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Process optimization for the production of ghee residue protein hydrolysates

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ABSTRACT

The nutritional potential of ghee residue (GR) still remains untapped. Hydrolysis of proteins is known to decrease allergenicity, improve their functionality, and also induces bitterness. Present investigation was aimed to optimize the production process of GR proteins hydrolysates through two stage enzymatic hydrolysis of 5× ultrafiltered (UF) retentate (12.56% total solids and 8.77% protein). First stage hydrolysis was optimized at 42°C temperature, 8.0 pH using 4% papain with 5 h hydrolysis time (14.48 degree of hydrolysis, %DH). Increased hydrolysis time, significantly increased the DH % without any bitterness in the hydrolysate. Second stage hydrolysis was carried out using three food grade enzymes viz. trypsin (0.2%), alcalase 2.4L (0.25%) and flavourozyme 1000L (0.25%), by adjusting pH of 5× UF retentate to 8.0, 7.0 and 7.0 at 38, 50 and 50°C, respectively. Period of hydrolysis was optimized for each enzyme to 8 h with 26.99, 29.22 and 37.44 (%DH), respectively. Increase in hydrolysis time (optimized 13 h), significantly increased the %DH and bitterness in optimized hydrolysate. Further, period of hydrolysis, type of enzymes and their interaction, significantly effected % DH. Optimized hydrolysate obtained with papain (fist stage hydrolysis), trypsin (0.2%), and flavourozyme 1000L enzymes (second stage) was slightly bitter (3.68 on 4-point sensory scale). This optimized process can be adopted for commercial production of GR protein hydrolysates to meet the ever growing demand of easily digestible foods that will also be helpful to combat the problem of protein energy malnutrition.

Key words: Ghee residue, Protein hydrolysates, Proteolytic enzymes, Ultrafiltration

Protein energy malnutrition (PEM) is a major cause of concern for children in our country (National Health Profile 2007). Ghee is produced by several methods such as by desi method, creamery butter method (Rikhari *et al*. 2012), pre-stratification only, its clarification (Tyagi *et al*. 2006) and by mechanization (Dodeja and Agrawala 2005). Ghee residue (GR), brown colour solid mass is a by-product of ghee manufacturing and its annual production is one tenth of the total amount of ghee produced in India. After extraction of fat, it is either used in animal feeds or being thrown as waste material (Janghu *et al*. 2014). It was used in the production of some confectionary items like *Burfi*, *Chocolate* and *Candies*; but its wide use on commercial scale is still awaiting. However, it is a rich source of milk proteins, nitrogenous compounds and has potential to be utilized as natural flavour enhancer and anti-oxidant (Hazra and Parmar 2014). Thus, owing to its chemical constituents, ghee residue possesses sound nutritional potential and can be valorized to meet the demands of humans as dietary supplement. Due to unawareness about the nutritional potential of ghee residue proteins; their extraction, hydrolysis and subsequent utilization in human dietary foods has not been tried. However, Munirathnamma (2013), first time developed a process for the extraction of GR proteins with better recovery and protein purity employing membrane based, pressure driven Ultrafiltration (UF) process. The GR proteins were concentrated and retained in the retentate solution by this process.

Proteins play a significant role in both formulation and processing of food materials as these impart nutritional, functional and sensorial attributes to the food (Rhicha *et al*. 2007). Functionality of milk proteins was reported to improve by hydrolysis (acid, alkali and enzymatic hydrolysis) or with physical methods like pulse electric field (PEF), heat treatment, ultrasonication, high pressure processing (HPP) and radiation. Chemical hydrolysis of proteins with acids and alkalis results in the destruction of essential amino acids such as tryptophan, methionine, cystine, cysteine and also needs higher operational time. Thus, enzymatic hydrolysis of milk proteins is the better option that offers mild hydrolysis conditions and strict control over the degree of hydrolysis. Enzyme plays a crucial role in hydrolysis (convert proteins to peptides and amino acids) and is selected on the basis of protein source and end user requirements (Pasupuleti and Braun 2010). Different factors such as protein source, type, activity and concentration of enzymes as well as process conditions and degree of hydrolysis determine the chain length of resultant

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peptides (Nathalie *et al*. 2006). It has been established that enzymatic hydrolysis has edge over acid and alkaline hydrolysis.

