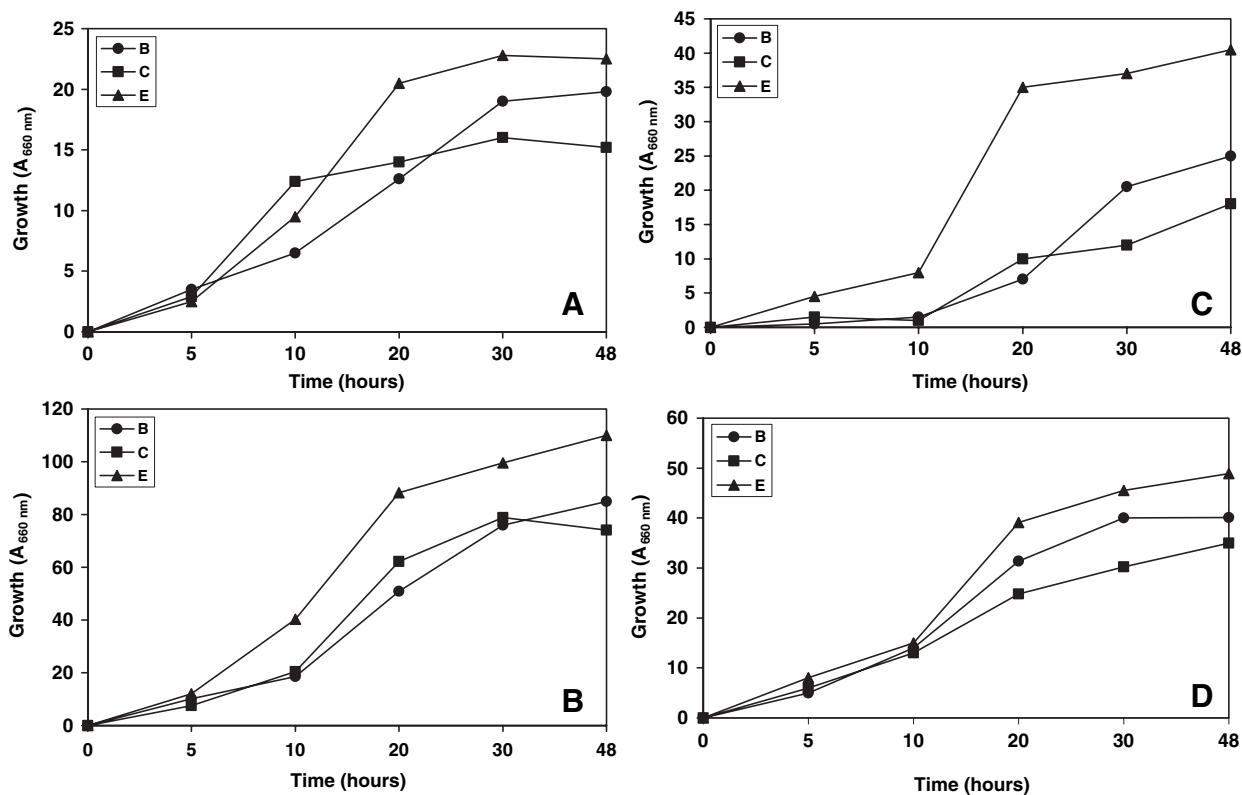


Fig. 1. Growth curves of test microorganisms (A) *E. coli* Ca-58, (B) *S. aureus* 65, (C) *P. aeruginosa* 2109, and (D) *S. dublin* 1953 in 1% solutions of (closed triangles) Eladin, (closed circles) blood hydrolysate, and (closed squares) Fluka casein hydrolysate. Ratios between A<sub>660nm</sub> in determined hours and A<sub>660nm</sub> in zero hour are presented (mean values from three determinations).

Gelatin, a product of denaturation or disintegration of collagen, however, is susceptible to most proteases.

Commercial subtilisin DY possesses wide specificity and hydrolyses peptide bonds at the carboxylic groups of at least 13 amino-acid residues [21]. This explains the high degree of hydrolysis of both calf skin and blood extracted proteins: 65% and 68%, respectively. Enzymatic hydrolysis is frequently used to improve functional and nutritional properties of proteins [22–24]. Acid hydrolysis allows high yields; however, this process results in high ash content in the final products because the neutralization step cannot be avoided [25].

