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Preparation of hydrolysates from bovine red blood cells and their debittering following plastein reaction

J. Synowiecki,[ab] R. Jagiełka[a] & F. Shahidi[b]

[a]*Department of Food Preservation and Technical Microbiology, Technical University of Gdańsk, Gdańsk, Poland*
[b]*Department of Biochemistry, Memorial University of Newfoundland, St. John's, NF, Canada A1B 3X9*

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Protein hydrolysates from bovine red blood cells were prepared using commercially available Alcalase. The best nitrogen recovery (69.2%) was achieved at a degree of hydrolysis (DH) of 25%. However, the product so obtained had a strong bitter taste (4.3 on a 5-point scale). At DH 16%, the nitrogen recovery was 65.9% and the bitter taste of product decreased (to 3.5). The hydrolysate contained 81.8% crude protein (N×6.25), 2.4% moisture and 15.8% sodium chloride and had excellent solubility characteristics in water in the pH range 2.6–9.5. The Hunter colour parameters of the hydrolysate showed that good release of haem compounds during the process had occurred. Incorporation of hydrolysates (up to 3%) in meat model systems enhanced their cooking yield by about 5%. The protein efficiency ratio value of the hydrolysate was 3.7 as compared with that of beef (2.8), pork (2.5) and cod (2.5) proteins. However, low contents of isoleucine and sulphur-containing amino acids gave an essential amino acid index of 67.3 for the product. The bitterness of hydrolysate was reduced considerably by plastein reaction with diethyl ester of glutamic acid. The resultant product had a crude protein (N×6.25) content of 96.3% and contained 30.6% of glutamic acid. The total free amino acid content in the product decreased from 18.6 mg g^{-1} hydrolysate to 1.85 mg g^{-1} plastein. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Protein hydrolysates have been reported to serve as novel nutritional products for incorporation into formulations for infants with allergy to intact proteins (Cordle *et al.*, 1991) or inborn errors of metabolism (Farrell *et al.*, 1987) and adults with impaired gastrointestinal function (Cordano & Cook, 1985). The gastrointestinal absorption of hydrolysates, especially di- and tripeptides, is more effective than of unhydrolysed proteins. Thus, use of protein hydrolysates would allow improvement of protein balance during childhood, pregnancy, physical training or sickness. Addition of hydrolysates can also improve essential amino acid composition of foods. Extensively hydrolysed proteins have high solubility under acidic conditions and heat treatment such as those experienced during pasteurization. The solutions of hydrolysates have very low viscosity, even at high concentrations. Hence, protein hydrolysates are unique ingredients for protein enrichment of soft drinks and juice-based beverages. In the meat industry, hydrolysates may be used to improve functional properties and possibly amino acid composition of meat products.

The development of bitterness may occur when food proteins are enzymatically hydrolysed. Peptides containing one or more hydrophobic amino acid residues such as leucine, isoleucine, valine, phenylalanine, tyrosine and tryptophan (Pedersen, 1994) are responsible for this phenomenon. In unhydrolysed proteins, the hydrophobic regions of polypeptide chains are embedded inside the molecules and cannot affect the taste sensors. Therefore, bitterness development depends both on the degree of hydrolysis and also on the amino acid composition of proteins. Novo Industri has reported the preparation of red blood cell hydrolysates (Olsen, 1985). Our preliminary investigations revealed that protein hydrolysates from bovine red blood cells had a strong bitter taste. Although this bitter taste may not be important when added to meat products in less than 3% (w/w), their use in soft drinks may be hindered.

The present study reports on the preparation and characteristics of bovine red blood cell hydrolysates and examines the influence of plastein reaction between the hydrolysates so prepared and glutamic acid. The taste, properties and composition of plasteins are then investigated.

