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# Production of enzymatically hydrolyzed soybean protein isolate by a novel neutral protease from *Enterobacter* sp. NRCM1 to be used as a base precursor of thermal process flavours

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## Abstract

A novel neutral protease from a newly isolated and identified bacteria *Enterobacter* sp. NRCM1 was used to produce the enzymatic hydrolyzed soybean protein isolate (E-SPM1). The protease was purified and biochemically characterized. The effect of hydrolysis time on degree of protein hydrolysis and released free amino acids was investigated. The enzymatic hydrolysates (E-SPM1$_{24}$, E-SPM1$_{48}$ and E-SPM1$_{72}$) produced after 24, 48 and 72 h, respectively were used as main precursors of beef-like flavours. The generated volatiles were determined by solid phase microextraction combined with gas chromatography- mass spectrophotometer (SPME- GC-MS). The results revealed that the optimum pH and temperature of protease activity were 7.0 and 50$^{o}$C, respectively. Sample E-SPM1$_{72}$ showed the highest degree of hydrolysis (43.71%) and content of free amino acids (5.78 g/100mL). The headspace volatiles of the model mixture based on E-SPM1$_{72}$ comprised the highest content of the most important compounds for meat aroma. The results of odour sensory analysis confirmed those of GC-MS for all investigated samples. The highest score of beefy note of sample E-SPM1$_{72}$ was mainly correlated to the significant increase of 2-methyl-3-furanthiol, the most potent aroma compound in beef flavour. The gradual increase of savoury note was consistent with that of the disulfide compounds, which possess savoury aromatic note.

*Keywords*: Soybean protein isolates; Enzymatic hydrolyzed protein; Protease bacteria; Beef-like flavour; Sensory evaluation.

## 1. Introduction

Enzymes, as natural catalysts, are widely used in various industrial fields and bioprocesses owing to their advantages such as low cost, ease production and less environmental pollution [1]. Many studies have been focused on detecting new enzymes having satisfactory properties for commercial applications [2-4]. Proteases comprise 65% of the total industrial enzymes market [5]. These enzymes catalyze the degradation of proteins by hydrolysis of the peptide bonds which link amino acids together in the peptide chains which forming the protein molecules [6, 7]. In industrial applications microbial proteases are very beneficial, compared to plant or animal proteases, due to their distinctive characteristics such as short generation time and large availability in nature [3]. Besides, they have been considered as ecofriendly because the appropriate production of them for commercial exploitation is non toxic and non pathogenic [5]. Proteases are widely used in various industrial fields such as foods, detergent formulation, pharmaceutical and leather tanning [4, 7, 8].

Thermal process flavours, which are produced by heating the appropriate precursors under controlled heating process *via* Maillard reaction, have been increasingly found application in analogue meat flavour [9, 10, 11]. The enzymatic hydrolyzed proteins and amino acids are considered as safe flavour precursors of thermal process flavours [12]. These flavours are mainly used to impart characteristic

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flavours of thermally treated food stuff such as nuts, coffee, cocoa and meat [13, 14]. Flavour of cooked meat has special importance in industrial processed food flavours [15]. In previous studies the effect of different thermal technologies on the production of beef-like flavour from the enzymatically hydrolyzed mushroom [10] has been investigated. Enzymatic hydrolysates of mushroom and soybean were used separately as substrates for the bioproduction of beef-like flavour by *Corynbacterium glutamicum* $1220^t$ [16].

Bacteria are considered the most important alkaline protease producers, the genus *Bacillus* being the most prominent source because of their ability to produce a high production of proteases having significant activity and stability at high temperature and pH [17, 18, 19]. The manufacturing of microbial protease from bacteria depends on the type of strain, composition of medium, procedure of cultivation, nutrient requirements, pH, temperature and incubation time [20].

In this study a novel inexpensive and active protease, from a newly isolated bacteria strain, was used for the production of SPI enzymatic hydrolysate to be used as a main precursor of beef-like flavour. To achieve this aim the proteolytic activity of different newly isolated bacteria was evaluated. The strains showed the highest activity were subjected to morphological and biochemical tests and that showed promising results was selected and identified. The protease of the selected protease bacteria was purified and used for the hydrolysis of SPI at optimum conditions. The hydrolysates obtained during different time of hydrolysis were used as base precursors for the production of beef-like flavours. A comparative study between the produced flavours was carried out concerning the odour profile and headspace volatiles.

## 2. Material and Methods

### 2.1. Substrates and chemicals

Soybean protein isolates (SPI) (80% protein) was obtained from Miro for Export & Import Co., Giza, Egypt. Mushroom (*Agricus bispours*) was obtained from bloshia mushroom company, Dokky, Egypt. White cheese was obtained from local market. Thiamine, cysteine, xylose, and standard n-paraffins ($C_8$- $C_{22}$) and authentic compounds were purchased from Sigma Aldrich Chemical Co. (st Louis, No, USA). All other chemicals used in the present study were of analytical grade and the solvents were purified and distilled before use.

### 2.2. Sampling, screening and isolation of bacteria

Three different sources; fermented cheese (C), SPI (S) and dried mushroom (M) powder were used for isolation of bacteria capable to produce protease enzymes. The collected samples were separately placed in flasks containing 100 mL sterile water and homogenized by shaking at 200 rpm for 15 min and a serial dilution was performed. Finally, 50 µL of the supernatant of each dilution was inoculated on casein medium agar and the spread plates were incubated at 37ºC for 48 h. The appeared colonies per plate of each sample were subjected to purification. The zone of hydrolysis was noted for each sample. The colonies that showed the highest clear zone (C2, S3, S4, M1, M2 and M3) were selected for further study. Each colony was grown on nutrient agar plate repeatedly and preserved on nutrient agar slant at 40ºC. Gelatin plates were employed for screening of protease producers [21].

### 2.3. Identification of promising bacterial isolate

The promising isolate, which produced a high amount of protease, was distinguished depending on biochemical, morphological and physiological characteristics of the potential producer as determined by embracing standard methods [22, 23]. Molecular identification was performed by using PCR amplification of 16S rDNA sequences. Chromosomal DNA was extracted and 16S rRNA gene was amplified by using the well-known primers F-(5′-GAGTTTGATCCTGGCTCAG-3′) and R-(5′-GGTTACCTTGTTACGACTT-3′). The PCR product of 16S rRNA was purified to remove contaminants and sequenced then the 16S rRNA sequence was compared with published 16S rRNA sequences of isolates in the NCBI databases using BLAST program (http://www.ncbi.nlm.nih.gov). Selected sequences of the greatest similarity to the 16S rRNA sequences of the isolate have been aligned and producing the phylogenetic tree. The 16S rRNA gene sequences of the isolate reported in this paper was deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases.