**Characterization of hydrolysates.** Some properties of the dry products Eladin and blood hydrolysate were determined and compared with those of the commercial casein hydrolysate, used as standard (Table 1). The obtained products have better characteristics than the standard especially higher amino nitrogen/total nitrogen ratio indicating a quality hydrolysed protein product. Casein hydrolysate was commonly used as growth substrate because of its high nutritional value and is readily available [3].

Table 2 lists the amino-acid composition of the obtained hydrolysates and casein hydrolysate. As can be seen, the new products are rich in amino acids, including nutritionally essential ones, such as arginine (6.37 in Eladin), lysine (11.2 in blood hydrolysate) and phenylalanine (5.54 in blood hydrolysate). The obtained hydrolysates have lower levels of glutamine, methionine, and isoleucine-but much higher levels of glycine, alanine, and histidine-than those of the reference peptone. Furthermore, compared with casein hydrolysate, Eladin has higher levels of proline, arginine, and blood hydrolysate as well as higher levels of asparagine, valine, leucine, phenylalanine, and lysine. The observed differences are not of importance for the inherent quality of the new products. The results of amino-acid composition were similar or better than those obtained for other fibrous proteins [26–29].

**Microbiologic test of hydrolysates.** In medical practice and the field of food microbiology, nonselective pre-enrichment of bacterial culture made in peptone water is widely adopted. Hence, we checked the new preparations as inexpensive peptones in bacterial growth media. All tested strains grew well in

1% water solutions of the obtained hydrolysates, a concentration commonly used in practice [30, 31]. Growth of the strains were followed up to 48 hours, and typical growth curves were observed. Growth curves of test microorganisms without adding NaCl and dextrose are presented in Fig. 1. The best growth in all of the media was shown by *S. aureus* 65 (Fig. 1B). For all tested strains, growth values at 48 hours reached with Eladin and blood hydrolysate were higher than those obtained with casein hydrolysate (Figs. 1A through 1D).

## Conclusion

A simple and low-cost method for effective hydrolysis of protein wastes has been developed. The procedure consists of extraction of high-molecular-weight protein from wastes, followed by enzymatic hydrolysis using inexpensive commercial bacterial proteinase. The proposed process could serve as a preliminary step in development of large-scale technology for the effective utilization of keratin and collagen wastes by converting them into valuable protein products. This could have positive ecological and financial effects. It was shown that the obtained protein hydrolysates could find widespread application in microbiologic practice as inexpensive alternative peptones in bacterial growth media. The new preparations could also find application as an enumeration medium for aerobic bacteria isolated from environmental samples.