MATERIALS AND METHODS

Protein hydrolysate from bovine red blood cells, separated from fresh whole blood by centrifugation at 3000*g*, was prepared using Alcalase 2.4L (Novo Industri, Denmark) according to the method developed by Novo. A suspension of red blood cells in water (1:3, v/v) was prepared and the resultant mixture was subjected to protein hydrolysis at pH 8.5 and 55°C. Degree of hydrolysis (DH) was calculated from the consumption of 4 N NaOH required to maintain a constant pH during the hydrolysis (Olsen, 1983). Enzyme inactivation was achieved by addition of 4 N HCl solution to the mixture to reach a pH of 4.0 at 55°C over a 30-min period. The hydrolysate was then centrifuged at 3000*g*, in order to remove haem residues, and then decolorized with charcoal (4%, w/v), neutralized to pH 7.0 and lyophilized.

Plastein reaction was conducted at 37°C and pH 5.5 for 24 h using papain (Sigma) activated with L-cysteine. The ratio of the enzyme to substrate, on a dry weight basis, was 1:50 (w/w). A 50% (w/v) solution of hydrolysate and diethyl ester of glutamic acid (2:1, w/w) in 20% (v/v) aqueous acetone was prepared. After incubation, the reaction mixture was treated with a five-fold volume of 0.2 N NaOH solution to hydrolyse the remaining ethyl ester linkages, dialysed against running tap water at +8°C for 24 h and lyophilized. The yield of plastein reaction (Y) was calculated according to the equation $Y = (m/m_o) \times 100\%$, where m and m_o are the mass of nitrogen (determined according to Kjeldahl; AOAC, 1990) in the product and the reaction substrate, respectively.

Individual amino acids were determined after digestion of the samples in 6 N HCl at 110°C as described by Blackburn (1968). The HCl was then removed under vacuum and dried samples were reconstituted using a lithium citrate buffer at pH 2.2. The amino acid composition of the hydrolysate was determined using a Beckman 121 MB amino acid analyser (Beckman Instruments, Palo Alto, CA). Cysteine and methionine were determined by performic acid oxidation prior to their digestion in 6 N HCl and were measured as cysteic acid and methionine sulphone, respectively (Blackburn, 1978). Analysis of tryptophan was carried out by hydrolysis of the sample under vacuum in 3 M mercaptoethane sulphonic acid at 110°C, as described by Penke *et al.* (1974).

The essential amino acid index (EAA) value was calculated according to the equation:

$$EAA = 100 \cdot \sqrt{\frac{X_1}{X_{s1}} \cdot \frac{X_2}{X_{s2}} \cdot \ \ldots \ \cdot \frac{X_n}{X_{sn}}}^{n}$$

where X_n and X_{sn} denote the contents of leucine, isoleucine, lysine, threonine (phenylalanine + tyrosine), tryptophan (methionine + cysteine) and valine in the sample and FAO/WHO standards, respectively (FAO/WHO/UNU, 1985). The protein efficiency ratio (PER) values were calculated from the equation developed by Lee *et al.* (1978):

$$PER = 0.0632 \sum_{i=1k} Xi - 0.1539$$

where

$$\sum Xi = Ile + Leu + Lys + Met + Phe + Thr$$

$$+Val + Arg + His + Trp$$

.For determination of free amino acids, 0.2 g of sample was dissolved in ice-cold 6% perchloric acid (1:2, w/v) using a Polytron homogenizer (Brinkman Model PT3000; Kinematica, Littan, Switzerland). After 30 min incubation, samples were centrifuged at 3000*g* for 10 min at 5°C. The pH of the extract was adjusted to 7.0 using a 33% KOH solution. The precipitated potassium perchlorate was removed by centrifugation at 3000*g* for 10 min. The supernatant was acidified with 10 N HCl to pH 2.2 and diluted with 0.3 M lithium citrate buffer, pH 2.2 (2:1, v/v). Free amino acids were analysed on a Beckman 121 MB amino acid analyser using Benson D-X 8.25 resin and a single column, according to the three-buffer lithium method, as described in the Beckman 121 MB-TB-0.17 Application Notes.