### 2.4. Conditions of Fermentation

The enzyme production had been carried out in optimized medium containing casein 1%, yeast extract 0.5% and NaCl 2.5%. Seed inoculum (1%, 24h old) was added to every Erlenmeyer flask (250 mL) containing 50 mL medium, the flasks were incubated at 30°C under static conditions. The cell free supernatant, obtained by means of centrifugation

(5000 rpm, 20 min), was used for the determination of the extracellular protease activity. The growth kinetics was determined by optical density measurement at 600 nm (UV-1600; Shimadzu Co., Kyoto, Japan) along with protease production by different isolates. The investigation was performed in medium containing1% SPI or dried mushroom, inoculated with 0.5 mL culture grown overnight. The medium (initial pH7) was incubated for 48 h at 37°C on a rotary shaker with shaking speed 150 rpm [24].

**2.5. Protease assay**

The protease activity was assayed according to Vishwanatha *et al.* [25] with some modifications. Briefly, enzyme solution was added to substrate solution (0.7% casein in phosphate 0.1 M buffer, pH 7.0) and the combination was incubated at 50°C for 10 min. The reaction was stopped by adding 2 mL of trichloroacetic acid mixture (2.0 % TCA in 0.22 M sodium acetate and 0.33 M acetic acid) and kept at room temperature for 20 min. The content was centrifuged and the absorbance of the filtrate was measured spectrophotometrically at 280 nm. Enzyme units were determined using tyrosine (0 -100 µg) as standard. Protein content was estimated using bovine serum albumin (BSA) as the standard according to Bradford method [26] and the concentration was expressed in mg/mL. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per milliliter in 1 min under the experimental conditions used. Specific activity of the sample was calculated by dividing the activity units by the protein content and expressed as Units /mg protein.

**2.6. Enzymatic hydrolysate production**

The enzymatic hydrolyses of soybean protein isolate (E-SPI) was carried out by dissolving 10 g SPI with 80 mL of phosphate buffer at pH 7. Each enzyme (20 mL) was added and the mixture was allowed to stand for 5 h at 50 ºC. Each hydrolysate was centrifuged and the precipitate was discarded. The suspension of each hydrolysate sample in buffer was subjected to analysis for free amino acids composition [10] by using Biochrom amino acid analyzer (Eppendrof-Biotronik, Germany).

**2.7. Partial purification of protease enzyme by ammonium sulfate**

The crude protease was fractionated *via* addition of solid ammonium sulfate ($(NH_4)_2SO_4$). The protease fractions, precipitated successively at 20–80% of $(NH_4)_2SO_4$ saturation, were separately accumulated by centrifugation at 5000 rpm for 20 min and dissolved in 10 mL 0.1 M phosphate buffer (pH 7) then dialyzed in opposition to the same buffer at 4°C for 3 days [27]. The protease activity and protein concentration were determined and the specific activity was calculated.

**2.8. Optimization of pH and temperature**

The optimal pH and temperature of the partial purified protease were determined adopting the protease assay mentioned above (2.5) with some variations. The 0.1 M phosphate buffer pH 7.0 was replaced by the following buffer: 0.1 M sodium acetate buffer (pH 4.0 – 5.5), 0.1 M phosphate buffer (pH 6 – 8) and 0.1 M glycine-NaOH buffer (pH 8.5 – 10.5). The reaction mixture was incubated at different temperatures starting from 30 to 70°C for 60 min.

**2.9. pH and thermal stability**

The pH stability of the partial purified protease was determined by incubating 2 mL of partial purified protease with 2 mL of 0.1 M sodium acetate buffer (pH 4.5 – 5.5), 0.1 M phosphate buffer (pH 6 – 8) and 0.1 M glycine-NaOH buffer (pH 8.5 – 10.5), respectively at 40°C without a substrate for 30 min according to Benmrad *et al.* [4]. The residual activity and temperature stability of the enzyme was determined by the standard assay method. The stability of enzyme temperature was performed by incubating 2 mL pure enzyme with 2.0 mL buffer (0.1 M phosphate buffer, pH 7) without a substrate at different temperatures (30 – 80°C) for 30 min.

**2.10. Degree of hydrolysis (DH)**

The degree of SPI hydrolysis, by the protease from M1 (PM1), at different reaction time 24, 48 and 72 h (at optimum pH and temperature), was determined. The degree of hydrolysis (DH) was defined as the ratio of free amino acids released in the hydrolysate to the total amount of amino groups in protein. DH(%) = amino acids(mg) / Total protein content(mg) × 100. The amount of free amino groups was determined by ninhydrine reaction [28].

**2.11. Preparation of beef-like flavour**

A mixture of cysteine (0.1 g), thiamine (0.1 g) and xylose (1.0 g) was dissolved in 20 mL of each enzymatic hydrolysate (E-SPM$1_{24}$, E-SPM$1_{48}$ and E-SPM$1_{72}$). The final volume was adjusted to 30 mL with phosphate buffer (0.5 M; pH 5) then transferred into 50 mL screw-sealed tube tightly capped and heated in a thermostatic oil bath with magnetic stirring (150 rpm) at 150°C for 60 min. After reaction, the tube was immediately cooled in ice-water to room temperature.

### 2.12. Odour sensory analysis

The odour profile of each generated flavour was subjected to qualitative descriptive odour sensory analysis. The panelists (10-male, 10-female) drawn from Food Technology and Nutrition Division, National Research Centre, Giza, Egypt were trained following the previous studies [10, 29]. Five descriptors, including overall acceptability, beefy, roasty, savoury, sulphurous were selected and used for the descriptive analysis of beef-like flavour. The panelist separately scored the intensity of each descriptive odour quality on a category scale 0.0 (not perceptible) to 10.0 (strongly perceptible). The analysis was carried out in triplicate.