## Literature Cited

- Ignatova Z, Christov P, Spassov G, Gousterova A, Borisov B, Nedkov P (1998) Characterization and some microbial applications of an enzyme hydrolysate obtained from pig pancreases. *Comptes Rendus de l'Academie Bulgare des Sciences* 51:75–78
- Parrado J, Millan F, Hernandez-Pinzon I, Bautista J, Machado A (1993) Sunflower peptones: Use as nitrogen source for the formulation of fermentation media. *Process Biochem* 28:109–113
- Reissbrodt R, Beer W, Muller R, Claus H (1995) Characterization of casein peptones by HPLC profiles and microbiological growth parameters. *Acta Biotechnol* 15:223–232
- Martone CB, Borla OP, Sánchez JJ (2005) Fishery by-product as a nutrient source for bacteria and archaea growth media. *Biores Technol* 96:383–387
- De la Broise D, Dauer G, Gildberg A, Guerard F (1998) Evidence of positive effect of peptone hydrolysis rate on *Escherichia coli* culture kinetics. *J Marine Biotechnol* 6:111–115
- Dalev P (1990) An enzyme-alkaline hydrolysis of feather keratin for obtaining a protein concentrate for fodder. *Biotechnol Lett* 12:71–72
- Atalo K, Gashe BA (1993) Protease production by a thermophilic *Bacillus species* which degrades various kinds of fibrous proteins. *Biotechnol Lett* 15:1151–1156
- Hood CM, Healy MG (1994) Bioconversion of waste keratins: Wool and feathers. *Resources Conservation and Recycling* 11:179–188
- Kurbanoglu EB, Kurbanoglu NI (2002) A new process for the utilization as peptone of ram horn waste. *J Biosci Bioeng* 94:202–206
- Nustorova M, Braikova D, Gousterova A, Vasileva-Tonkova E, Nedkov P (2006) Chemical, microbiological and plant analysis of soil fertilized with alkaline hydrolysate of sheep's wool waste. *World J Microbiol Biotechnol* DOI 10.1007/s11274-005-9045-9
- Nilsang S, Lertsiri S, Suphantharika M, Assavanig A (2005) Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. *J Food Eng* 70:571–578
- Nedkov P (1986) A preparative method for producing partially purified alkaline protease. *Communications of Department of Chemistry, Bulgarian Academy of Sciences* 19:246–253
- Johnson AJ, Kline DL, Alkjaersig N (1969) Assay methods and standard preparations for plasmin, plasminogen and urokinase in purified systems. *Thromb Diath Haemorrh* 21:259–272
- Tzokov S, Nedkov P, Dalev P (1996) Clarification and raising the gelling ability of gelatin solutions. *Biotechnol Biotechnol Equip* 10:59–64
- Adler-Nissen J (1979) Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J Agric Food Chem* 27:1256–1262
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Bradstreet RB (1965) *The Kjeldahl method for organic nitrogen*. New York, NY: Academic
- AOAC International (formerly the Association of Official Analytical Chemists) (1995) *Official methods of analysis*. Arlington VA: AOAC
- The United States Pharmacopeia Convention (1982) *Protein hydrolysate injection*. USP, XXI, pp 911–912
- Gerhardt P (1981) *Manual of methods for general bacteriology*. Washington, DC: American Society for Microbiology, pp 442–490
- Nedkov P, Lilova A (1985) A method for determining small differences in the specificity of proteolytic enzymes. *Communications of Department of Chemistry, Bulgarian Academy of Sciences* 18:435–442
- Bajza Z, Vrcek V (2001) Thermal and enzymatic recovering of proteins from untanned leather waste. *Waste Manag* 21:79–84
- Guerard F, Guimas L, Binet A (2002) Production of tuna waste hydrolysates by a commercial neutral protease preparation. *J Mol Catalysis B Enzyme* 19–20:489–498
- Pedroche J, Yust MM, Lqari H, Girón-Calle J, Vioque J, Alaiz M, et al. (2004) Production and characterization of casein hydrolysates with a high amino acid Fischer's ratio using immobilized proteases. *Int Dairy J* 14:527–533
- Dufosse L, De La Broisse D, Guerard F (1997) Fish protein hydrolysates as nitrogen sources for microbial growth and metabolite production. *Recent Research Developments in Microbiology* 1:365–381
- Baden HP, Kubilus J (1983) Fibrous proteins of bovine hoof. *J Invest Dermatol* 81:220–224
- Kida K, Morimura S, Noda I, Nishida Y, Imai T, Otagiri M (1995) Enzymatic hydrolysis of the horn and hoof of cow and buffalo. *J Ferment Bioeng* 80:478–484
- Morimura S, Nagata H, Uemura Y, Fahmi A, Shigematsu T, Kida K (2002) Development of an effective process for utilization of collagen from livestock and fish waste. *Process Biochem* 37:1403–1412
- Kurbanoglu EB, Algur OF (2004) A new medium from ram horn hydrolysate for enumeration of aerobic bacteria. *Turkish Journal of Veterinary and Animal Sciences* 26:115–123
- MacFaddin JF (1985) *Media for isolation-cultivation-identification-maintenance of medical bacteria*, vol. 1. Baltimore, MD: Williams & Wilkins
- Vanderzant C, Splittstoesser DF (eds) (1992) *Compendium of methods for the microbiological examination of foods*, 3rd ed. Washington, DC: American Public Health Association