Protein solubility was determined by spectrophotometric turbidity measurements as described by Yamashita *et al.* (1975). Eighty milligrams of sample were suspended in 40 ml of water and the pH of the mixture was adjusted with 0.1 N NaOH or HCl. After adjusting the volume to 50 ml, the percentage solubility (X) was calculated as $X(\%) = (21.2 - OD)/0.211$, where OD is the optical density of the protein solution at 600 nm.

The tristimulus Hunter colour parameters **L** (lightness: 100, white; 0, black), **a** (red, +; green, −) and **b** (yellow, +; blue, −) of the samples were measured using a Colormet colorimeter (Instrumar Engineering, St John's, NF). The unit was standardized with a B-143 white calibration tile having a Hunter **L** value of 94.5 ± 0.2, an **a** value of -1.0 ± 0.1, and a **b** value of 0.0 ± 0.2.

Taste evaluation of a 20% aqueous solution of the samples was conducted using a six-member trained panel and a 5-point scale (1, no bitterness; 2, weakly bitter; 3, mildly bitter; 4, strongly bitter; 5, very strongly bitter).

Analysis of variance and Tukey's studentized range tests (Snedecor & Cochran, 1980) were used to determine differences in mean values of three to six replicates of each measurement. Significance was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Degree of hydrolysis (DH) had a marked influence on nitrogen recovery and bitterness of bovine red blood cell

Table 1. Sensory scores of bovine red blood cell hydrolysates and plastein products

DH (%)	Bitterness scores	
	Hydrolysate	Plastein
16	3.5 ± 0.1^{ax}	2.0 ± 0.1^{bx}
19	4.0 ± 0.2^{ay}	2.1 ± 0.2^{bx}
25	4.3 ± 0.2^{y}	–

Results are mean values of six sensory evaluation replicates ± standard deviation.
Values in each row (a,b) and column (x,y) with different superscripts are significantly different ($P < 0.05$) from one another.
Scores: 1, no bitterness; 2, weak bitterness; 3, mild bitterness; 4, strong bitterness; 5, very strong bitterness.
DH, degree of hydrolysis.

Table 3. Hunter colour parameters of bovine red blood cell hydrolysate (DH 19%) and plastein

Colour specification	Hydrolysate	Plastein
L	73.6 ± 0.2^{a}	87.2 ± 0.3^{b}
a	0.7 ± 0.1^{a}	-0.5 ± 0.0^{b}
b	19.1 ± 0.5^{a}	17.1 ± 0.8^{b}
Hue	87.9 ± 0.2^{a}	-88.3 ± 0.1^{b}
Chroma	19.1 ± 0.5^{a}	17.1 ± 0.6^{b}

Results are mean values of four measurements ± standard deviation.
Values in each row with different superscripts are significantly different ($P < 0.05$) from one another.

hydrolysate (BRBCH). The choise of Alcalase was made based on its specificity for terminal hydrophobic amino acids, which generally leads to the production of non-bitter hydrolysates (Adler-Nissen, 1986). The best nitrogen recovery was 69.2 ± 1.0% of that present in the red blood cell fraction at a DH of 25%. At DH 16%, the nitrogen recovery was 65.9 ± 0.4% and some unhydrolysed proteins were separated along with haem residues during the centrifugation step.

Results in Table 1 indicate that products with a high DH value possess a strong bitter taste; thus, the DH should not exceed 19%. However, when DH was below 17%, complete separation of haem from the hydrolysate solution was not possible. The highest loss of nitrogenous compounds occurred during haem separation and bleaching of the hydrolysate solution with charcoal (Table 2). The observed differences, on a dry weight basis, were due to the addition of NaOH to the solution for maintaining a constant pH during hydrolysis and formation of sodium chloride during inactivation of enzyme and neutralization of the product. The BRBCH (DH 19%) contained 81.8% crude protein (N×6.25), 2.4% moisture and 15.8% sodium chloride. The Hunter colour parameters of products (given in Table 3) show good release of haem pigments during the hydrolysis process and subsequent unit operations.