### 2.13. Solid phase microextraction (SPME)

A divinylbenzene/carboxen/polydimethyl siloxane (DVB/CAR/ PDMS) fiber (2 Cm, coating thickness: 50/30 mm) was used in solid phase microextraction analysis (SPME; Supleco, 57348-U, Bellefonte, PA, USA). This fiber showed a high ability to extract the target volatile compounds [30, 31]. The optimal extraction conditions (time and temperature) of the volatile compounds were investigated [32]. For all investigated compounds the optimum conditions for extraction were 60 min at 60ºC. Each sample (50 mL) was placed in a 100 mL headspace vial sealed with a PTFE faced silicon septum (Supelco, Bellefonte, PA, USA). Extraction was carried out by exposing the SPME fiber to the headspace of each sample for 60 min at 60°C, then it was inserted into the GC injection port for desorption (260°C / 5 min in splitless mode). Before use, the fiber was conditioned in the injection port of the GC (270°C / 1 h) as recommended by manufacture. Extraction was carried out in triplicate for each sample.

### 2.14. Gas chromatography – mass spectrometry (GC–MS) analysis

The GC-MS analysis was performed according to Fadel *et al.* [32]. A gas chromatography (Hewlett-Packard model 5890, USA) coupled to a mass spectrometer (Hewlett-Packard-MS 5970, USA) was used for the qualitative and quantitative analysis of the volatile compounds in the head space of each sample. The volatile compounds were separated on a DB5 column (60 m, 0.32 mm i.d., 0.25 µm film thickness). The isolated peaks were identified by matching with data from the library of mass spectra (National Institute of Standard and Technology, NIST), comparison with those of authentic compounds and published data [13, 33, 34]. The amount of each individual compound was expressed as total ion chromatograms (TIC)

### 2.15. Statistical analysis

Data were analyzed using the analysis of variance (ANOVA) by using SPSS program for windows (Version 21) (SPSS, IBM Corporation, Armonk, New York, USA). The multiple range least significant difference test L.S.D. and Duncan multiple range tests were applied to the results ($P< 0.05$) [35].

## 3. Results and Discussion

### 3.1. Isolation, screening and identification of protease-producing bacteria

Among the 11 bacteria isolated from the different local sources; fermented cheese, SPI and dried mushroom powder, M1 showed the highest enzyme production (data not shown). The selected colony M1 exhibited a white creamy, smooth, circular, and was Gram-negative and short rods shaped. The identification was confirmed by molecular analyses based on 16S rDNA. A portion of the 16S rRNA gene (~1004 bp) was amplified from the genomic DNA and the amplicon was subjected to sequencing followed by homology search analysis. The sequence showed 100% similarity (accession no: KR778808.1) with already available sequences of *Enterobacter* sp. in the GenBank. On the basis of morphology and 16S rRNA and ITS sequence homology, the isolate is designated as a new strain of *Enterobacter* sp. NRCM1. The homology analysis was established using BLAST software. The phylogenetic tree was constructed using the dendrogram method implemented in Fig. 1.

### 3.2. Composition of free amino acids released by proteases from different bacteria

The composition of free amino acids released in the SPI enzymatic hydrolysates (E-SPC2, E-SPM1 and E-SPS3) produced by the proteases from the more active bacterial strains (C2, M1 and S3) is shown in Table 1. The results revealed that E-SPM1 comprised the highest content of free amino acids (1.83 g/100 mL) followed by E-SPS3 (1.47 g/100 mL) and E-SPC2 (0.53 g/100 mL). Among the 15 free amino acids found in the hydrolysates, glutamic acid comprised the highest content in E-SPM1 followed by aspartic acid, leucine and arginine.

### 3.3. Purification of protease from M1 (PM1)

Partial purification of PM1 was performed by

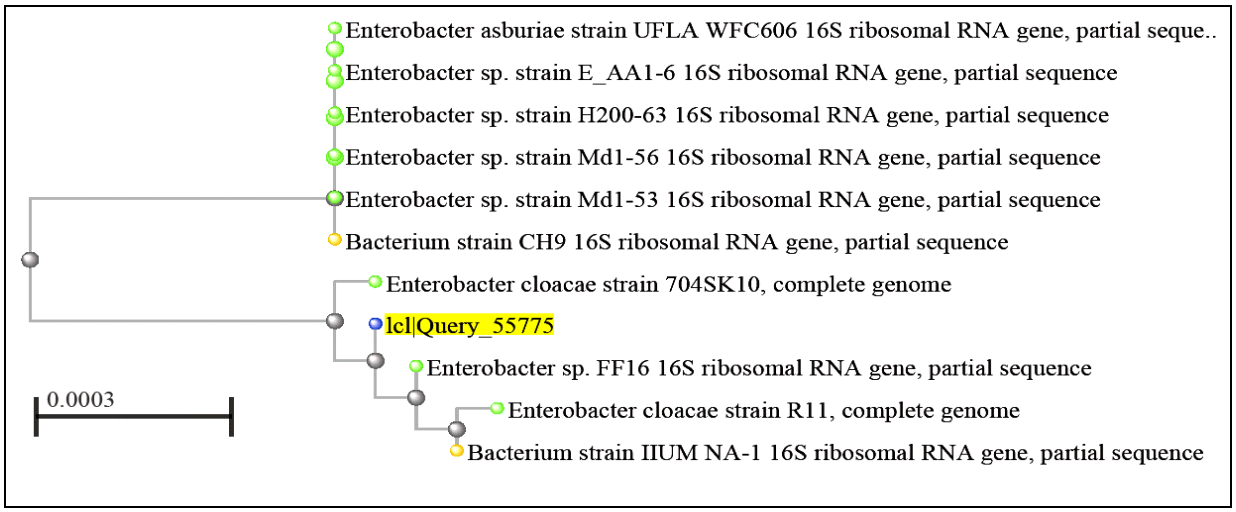


**Fig. 1.** Phylogenetic tree of the partial sequence of 16S rRNA of the local isolate M1 with respect to closely related sequences available in GenBank databases