Degree of hydrolysis (DH) is the extent of the hydrolysis of proteins and expressed in %. Important parameters considered during the optimization of hydrolysis reaction are pH, concentration of enzyme, temperature, and time of hydrolysis (He *et al*. 2005). As enzymes are highly specific in their action that limits the higher DH with single enzyme, higher DH during hydrolysates are obtained employing enzymes with broader specificity. The bitterness in the hydrolysates is also the results of enzymes specificity. Certainly, development of bitterness was considered as the main hurdle during production of protein hydrolysates, which results from the formation and accumulation of bitter tasting peptides with a high content of hydroprobhic amino acids (Fitz Gerald *et al.* 2006). Therefore, the combined use of exoproteases, microbial proteases or papain were emphasized (Richa *et al*. 2007). Further, Seo *et al*. (2008) reported that two stage hydrolysis of proteins with endopeptidase papain and exopeptidases flavourozyme 1000L and alcalase 2.4 Lup to 8 h hydrolysis could reduce the bitterness in the hydrolysates. However, the GR proteins hydrolysis and bitterness reduction (if any) has not been reported till date.

Therefore, present investigation was undertaken to produce ghee residue proteins hydrolysates from UF retentate solution and, optimization of enzymes (first stage-papain, second stage- trypsin, alcalase 2.4L and flavorozyme 1000L) to obviate bitterness from the resultant hydrolysates.

MATERIALS AND METHODS

Enzymatic hydrolysis of GR UF retentate: For enzymatic hydrolysis, GR proteins were extracted using the ultrafiltration process (UF 5× retentate) as described by Munirathnamma (2013). For experiments, ghee residue was procured from Experimental Dairy of ICAR-National Dairy Research Institute, Karnal, Haryana, India. The quantity of UF retentate required for enzymatic hydrolysis was decided on the basis of sample required for sensory analysis. Enzymatic hydrolysis of UF retentate was carried out in 250–500 ml beakers. For first stage hydrolysis, the pH of UF retentate (12% total solids) was adjusted to 8.0 and 300 ml solution was poured into 500 ml beakers and maintained at 42±1°C in a water bath. About 4% papain (on protein basis), was added to the UF retentate solution as earlier reported by Khanna and Gupta (1991). After every 1 h of hydrolysis, the pH of UF retentate was readjusted to 8.0 using 2.0 N NaOH and 2.0 N HCl. For determining the DH (%) and evaluating the sensory quality of the product; 20 ml of samples were drawn at interval of 1, 2, 3, 4 and 5 h.

For two-stage hydrolysis, pH of the first stage hydrolysates were readjusted to 8.0, 7.0 and 7.0 with 2.0 N NaOH and 2.0 N HCl; maintained at temperature 38±1°C, 50±1°C and 50±1°C and hydrolysis was carried out by the addition of 0.016, 0.25 and 0.25% (on protein basis) of trypsin, alcalase 2.4L and flavorozyme 1000L, respectively.

To determine the DH (%) and evaluate the sensory quality of the product, 20 ml of samples were drawn at varying interval of 1–8 h. The DH (%) was determined according to the method reported by Adler-Nissen (1986) and enzymes were inactivated carried out by heating hydrolysates in boiling water bath at 80°C. Thereafter, the liquid GR protein hydrolysate was further treated to remove insoluble matter as suggested by Khanna and Gupta (1991).

Enzymes and chemicals used: Papain and trypsin were procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, while alcalase 2.4L and flavourzyme 1000L were procured from Novozymes South Asia Pvt. Ltd., Bengaluru, Karnataka. All the chemicals and reagents used for analytical purpose in the investigation were of analytical reagents (AR) grade and were procured from SD Fine Chemical Pvt. Ltd., Mumbai.

Determination of pH: In the present investigation, pH meter (*PHAN LABINDIA* Labtek Engg. Pvt. Ltd., India) was used for the pH determination of GR solution and UF retentate. About 50 ml of sample was taken at 20°C for pH determination. Prior to pH measurement, the pH meter was calibrated with standard buffers of pH 4.0, 7.0 and 9.2.

Measurement of degree of hydrolysis (% DH) of proteins: DH was determined using the method of Adler-Nissen (1986).

Sensory evaluation of hydrolysates: The degree of bitterness in the GR proteins hydrolysates were evaluated by a trained panel of 8 experienced judges from Dairy Technology Division of ICAR-National Dairy Research Institute, Karnal, Haryana. Judges were asked to score the samples for sensory score grading on a 4 point scale as suggested by Khanna and Gupta (1991).

Statistical analysis: The data obtained during the study were statistically analysed for analysis of variance (ANOVA) using SPSS Software version 2.0. Mean±SE was also calculated, wherever required and critical difference (CD) values were also calculated.

RESULTS AND DISCUSSION

Optimization of first stage hydrolysis of GR protein by papain: For the production of any hydrolysed product, the selection of proteolytic enzyme is crucial because it relates their specific action on protein, which influences the composition of digested product. In this part of investigation, experiments were carried out under laboratory conditions and attempts were made to determine the optimum period of hydrolysis for both first and second stage of hydrolysis. Trials were conducted for enzymatic hydrolysis of ghee residue UF retentate containing 12.52% total solids (Table 1) using 4% papain in a set of beakers placed in temperature controlled water bath for a period of

Table 1. Chemical composition of 5× UF retentate solution*

Constituents	UF retentate (5×)
Total solids (%)	12.52±0.03
Fat (%)	3.30±0.02
Protein (%)	8.77±0.03
Lactose (%)	0.25±0.02
Ash (%)	0.20±0.01

* Mean ± SE (n=3).