The amino acid composition of the hydroysate indicated the presence of somewhat higher amounts of glutamic acid, cysteine, glycine and serine and smaller amounts of leucine, methionine, phenylalanine and tryptophan than those present in the unhydrolysed bovine globin (Table 4). These differences may possibly originate from the loss of unhydrolysed residues which might have been removed with the haem by-product. The essential amino acid index (EAA) of the hydrolysate (63.2) was similar to that of bovine globin (67.3) and lower than that for proteins from bovine longissimus dorsi (101.7). The lower contents of isoleucine, methionine and cysteine in globin compared with those in bovine muscle proteins (Schweigert, 1987) or FAO/WHO standards (FAO/WHO/UNU, 1985) may explain the observed results. The PER for BRBCH was 2.7 compared with those for beef (2.8), pork (2.5) and cod (2.9). However, globin contains 35.8% hydrophobic amino acids, which is more than the amount for proteins from beef (27.5%) and pork (25.9%) (Schweigert, 1987; Shahidi *et al.*, 1984). The latter amino acids in the free form or in short-chain peptides cause bitterness of products (Pedersen, 1994). As a consequence, the BRBCH had a bitter taste which increased with increasing DH (Table 1).

The solubility of BRBCH, determined in the pH range 2.5–9.5, attained a minimum of 93.52% at pH 2.6 (Table 5). Therefore, the product exhibited good solubility characteristics over a wide pH range. The addition of 3% of BRBCH to comminuted beef containing 3% sodium chloride resulted in a decrease of drip water and a corresponding increase of approximately 5% in the cooking yield of the meat.

The bitterness of BRBCH was effectively reduced by approximately 50% from its initial value as a result of papain-catalysed plastein reaction with glutamic acid (Table 1). Glutamic acid was used as its diethyl ester

Table 2. Mass and nitrogen balance of hydrolysate at DH 19% using Alcalase 2.4L

Material	Mass balance (%, dry weight basis)	Nitrogen balance (%)
Red blood cells	100	100
Hydrolysed blood cells	109 ± 0.9	100
Hydrolysate after haem separation	86.2 ± 0.3	73.1 ± 0.5
Haem residue	33.5 ± 0.1	26.9 ± 0.1
Hydrolysate after bleaching with charcoal	77.2 ± 0.3	66.2 ± 0.2
Compounds adsorbed on charcoal	9.0 ± 0.1	6.9 ± 0.0
Lyophilized hydrolysate	79.1 ± 0.3	65.9 ± 0.1

Results are mean values of three batch operations ± standard deviation.
DH, degree of hydrolysis.

Table 4. Amino acid composition (%N×6.25) of bovine red blood cell hydrolysate (BRBCH) at DH 19% and plastein as compared with bovine globin

Amino acid	Bovine globin	BRBCH	Plastein
Alanine	8.6	8.64±0.02	4.92±0.07
Arginine	3.6	3.49±0.01	1.79±0.01
Aspartic acid	10.0	11.2±0.20	12.1±0.03
Cysteine	0.1	1.03±0.01	1.22±0.09
Glutamic acid	6.8	7.96±0.11	30.7±0.36
Glycine	3.7	5.18±0.04	3.85±0.02
Histidine	7.8	7.92±0.16	5.05±0.02
Isoleucine	0.2	0.54±0.00	0.37±0.03
Leucine	13.8	11.2±0.53	8.47±0.03
Lysine	10.5	10.2±0.23	8.03±0.00
Methionine	1.7	0.85±0.01	0.88±0.02
Phenylalanine	7.9	5.53±0.05	3.80±0.06
Proline	3.5	3.33±0.02	3.13±0.01
Serine	3.0	5.18±0.15	3.38±0.01
Threonine	3.8	3.70±0.02	1.96±0.02
Tryptophan	2.0	0.78±0.01	0.51±0.02
Tyrosine	2.5	1.94±0.00	1.23±0.04
Valine	9.4	9.28±0.01	6.92±0.04