**Table 1.** Composition of free amino acids in the SPI hydrolyzed by proteases from different bacteria

| Amino acids | E-SPC2 | E-SPM1 | E-SPS3 |
|---|---|---|---|
| | g/100mL | | |
| Aspartic | 0.07 ± 0.03 | 0.20 ± 0.06 | 0.17 ± 0.08 |
| Threonine | 0.03 ± 0.01 | 0.10 ± 0.04 | 0.05 ± 0.02 |
| Serine | 0.03 ± 0.01 | 0.09 ± 0.04 | 0.07 ± 0.03 |
| Glutamic | 0.10 ± 0.05 | 0.28 ± 0.08 | 0.32 ± 0.10 |
| Glycine | 0.03 ± 0.01 | 0.11 ± 0.05 | 0.07 ± 0.03 |
| Alanine | 0.04 ± 0.02 | 0.11 ± 0.05 | 0.08 ± 0.04 |
| Valine | 0.03 ± 0.01 | 0.12 ± 0.05 | 0.06 ± 0.03 |
| Isoleucine | 0.02 ± 0.00 | 0.09 ± 0.04 | 0.07 ± 0.03 |
| Leucine | 0.04 ± 0.02 | 0.16 ± 0.05 | 0.10 ± 0.04 |
| Tyrosine | 0.02 ± 0.00 | 0.08 ± 0.03 | 0.06 ± 0.02 |
| Phenylalanine | 0.02 ± 0.01 | 0.11 ± 0.05 | 0.07 ± 0.03 |
| Histidine | 0.01 ± 0.00 | 0.05 ± 0.02 | 0.04 ± 0.02 |
| Lysine | 0.04 ± 0.02 | 0.12 ± 0.05 | 0.12 ± 0.05 |
| Arginine | 0.03 ± 0.01 | 0.13 ± 0.06 | 0.11 ± 0.05 |
| Proline | 0.02 ± 0.00 | 0.08 ± 0.04 | 0.09 ± 0.05 |
| Total amino acid | 0.53 ± 0.16 | 1.83 ± 0.29 | 1.48 ± 0.27 |

**E-SPC2:** Enzymatic hydrolyzed SPI hydrolyzed by protease produced by bacteria isolated from fermented cheese
**E-SPM1:** Enzymatic hydrolyzed SPI hydrolyzed by protease produced by bacteria isolated from fermented muchroom
**E-SPS3:** Enzymatic hydrolyzed SPI hydrolyzed by protease produced by bacteria isolated from fermented soybean
Results are the mean of three replicates

ammonium sulfate ($(NH_4)_2SO_4$) precipitation at different concentrations. The fractions collected at levels of saturation of 20, 40, 60 and 80% ($(NH_4)_2SO_4$, w/v) were assayed for specific protease activity after dialysis. Fractions collected at 20 % comprised the highest percent of protease activity (296.22 U/mL) followed by 40 % (107.27 U/mL) with total protein content 3.16 and 1.85 mg/mL, respectively (Table 2). As show in Table 2, the fraction corresponds to 20% ammonium sulfate contained 81.8% protease activity and caused 1.7 fold increases in specific activity compared with the un-concentrated supernatant.

### 3.4. Characterization of purified protease PM1

#### 3.4.1. Effect of pH on enzyme activity and stability

The effect of pH on the protease activity was studied by using SPI as a substrate under the standard assay conditions. The protease displayed activity over a broad range of pH (6.0 – 9.0), with an optimum at pH 7.0 (Fig. 2). The relative activities at pH 6.0 and 9.0 were 85.3 and 76.5%, respectively. The pH stability profile of protease illustrated in Fig. 2 indicated that the partial purified enzyme was highly stable in the pH range of 7.0 – 9.0. Partial purified protease was, therefore, identified as a neutral protease, which is consistent with the protease from *Aspergillus flavipes* and *Aspergillus brasiliensis* [36, 37]. Furthermore, compared to the commercial neutral protease, this partially purified protease demonstrated better stability under the conditions of weak acid and weak alkalinity. It was further confirmed that this protease meets the basic requirements of the food industry.

**Table 2.** Ammonium sulfate fractionation pattern of protease by *Enterobacter* sp. NRCM1

| Purification steps | Total activity (U/mL) | Total protein (mg/mL) | Specific activity (U/mg protein) | Recovery (%) | Purification fold |
|---|---|---|---|---|---|
| Crude enzyme | 362.15 | 6.57 | 55.12 | 100.00 | 1.00 |
| $(NH_4)_2SO_4$ Saturation (%) | | | | | |
| 20 | 296.22 | 3.16 | 93.74 | 81.80 | 1.70 |
| 40 | 107.27 | 1.85 | 57.98 | 29.60 | 1.00 |
| 60 | 41.55 | 0.66 | 62.20 | 16.90 | 1.10 |
| 80 | ----- | 0.85 | ----- | ---- | ---- |

### 3.4.2. Effect of temperature on enzyme activity and stability

The enzymatic activity was detected over a broad range of temperature (30 – 70°C). The optimum temperature recorded for protease activity was 50°C, using SPI as a substrate as shown in Fig. 3. These results are in line with other previously investigated proteases from bacterial origins such as *Bacillus pumilus* CBC [38] and *Bacillus licheniformis* strains [39, 40]. Shankar *et al.* [41] and Al-Ghanimi *et al.* [42] reported that the protease from *Beauveria* sp was active in the temperature range of 30 - 60°C with an optimum at 50°C. As shown in Fig. 3 the protease enzyme was stable up to 70°C and maintained about 80% of its activity at 80°C. These results indicate that the partial purified protease form NRCM1 is a thermostable protease enzyme. Thermostability is a key characteristic of protease enzymes required in various industries. Since NRCM1 possess thermostability over a wide range of temperature, it can potentially be used in many industries [43]. The optimum activity of protease from *Halococcus agarilyticus* GUGFAWS-3 was between 20 and 80ºC, 0 - 5 M NaCl, and pH 3 - 13. The enzyme was thermostable at 70ºC with 50.26 ± 2.40% of relative enzyme activity at 75 min [44].

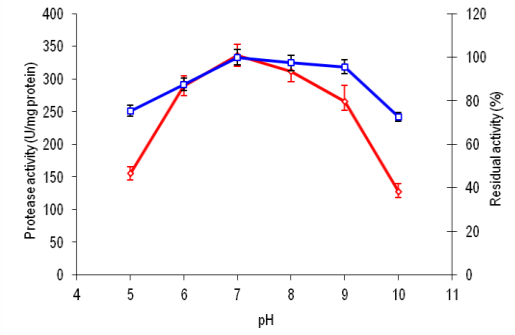


**Fig. 2.** Effect of pH on protease activity and stability (Protease activity ○ and protease stability □).
The bars showed standard deviations of triplicate samples.

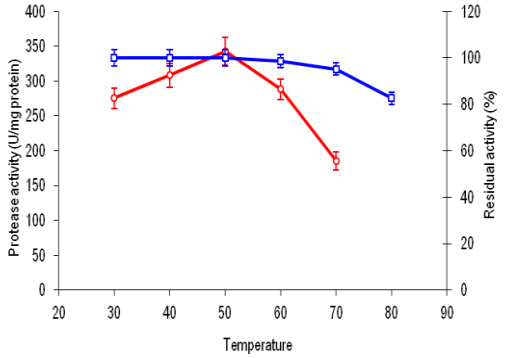


**Fig. 3.** Effect of temperature on protease activity and stability (Protease activity ○ and protease stability □).
The bars showed standard deviations of triplicate samples.