7 h. During hydrolysis process, samples were drawn at every 1 h interval and subjected to DH determination. The % DH, thus obtained were statistically analysed. Increase in hydrolysis time had a significant (P<0.01) effect on DH when 4% papain was used for first stage of hydrolysis. It was observed that the time taken for hydrolysis of UF retentate beyond 5 h of hydrolysis, the rate of increase in DH declined substantially. After 5 h of hydrolysis, the DH with 4% papain was 14.48% which marginally increased to 14.91% after 7 hours of hydrolysis. Khanna and Gupta (1991) also reported 19.2% DH, with papain protease from papaya. On the basis of DH values, 5 h hydrolysis period was considered to be optimum. The use of 4% papain resulted in 14.48% DH value at this hydrolysis period. As the rate and degree of enzymatic hydrolysis vary from enzyme to enzyme, so the period of hydrolysis needs to be optimized separately for each enzyme. Keeping other factors constant, DH usually increases up to certain time period, followed by decline afterwards (Nathalie *et al*. 2006).

Effect of period of hydrolysis on sensory score of hydrolysates in first stage: To determine the effect of hydrolysis period and enzyme treatment on the bitterness of produced hydrolysate during first stage hydrolysis, samples were drawn at every one hour intervals and subjected to sensory evaluation. Statistical analysis of sensory data revealed that period of hydrolysis, judges and their interaction did not have any significant (P>0.05) effect on the sensory grading of first stage hydrolysate with 4% papain. Thus, with increase in hydrolysis time, DH increased, but sensory score remained constant. Decline in sensory score of hydrolysate sample was very steep during first hour, but afterwards that remained constant (Fig. 1). Hydrolysate obtained almost equal sensory scores in the range of 3.98–3.99 (i.e. did not have any bitterness), during its first stage hydrolysis with papain and 14.56% DH.

Optimization of second stage hydrolysis time with three food grade enzymes: Two-stage hydrolysis may be costly, but specificity of two different enzymes groups would definitely improve the DH. Thus, in this part of the study, optimization of four different enzymes viz. papain (broad specificity to hydrolyse proteins), trypsin (a serine protease), alcalase 2.4L (a proteolytic enzyme) (Chen *et al.*1993) and flavourozyme 1000L (fungal protease/peptidase) was done. For effective and economical production of protein hydrolysates, an attempt was made using papain in first stage followed by trypsin, alcalase 2.4L or flavourozyme 1000L enzymes for second stage of hydrolysis. The duration of first stage hydrolysis was fixed as 5 h (optimized previously) and second stage of hydrolysis was extended up to 10 h more. The hydrolysis was accomplished with papain enzyme in first stage and trypsin, alcalase 2.4L and flavourozyme 1000L for second stage to find their effect on DH and bitterness of the hydrolysate. This hydrolysis of GR UF retentate was performed using papain (4%) in first stage and trypsin (0.2%), alcalase 2.4L (0.25%) and flavourozyme 1000L (0.25%) in second stage, as suggested by Rhicha *et al*. (2007). The % DH values obtained for different enzymes used in second stage hydrolysis were 26.99, 29.22 and 37.44, respectively. Highest degree of hydrolysis (37.44%) was obtained with 0.25% concentration of flavourozyme 1000L, while minimum (26.99%) DH was exhibited by 0.2% trypsin. It was observed that with the increase in hydrolysis time, % DH also increased during second stage hydrolysis (Fig. 2). Further, it was also observed that period of hydrolysis, different enzymes as well as their interaction had significant (P<0.01) effect on the % DH. The degree of hydrolysis increased steeply up to first 8 h of hydrolysis but after that remained marginal. On the basis of above mentioned results and the economy, convenience and simplicity of the operational procedure, use of papain in first stage coupled with trypsin, alcalase 2.4L and flavourozyme 1000L in second stage of hydrolysis

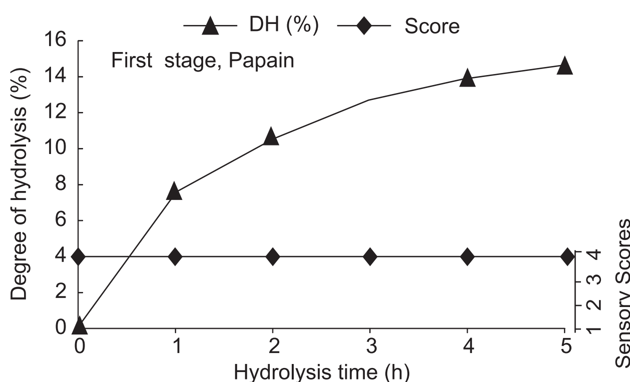

Fig. 1. Effect of period of hydrolysis on degree of hydrolysis and sensory score in first stage of hydrolysis.