Results are mean values of three replicates ± standard deviation.
From Tybor *et al.* (1975).

derivative, because according to Yamashita *et al.* (1975) the free compound was quite unreactive. After completion of the reaction, the glutamic acid content of the product was increased by a factor of 3.9, from 7.96% in BRBCH to 30.67% in the final plastein (Table 4). Lower content of free glutamic acid in plastein (0.46 mg g^{-1}) than in hydrolysate (1.04 mg g^{-1}) shows that all added glutamic acid was effectively incorporated into the peptide molecules (Table 6). As a result, the content of crude protein (N×6.25) was increased from 81.8% in the starting BRBCH to 96.3% in the plastein product. The differences in the amino acid composition of hydrolysate and plastein product were mainly due to the incorporation of high amounts of glutamic acid, which results in a relative decrease in the proportion of other amino acids in the product (Table 4). As a consequence, the EAA and PER of plastein decreased to 46.0 and 2.2, respectively. The plastein reaction also decreased the total content of free amino acids from 18.57 mg g^{-1} hydrolysate to 1.85 mg g^{-1} plastein. Therefore, some of the free amino acids participated in the plastein reaction (Table 6).

Table 5. Effect of pH on solubility (%) of bovine red blood cell hydrolysate (BRBCH) and plastein

pH	BRBCH	Plastein
2.6	93.52±0.20	99.45±0.20
4.0	93.97±0.15	98.11±0.05
4.5	94.41±0.07	97.75±0.11
6.0	94.75±0.01	97.90±0.02
7.0	95.50±0.09	98.23±0.04
8.0	97.53±0.14	98.81±0.09
9.5	98.07±0.06	99.93±0.12

Results are mean values of three determinations + standard deviation.

Table 6. Free amino acids (mg g^{-1} sample) of bovine red blood cell hydrolysate (BRBCH) at DH 19% and plastein

Amino acid	BRBCH	Plastein
Alanine	1.75±0.26	–
Aspartic acid + asparagine	1.16±0.11	0.10±0.02
Cysteine	0.84±0.01	0.10±0.01
Glutamic acid + glutamine	1.04±0.16	0.46±0.03
Glycine	0.45±0.12	–
Leucine	7.04±0.13	0.47±0.02
Lysine	2.13±0.01	0.26±0.03
Methionine	0.37±0.02	0.27±0.09
Phenylalanine	0.73±0.03	0.03±0.01
Serine	0.83±0.04	–
Threonine	0.72±0.04	–
Tyrosine	0.03±0.01	–
Valine	1.47±0.15	0.18±0.01

Results are mean values of three replicates ± standard deviation.

The increased amount of total hydrophilic amino acids had a positive influence on the solubility of plastein in water and also improved other functional properties of the products (Table 5). The plastein also had a slightly better solubility in the pH range 2.6–9.5 than that of BRBCH, but had a minimum solubility between pH 4.5 and 6.0 (about 97.7–97.9%). Plastein reaction also improved the colour of the product (Table 3) as the Hunter L value of 87.2 indicates an almost white-coloured product.

In conclusion, the best nitrogen recovery from bovine red blood cells was achieved for a DH of 19–25%, but products with DH > 19% had a bitter taste. Plastein reaction with glutamic acid diethyl ester resulted in a relative decrease in the proportion of hydrophobic amino acids and hence improved the taste of the product, which had excellent solubility characteristics over a wide pH range. The product possessed an almost white colour. Further investigations on plastein reaction with isoleucine and sulphur-containing amino acids may prove beneficial in improving the nutritional value of the hydrolysates from bovine red blood cells. The haem by-product may be used for the production of the preformed cooked cured-meat pigment (Shahidi & Pegg, 1991).

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