### 3.5. Effect of degree of hydrolysis on free amino acids composition of E-SPIM1

The free amino acids play an important role in the formation of thermal process flavours [9]. The degree of hydrolysis DH of SPI by PM1 for 24, 48 and 72 h were 29.5, 36.8 and 43.71 %, respectively. These results confirm the high proteolytic activity of PM1. In previous studies [45, 46] the DH of soybean protein by immobilized enzymes was in the range of 1-39.5%. The content of the free amino acids identified in the E-SPM1 hydrolyzed for 24 (E-SPM1$_{24}$), 48 (E-SPM1$_{48}$) and 72h (E-SPM1$_{72}$) at the optimum pH and temperature (7.0, 50ºC, respectively) are shown in Table 3. The results revealed that purification and hydrolysis of E-SPM1 at optimum conditions gave rise to a remarkable increase in the total content of free amino acids from 1.83 g/100mL (Table 1) to 2.68, 4.12 and 5.78 g/100mL after hydrolysis for 24, 48 and 72 h, respectively. This finding confirms the result of DH, mentioned above. A total of 15 amino acids were found in the hydrolysates among them glutamic was the abundant amino acid, it comprised the highest content in all investigated hydrolysates. Generation of 2-methyl-3-furanthiol, the key aroma compound of beef flavour, from a model system

containing cysteine and xylose was enhanced by addition of glutamic acid [47]. Aspartic acid was the second abundant amino acid, it comprised approximately 12% of the total content of amino acids in the hydrolysates. The other abundant amino acids identified in E-SPM1$_{72}$ were arginine, lysine and leucine which collectively accounted for more than 23% of the total amino acids. Lysine considered the main precursor of alkylpyrazines generated by thermal reaction [48]. Whereas, the potent odourant, 2-methylbutanal is the strecker degradation of leucine [49]. Arginine, alanine and phenylalanine may play an important role in thermal process flavours [9].

**Table 3.** Composition of free amino acids in enzymatic hydrolyzed SPI (E-SPIM1) at different time of hydrolysis.

| Amino acid | E-SPM1$_{24}$ | E-SPM1$_{48}$ | E-SPM1$_{72}$ |
|---|---|---|---|
| | g/100mL | | |
| Aspartic | 0.33 ± 0.02 | 0.50 ± 0.03 | 0.71 ± 0.03 |
| Threonine | 0.10 ± 0.01 | 0.17 ± 0.01 | 0.22 ± 0.01 |
| Serine | 0.12 ± 0.01 | 0.21 ± 0.02 | 0.27 ± 0.01 |
| Glutamic | 0.57 ± 0.03 | 0.84 ± 0.04 | 1.06 ± 0.05 |
| Glycine | 0.12 ± 0.01 | 0.19 ± 0.01 | 0.28 ± 0.01 |
| Alanine | 0.13 ± 0.01 | 0.22 ± 0.01 | 0.34 ± 0.02 |
| Valine | 0.13 ± 0.01 | 0.23 ± 0.01 | 0.30 ± 0.01 |
| Isoleucine | 0.12 ± 0.01 | 0.20 ± 0.01 | 0.26 ± 0.01 |
| Leucine | 0.20 ± 0.01 | 0.31 ± 0.02 | 0.42 ± 0.02 |
| Tyrosine | 0.07 ± 0.00 | 0.10 ± 0.00 | 0.20 ± 0.01 |
| Phenylalanine | 0.13 ± 0.01 | 0.19 ± 0.01 | 0.29 ± 0.01 |
| Histidine | 0.08 ± 0.00 | 0.13 ± 0.01 | 0.17 ± 0.01 |
| Lysine | 0.28 ± 0.01 | 0.47 ± 0.03 | 0.45 ± 0.02 |
| Arginine | 0.15 ± 0.01 | 0.17 ± 0.01 | 0.48 ± 0.03 |
| Proline | 0.15 ± 0.01 | 0.19 ± 0.01 | 0.33 ± 0.02 |
| Total | 2.68 ± 0.12 | 4.12 ± 0.19 | 5.78 ± 0.27 |

**E-SPM1$_{24}$:** Enzymatic hydrolyzed SPI hydrolyzed by protease PM1 at 24h.
**E-SPM1$_{48}$:** Enzymatic hydrolyzed SPI hydrolyzed by protease PM1 at 48h.
**E-SPM1$_{72}$:** Enzymatic hydrolyzed SPI hydrolyzed by protease PM1 at 72h.
Results are the mean of three replicates

### 3.6. Headspace volatiles of beef-like flavour

The efficiency of E-SPIM1 produced after 24, 48 and 72 h (E-SPM1$_{24}$, E-SPM1$_{48}$ and E-SPM1$_{72}$, respectively) as main precursors of characteristic beef-like flavours was evaluated. The headspace volatiles generated from each hydrolysate were isolated by HS-SPME and subjected to GC-MS analysis (Table 4). The total content of the identified volatile compounds showed a gradual increase by increasing the time of hydrolysis. Most of the identified compounds were reported previously as Maillard reaction products derived from meat model systems containing cysteine with pentose or based on E-HVP with other flavour precursors [13, 29, 31].

Among the 58 volatile compounds reported in Table 4, 25 are sulfur containing compounds. 2-Methyl-3-furanthiol (compound **17**) was the major sulfur containing compound generated from E-SPM1$_{24}$, it showed a significant increase by increasing the time of hydrolysis. This compound is the most potent odourant of beef aroma [9]. Early, compound **17** had been proposed to be a degradation product of thiamine [50]. Addition of cysteine to E-HVP gave rise to generation of high yield of **17** [10, 13, 33]. Cerny [51] reported that thiamine and cysteine have similar part as precursors of 2-methyl-3-furanthiol, it was proposed to be formed in the reaction of hydrogen sulfide (degradation product of cysteine and thiamine) with 4-hydroxy-5-methyl-3(2H)furanone (a sugar degradation product) [52].