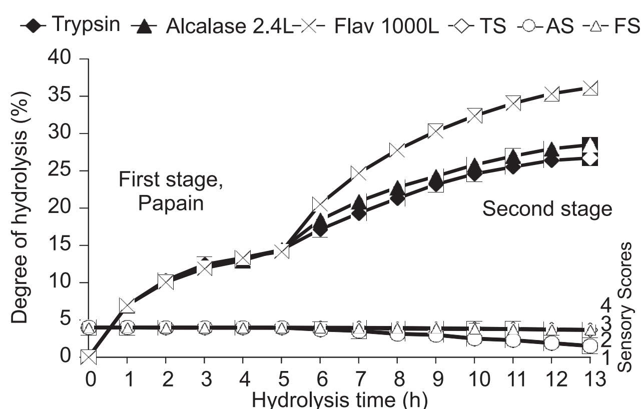

Fig. 2. Effect of different enzymes on degree of hydrolysis and sensory score in first and second stage.

with total 13 h (5 h for first stage and 8 h for second stage) of hydrolysis was selected as optimum to produce enzymatic GR protein hydrolysates.

Effect of period of second stage hydrolysis with food grade enzymes on sensory scores: The main hurdle in the development of protein hydrolysates is their bitterness as reported by FitzGerald *et al*. (2006) because of the formation and accumulation of bitter tasting peptides with a high content of hydroprobhic amino acids. Hence, experiments were conducted for 13 h hydrolysis period with two stage hydrolysis to check whether period of hydrolysis, type and concentration of enzymes has any effect on the reduction of bitterness or not. Effect of different enzymes on both DH and sensory score of the hydrolysate are presented in Fig. 2. During second stage, period of hydrolysis, different enzymes and judges along with their interactions had significant ($P<0.01$) effect on sensory score. The type of proteolytic enzymes used markedly affect the taste of the resulting product. Proteases with endopeptidase activities have been reported to give rise to bitter peptides. Proteins are noted for giving rise to an intensely bitter taste due to liberation of bitter tasting peptides when hydrolysed with endopeptidases (Adler-Nissen 1976). The bitterness was found to develop right from the initiation of hydrolysis process with alcalase 2.4L as the sensory score reduced to 1.51 at end of hydrolysis as against its initial value (3.98). The sensory scores for trypsin and flavourozyme 1000L hydrolysate was observed to decrease to 3.68 at the end of hydrolysis from their initial scores (3.98). It was observed that during second stage hydrolysis, the degree of hydrolysis increased steeply up to first 8 h (Fig. 2). Seo *et al*. (2008) also observed that extended period of hydrolysis for 8 h in two stages with endopeptidase papain and exopeptidases flavourozyme 1000L and alcalase 2.4L reduced the bitterness of the hydrolysates.

The 5× UF retentate containing 12.56% total solids and 8.77% protein content was subjected to enzymatic hydrolysis with different food grade enzymes. Single stage hydrolysis of GR UF retentate solution with 4% papain resulted in marked increase in % DH up to 5 h; beyond 5 h of hydrolysis, the rate of increase in DH declined substantially. Increase in hydrolysis time had a significant ($P<0.01$) effect on DH in first stage hydrolysis without any bitterness in sensory scores. Second stage hydrolysis with trypsin (0.2%), alcalase 2.4L (0.25%) and flavourozyme 1000L (0.25%) increased the % DH values to 26.99, 29.22 and 37.44, respectively after 13 h hydrolysis period. The hydrolysates produced with papain (first stage) and alcalase 2.4L enzymes (second stage) were bitter in taste. However, GR protein hydrolysates produced using papain (first stage) and either trypsin or flavourozyme 1000L enzyme (second stage) were free from bitterness and obtained 3.68 scores on 4-point sensory scale. Thus, based on sensory evaluation, economy, convenience and simplicity of the operational procedure; papain (first stage) and either trypsin or flavourozyme 1000L enzymes (second stage) successfully produced GR protein hydrolysates. These hydrolysates can be used as an active ingredient in the formulation of medicine, specialist beverages with high nitrogen content, pre-digested nutrition for enteral/parenteral and general/specific population segments, energy drink, sport drink, diet of aged people and weight controlling diets. Thus, this approach might be employed for the large scale production of protein hydrolysate from ghee residue to cater the ever growing demand of quality protein products will also help to reduce the problem of protein energy malnutrition.

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