3-Mercapto-2-butanone (Compound **12**) was identified in volatiles of E-SPIM1$_{24}$ with relatively low concentration, however it showed a dramatic increase by increasing the time of hydrolysis. This compound possesses a meaty and cooked rice aroma [34] and is considered as one of the most contributor compounds to process flavours from several Maillard reaction model systems containing cysteine with different sugars [52]. Compound **12** is probably formed from the reaction of its corresponding alkanedione (2,3-butanedione, compound **2**), which is a breakdown product of sugar, with hydrogen sulfide (strecker product of cysteine) at low pH [52]. As shown in Table 4, the increase in concentration of compound **12** was associated with a decrease in the content of compound **2**. It was the major identified compound in the headspace volatiles generated from soybean nanoparticles with other flavour precursor of meat flavour [29] and was the abundant compound in the headspace volatiles of beef soup [53].

2-Furfurylthiol (**20**), 2-methyl-3-(methylthio)furan (**22**), 2-methyl-3-furfurylmethyldisulfide (**39**), 2-furfurylmethyldisulfide (**40**), 1-(2-methyl-3-furylthio)ethanthiol (**45**), 2-methyl-3-furylmethyltrisulfide (**46**), 2-furylmethyltrisulfide (**47**), 3[(2-methyl-3-furyl)dithio]-2-butanone (**48**), bis(2-methyl-3-furyl)disulfide (**49**), 3-[(2-methyl-3-furyl)dithio]-2-pentanone (**50**), 2-(2-furylmethyldithio)-2-butanone (**51**), 2-(2-furylmethyldithio)-3-pentanone (**52**), bis(2-furylmethyl)-disulfide (**53**), 2-methyl-3-[(2-methyl-3-thienyl)]dithiofuran (**54**) and bis(2-furylmethyl)-trisulfide (**57**) are the sulfur containing furan

compounds. Compounds **17** and **20** are important odourants in cooked beef, chicken broth as well as commerecial meat flavourings [54, 55]. Compound **20** was proposed to be formed by the reaction of hydrogen sulfide with furfural (a sugar degradation product) [56]. Compound **45** was found in the volatiles of model meat reaction mixture, xylose/ cysteine/ glycine at pH 7.0 [31] and considered as one of the potent odourant of meat.

Compounds **9**, **39**, **40**, **48**, **49**, **50**, **51**, **52**, **53**, **54**, **55** and **56** are the identified disulfide compounds, their very low thresholds impart a high potency in the odour profile flavourings [57]. Among them compounds **48** - **53** are dimmers of their corresponding thiols. Compound **49**, was found at high concentration in the volatiles of E-SPM1$_{72}$, it is a dimmer of compound **17** which easily oxidize to **49** [49]. Compounds **17** and **49** had been reported as most contributors of beef aroma [9], they have various characteristic aromas described as "sweet meat", "beef broth" to pungent and/ or "sulphurous" [30].

Among the twelve identified thiophenes (compounds **10**, **16**, **24**, **25**, **30**, **31**, **34**, **36**, **37**, **42**, **43** and **44**) the two thiophenethiols **31** and **34** were detected with total yield 0.20, 0.30 and 0.60 x $10^6$ in the E-SPM1$_{24}$, E-SPM1$_{48}$ and E-SPM1$_{72}$, respectively (Table 4). Furan and thiophene thiols have been reported as important volatiles in cooked meat [58] and model meat systems [51, 59, 60]. Compounds **10**, **24**, **25** and **30** were found in the volatiles of meat-like flavours generated from a model meat system based on enzymatic hydrolyzed mushroom protein [10]. Compounds **10** and **25** were identified among the volatiles of a meat model system containing 21 amino acids with ribose [61]. Compound **30** was thought to impart an undesirable aroma to the flavour generated from extruded hydrolyzed soybean protein [33].

Four thiazoles were detected in the present study (compounds **15**, **21**, **27** and **28**) with total concentration 0.66, 3.98 and 1.92 x $10^6$ in E-SPM1$_{24}$, E-SPM1$_{48}$ and E-SPM1$_{72}$, respectively (Table 4). Compounds **15** and **28** possess roasty popcorn note whereas compounds **21** and **27** possess meaty note [13, 31].

The total amount of the seven identified pyrazine compounds (compounds **13**, **26**, **29**, **32**, **33**, **35** and **38**) in headspace volatiles of E-SPM1$_{24}$, E-SPM1$_{48}$ and E-SPM1$_{72}$ were 1.38, 4.22 and 3.52 x $10^6$, respectively. This increase in total pyrazine content may be attributed to the increase in the total content of the basic amino acids (Table 4) by increasing time of enzymatic hydrolysis of SPI. The thermally generated pyrazines were thought to impart a beef-like aroma to the beef-like flavour produced from meat model mixtures based on E-HVP [10, 29, 33]. Ba *et al.* [60] correlated the low amount of pyrazines in the volatiles of a model mixture containing 21 amino acids / ribose to the presence of sulfur containing amino acids (such as cysteine and methionine) which are the precursors the sulfur containing volatile compounds.

### 3.7. Relation between the odour profile and composition of the beef-like aroma

Odour profile analysis was conducted to explore the effect of time of enzymatic hydrolysis on the efficiency of SPI hydrolysates as main precursors of beef flavour. The sensory attributes, beefy, roasty, savoury, sulphurous as well as the overall acceptability were selected and scored by panelists. Three replicates were applied to assess the results (Fig. 4). Sample E-SPM1$_{72}$ showed the highest scores of beefy, roasty, savoury and overall acceptability followed by E-SPM1$_{48}$ whereas E-SPM1$_{24}$ showed much less scores. Opposite trend was found for the sulphurous note. These finding confirm the fact that the results of GC-MS analysis should be positively correlated with the odour sensory characteristics of the meat-like flavours [9]. The thiol, sulfide or disulfides groups substituted furans at the 3- position have been reported to be the major contributors to the aroma profile of meat-like flavours [9]. In the present work these compounds (**17**, **22**, **39**, **45**, **46**, **48**, **49** and **50**) showed the highest content 21.14 x $10^6$ in sample E-SPM1$_{72}$ followed by E-SPM1$_{48}$ 10.18 x $10^6$ and E-SPM1$_{24}$ 4.12 x $10^6$ (Table 4).

The highest savoury note of E-SPM1$_{72}$ compared with E-SPM1$_{48}$ and E-SPM1$_{24}$ agreed with the total yield of the disulfide compounds in the volatiles of these samples (Table 4). These compounds possess savoury aromatic note and are considered dehydrogenation products among furanthiols, thiophenthiols and α-mercaptoketones [61, 62].

Sample E-SPM1$_{72}$ showed the highest roasty note compared with E-SPM1$_{48}$ and E-SPM1$_{24}$, this may be

**Table 4.** Composition of volatile compounds identified in headspace of beef-like flavour model mixture based on E-SPM1 hydrolyzed at different time (24, 48 and 72 h).

| Peak No. | KI[a] | Volatile Compounds[b] | Peak area[c] X $10^6$ | | | Identification methods[d] |
|---|---|---|---|---|---|---|
| | | | E-SPM1$_{24}$ | E-SPM1$_{48}$ | E-SPM1$_{72}$ | |
| 1 | 603 | 2-Methanthiol | 1.30 ± 0.07 | 2.78 ± 0.14 | 2.00 ± 0.10 | B |
| 2 | 612 | 2,3-Butanedione | 29.18 ± 1.47 | 18.68 ± 0.94 | 13.34 ± 0.67 | B |
| 3 | 647 | 2-Methylbutanal | 0.50 ± 0.03 | 0.16 ±0.01 | 0.16 ± 0.01 | A |
| 4 | 656 | 3-Methylbutanal | 0.92 ± 0.05 | ----- | 0.06 ± 0.00 | A |
| 5 | 685 | 2-Pentanone | 1.04 ± 0.05 | 0.02 ± 0.00 | ----- | A |
| 6 | 717 | 3-Pentanone | ----- | 1.02 ± 0.05 | 1.00 ± 0.05 | B |
| 7 | 725 | 2,3-Pentanedione | ----- | 0.06 ± 0.00 | ----- | B |
| 8 | 735 | 3-Hydroxy-2-butanone | 0.14 ± 0.01 | 0.02 ± 0.00 | 0.02 ± 0.00 | B |
| 9 | 769 | Dimethyl disulfide | 0.44 ± 0.02 | ----- | ----- | B |
| 10 | 780 | 2-Methylthiophene | 0.34 ± 0.02 | ----- | 0.12 ±0.01 | A |
| 11 | 801 | 4,5-Dihydro-2-methyl-3(2H) furanone | 0.20 ± 0.01 | 0.04 ± 0.00 | 0.02 ± 0.00 | A |
| 12 | 818 | 3-Mercapto-2-butanone | 0.86 ± 0.04 | 14.22 ± 0.72 | 21.34 1.08 | B |
| 13 | 829 | 2-Methylpyrazine | 0.16 ± 0.01 | 0.04 ± 0.00 | 0.02 ± 0.00 | A |
| 14 | 839 | 2-Furfural | 0.20 ± 0.01 | 0.03 ± 0.00 | ----- | A |
| 15 | 841 | 2-Methylthiazole | ----- | 0.02 ± 0.00 | ----- | B |
| 16 | 854 | 2-Ethylthiophene | 0.08 ± 0.00 | 0.02 ±0.00 | ----- | B |
| 17 | 871 | 2-Methyl-3-furanthiol | 2.44 ±0.12 | 5.20 ± 0.11 | 8.52 ± 0.43 | B |
| 18 | 897 | 3-Mercapto-2-pentanone | 0.34 ± 0.02 | 0.08 ± 0.00 | 0.14 ± 0.01 | B |
| 19 | 901 | 2-Acetylfuran | ----- | ----- | 0.14 ± 0.01 | B |
| 20 | 914 | 2-Furfurylthiol | 0.04 ± 0.00 | 0.12 ± 0.01 | 0.14 ± 0.01 | B |
| 21 | 938 | 4,5-Dimethylthiazole | ----- | ----- | 0.04 ± 0.00 | B |
| 22 | 945 | 2-Methyl-3-(methylthio)furan | 0.16 ± 0.01 | 0.26 ± 0.02 | 0.60 ± 0.03 | B |
| 23 | 955 | Dimethyltrisulfide | 1.48 ± 0.08 | 1.40 ± 0.07 | 1.54 ±0.08 | B |
| 24 | 981 | 4,5-Dihydro-2-methyl-2(2H) thiophene | 0.56 ± 0.03 | ----- | ----- | B |
| 25 | 995 | 2-Methyltetrahydrothiophene-3-one | 0.20 ± 0.01 | 0.04 ± 0.00 | ----- | B |
| 26 | 1001 | 2-Ethyl-3-methylpyrazine | 0.52 ± 0.03 | 2.12 ± 0.11 | 1.16 ± 0.06 | B |
| 27 | 1015 | 4-Ethyl-5-methylthiazole | 0.26 ± 0.02 | 3.56 ± 0.18 | 1.70 ± 0.09 | B |
| 28 | 1028 | 2-Acetythiazole | 0.40 ± 0.02 | 0.40 ± 0.02 | 0.18 ± 0.01 | B |
| 29 | 1032 | Trimethylpyrazine | 0.02 ± 0.00 | 0.24 ± 0.01 | 0.22 ± 0.01 | B |
| 30 | 1038 | 2-Methyl-3(2H)thiophene | 0.38 ± 0.02 | 0.40 ± 0.02 | 0.04 ± 0.00 | A |
| 31 | 1061 | 2-Methyl-3-thiophenethiol | 0.16 ± 0.01 | 0.24 ± 0.01 | 0.20 ± 0.01 | A |
| 32 | 1066 | 2-Ethyl-3,5-dimethylpyrazine | 0.16 ± 0.01 | 1.46 ± 0.07 | 1.66 ± 0.08 | B |
| 33 | 1083 | 2,3-Diethylpyrazine | 0.08 ± 0.00 | 0.06 ± 0.00 | 0.08 ± 0.00 | B |
| 34 | 1092 | 2-Thiophenemethanthiol | 0.04 ± 0.00 | 0.06 ± 0.00 | 0.40 ± 0.02 | B |
| 35 | 1106 | 2-Ethyl-3,6-dimethylpyrazine | 0.34 ± 0.02 | 0.30 ± 0.02 | 0.24 ± 0.01 | B |
| 36 | 1115 | 2-Formyl-5-methylthiophene | 0.34 ± 0.02 | 0.03 ± 0.00 | ----- | B |
| 37 | 1130 | 2-Formyl-3-methylthiophene | 0.12 ± 0.01 | 0.20 ± 0.01 | 0.16 ± 0.01 | B |
| 38 | 1157 | 2,3-Diethyl-5-methylpyrazine | 0.10 ± 0.01 | ----- | 0.14 ± 0.01 | B |
| 39 | 1182 | 2-Methyl-3-furfurylmethyldisulfide | 0.22 ± 0.01 | 0.24 ± 0.01 | 0.38 ± 0.02 | B |
| 40 | 1230 | 2-Furfurylmethyldisulfide | 0.04 ± 0.00 | 0.18 ± 0.01 | 0.18 ± 0.01 | B |
| 41 | 1252 | 3-Methyl-1,2,4-trithiane | ----- | 0.52 ± 0.03 | 0.24 ± 0.01 | B |
| 42 | 1260 | 2,5-Thiophenedicarboxyldehyde | 0.64 ± 0.03 | 0.08 ± 0.00 | ----- | B |
| 43 | 1268 | 2-Acetyl-2,5-dimethylthiophene | 0.20 ± 0.01 | 0.18 ± 0.01 | 0.14 ± 0.01 | B |
| 44 | 1307 | Ethyl benzothiophene | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.14 ± 0.01 | B |
| 45 | 1325 | 1-(2-Methyl-3-furylthio)ethanthiol | 0.34 ± 0.02 | 0.08 ± 0.00 | 0.06 ± 0.00 | B |
| 46 | 1388 | 2-Methyl-3-furylmethyltrisulfide | 0.04 ± 0.04 | ----- | 0.22 ± 0.01 | B |
| 47 | 1457 | 2-Furylmethyltrisulfide | ----- | 0.02 ± 0.00 | 0.02 ± 0.00 | B |
| 48 | 1518 | 3[(2-Methyl-3-furyl)dithio]-2-butanone | 0.14 ± 0.01 | 0.06 ± 0.00 | 0.06 ± 0.00 | B |
| 49 | 1547 | bis(2-Methyl-3-furyl)disulfide | 0.60 ± 0.03 | 4.22 ± 0.21 | 11.10 ± 0.56 | B |
| 50 | 1561 | 3-[(2-Methyl-3-furyl)dithio]-2-pentanone | 0.18 ± 0.02 | 0.12 ± 0.01 | 0.20 ± 0.01 | B |
| 51 | 1594 | 2-(2-Furylmethyldithio)-2-butanone | ----- | 0.12 ± 0.01 | 0.18 ± 0.01 | B |
| 52 | 1662 | 2-(2-Furylmethyldithio)-3-pentanone | ----- | 0.04 ±0.00 | 0.02 ± 0.00 | B |
| 53 | 1701 | bis(2-Furylmethyl)-disulfide | 0.04 ± 0.00 | 0.04 ±0.00 | 0.08 ± 0.00 | B |
| 54 | 1720 | 2-Methyl-3-[(2-methyl-3-thienyl)]dithiofuran | 0.04 ± 0.00 | 0.06 ± 0.00 | 0.08 ±0.00 | B |
| 55 | 1769 | (2-Methyl-3-thienyl)disulfide | 0.52 ± 0.03 | 0.30 ± 0.02 | 0.30 ± 0.02 | B |
| 56 | 1877 | bis(2 or 3-Thienyl)disulfide | 0.40 ± 0.02 | 1.20 ± 0.06 | 0.54 ± 0.03 | B |
| 57 | 1937 | bis(2-Furyl methyl)trisulfide | 0.04 ± 0.00 | 0.06 ± 0.00 | ----- | B |
| 58 | 1944 | 2-Thiophene methanol | ----- | ----- | 0.10 ± 0.01 | A |
| | | Total | 47.02 ± 2.37 | 60.87 ± 3.06 | 69.42 ± 3.50 | |

[a]Retention indices.
[b]Compounds listed according to their elution on DB5 column.
[c]Results were the means of three replicates of the peak total ion current TIC (peak area × $10^6$).
[d]Volatile compounds identification was performed as follows: A – mass spectrum and retention index were consistent with those of an authentic standard, B – mass spectrum was identical with that of NIST mass spectrum database, and retention index was consistent with that of the literature.
**E-SPM1$_{24}$:** Enzymatic hydrolyzed SPI hydrolyzed by protease PM1 at 24h.
**E-SPM1$_{48}$:** Enzymatic hydrolyzed SPI hydrolyzed by protease PM1 at 48h.
**E-SPM1$_{72}$:** Enzymatic hydrolyzed SPI hydrolyzed by protease PM1 at 72h.

correlated to the highest content (0.62 x $10^6$) of the compounds containing 2-furylmethyl group [58] in the headspace volatiles of this sample compared with the other samples, E-SPM1$_{48}$ (0.58 x $10^6$) and E-SPM1$_{24}$ (0.16 x $10^6$). In addition, the high yield of pyrazines in E-SPM1$_{48}$ and E-SPM1$_{72}$ are responsible for their high roasty note (Table 4 and Fig. 4).

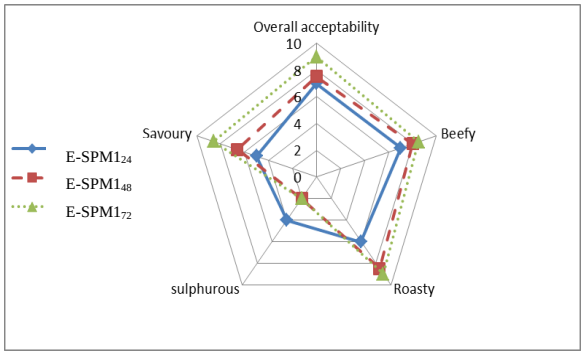


**Fig. 4.** Profile of beef –like aroma generated from model mixtures based on SPI hydrolyzed with protease produced by M1 strain during 24, 48 and 72 h hydrolysis time.

## 4. Conclusions

A novel protease from *Enterobacted* sp. NRCM1 (M1), newly isolated from mushroom, was purified and biochemically characterized. It showed high degree of hydrolysis and catalytic efficiency. The enzymatic SPI hydrolysate produced after 72 h (E-SPM1$_{72}$) by this enzyme showed the highest degree of hydrolysis and highest content of free amino acids compared with the hydrolysates obtained after 48 and 24 h (E-SPM1$_{48}$ and E-SPM1$_{24}$, respectively). Besides, the beef-like flavour generated from a model mixture based on E-SPM1$_{72}$ showed the best odour sensory characteristics and highest yield of the most potent odourant compounds of beef flavour, compared with those from the other investigated hydrolysates. A quit agreement was found between the results of odour profile and composition of headspace volatiles. The novel protease from *Enterobacted* sp. NRCM1 (M1), offers a new promising opportunities for the production of a high yield of enzymatic hydrolyzed vegetable proteins (E-HVP) which can be used as main precursors of inexpensive thermal process flavours, especially meat- like flavour.

## 5. Conflicts of interest

There are no conflicts to declare.

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