International Conference on
"Novel Approaches in Agro-ecology, Forestry, Horticulture, Aquaculture,
Animal Biology and Food Sciences for Sustainable Community Development"
(Agro-tech-2018)

PROTEOLYTIC ACTIVITY OF MICROBIAL PROTEASES IN BUFFALO SODIUM CASEINATE AT 50^0C

Alka Parmar and Rajesh Kumar Bajaj

Dairy chemistry Division, National Dairy Research Institute,
Karnal, Haryana, India

Abstract—*Casein from buffalo milk shows high homology to that from cow milk, therefore, potential precursors for bioactive peptides with diversified functionality. In present study proteolytic activity, degree of hydrolysis, and protein content (NaOH soluble precipitate) of alcalase and flavourzymes hydrolyzed buffalo sodium caseinates with substrate concentration 50mg and 10mg (reaction mixture) at 50^0C were checked. Out of two concentrations, 50mg showed higher effects compaired to 10mg concentration. Further, Proteolytic activity of hydrolysates determined using OPA method (Serine equivalents) and Lowry method (Tryptone equivalents). For alcalase, proteolytic activity was 135.149±1.89 nkat/ml, 33.883±0.737 nkat/ml and for flavourzyme 87.264±1.89 nkat/ml, 22.226±0.737 nkat/ml using OPA and Lowry method respectively. Degree of hydrolysis was 13.255±0.169% for alcalase and 7.016 ±0.737% for flavourzyme after 15 min hydrolysis. Alcalase showed higher proteolytic activity compaired to flavourzyme in 15 min of assay time.*

Keywords*: O-phthaldihyde (OPA), Buffalo caseinate, Proteolytic activity, Degee of hydrolysis, alcalase (Alc), Flavourzyme (Flv).*

1. INTRODUCTION

According to the World Health Organization (WHO), cardiovascular diseases (CVDs) are the number one cause of death globally, 80% of which occurs in lower and middle income countries with an estimated 23.6 million people likely to die of CVDs, mainly heart disease and stroke, by 2030. Industrial food-grade proteinases such as Alcalase, Flavourzyme and Protamex derived from microorganisms, as well as enzymes from plant (e.g. papain) and animal sources (e.g., pepsin and trypsin) as shown in figure 1, have been widely used in producing bioactive peptides (Kristinsson & Rasco, 2000). Hydrolysis of proteins involves the cleavage of these peptide bonds, resulting in breakdown of proteins to peptides and amino acids. These products of protein hydrolysis, which are normally mixtures of peptides and amino acids, are called protein hydrolysates.

Table 1: List of different digestive and microbial enzymes (Qian *et al.*, 2007; Liu & Zhao., 2010)

Enzyme	Source	pH range	Optimum Temperature	Type of Protease	Specificity
DIGESTIVE ENZYME					
Pepsin	Bovine,porcine	1-4	37	Endo-peptidase	Mainly hydrophobic – COOH and NH2
Trypsin	Bovine,porcine	7-9	37	Endo-peptidase	Lys-,arg-COOH
Chymotrypsin	Bovine,porcine	8-9	37	Endo-peptidase	Phe-,tyr-,trp-COOH
MICROBIAL ENZYME					
Alcalase	*B.licheniformis*	6-10	50	Endo-peptidase	Mainly hydrophobic – COOH
Neutrase	*B.amyloliquefaciens*	6-8	50	Endo-peptidase	Leu-,phe-NH2
Flavourzyme	*A.oryzae*	6-8	50	Endo-peptidase & Exo-peptidase	Mainly hydrophobic – COOH

Enzymatic hydrolysis of milk proteins can modify both the techno-functional and bio-functional properties of the resultant hydrolysates. Exogenous and endogenous enzymes due to their specificity provide an attractive approach for generation of bioactive peptides from buffalo casein. The present paper mainly focus on the methods which suits bests for checking proteolytic activity of buffalo caseinate samples using microbial proteases (Parmar, *et al.,* 2015)

2. MATERIALS AND METHODS

Materials

Buffalo casein was prepared using fresh pooled buffalo (murrah) milk sample from cattle yard, National Dairy Research Institute, karnal, India. It was separated by isoelectric precipitation of buffalo casein, the precipitates were filtered through muslin cloth, wash the same thrice with distilled water and the wet casein was collected, and dispersed in distilled water and dissolved by adding 1N NaOH solution with thorough stirring till it reached Ph 7.0. The enzymes used were Flavourzyme 1000 L Alcalase 2.4 L (Novozymes) were gift samples from M/s. (OPA) o-Phthalaldehyde (s.d. Fine- chem. Ltd., Mumbai, India).β-mercaptoethanol (HIMEDIA).

Proteolytic activity of the enzyme

The proteolytic activity of proteases (alcalase and flavourzyme) was determined using two methods: 1) Hrckovam, (2002) with slight modification and 2) Stressler *et al.,* 2013. In method 1 and 2 sodium caseinate (10mg/ml, pH 7.5) as a substrate was used. In first method: freshly prepared substrate solution (1ml) was incubated at 50^0C for 5min then 0.2ml of enzyme solution (1:100 times diluted) and 0.8ml of phosphate buffer (pH 7.5) was added. Solution was incubated at 50^0C for 15 min, 3ml of 5% trichloroacetic acid was added to terminate reaction .In method 2 The substrate solution (5 ml) and 0.5 ml (1:100 times diluted) of enzyme solution 0.5 ml of 2M aqueous trichloroacetic acid (TCA) solution was added, The resulting precipitate and supernatent was removed by centrifugation (8000g per 10min). TCA soluble portion was used to estimate proteolytic activity by Lawry's technique expressed by tryptone equivalents and OPA technique expressed by serine equivalents. Proteolytic activity was expressed in nkat/ml. One katal of proteolytic activity was defined as the amount of enzyme required to release 1 mol of serine equivalents per second.

$$\text{Method-1: nkat/ml Enzyme} = \frac{(\mu\text{mole Serine equivalents released}) \times (V_1)}{(V_2) \times (T) \times (V_3) \times 60 \times 1000}$$

$$\text{Method-2: nkat/ml Enzyme} = \frac{(\mu\text{mole Tryptone equivalents released}) \times (V_1)}{(V_2) \times (T) \times (V_3) \times 60 \times 1000}$$

Where:

V_1 = Total volume (in milliliters) of assay
T = Time of assay (in minutes)
V_2 = Volume of Enzyme (in milliliters) of enzyme
V_3 = Volume (in milliliters) used in Colorimetric Determination

Degree of hydrolysis by o-Phthalaldehyde (OPA) method

Degree of hydrolysis for buffalo casein hydrolysates was determined using method given by Parmar *et al.,* (2015)**.** The OPA solution was made by combining the following reagents and diluting to a final volume of 50 ml with water: 25 ml of 100 mM sodium tetra borate; 2.5 ml of 20% (wt/wt) SDS; 40 mg of OPA (dissolved in 1 ml of alcohol); and 100 µl of β-mercaptoethanol. This reagent was prepared daily. To assay proteolysis with milk proteins as substrates, a small aliquot (usually134 µl) was added directly to 1.0 ml of OPA reagent in a 1 ml quartz cuvette, the solution was mixed briefly by inversion and incubated for 2 min at ambient temperature, and take the absorbance at 340 nm.

Serine $-NH_2$: $(A_{340Sample}-A_{340Blank}) / (A_{340Standard}-A_{340Blank})*0.951meq/l*D*/ P$

Where: D = Sample dilution & P =Protein content

$DH\% = h / h_{tot}*100\%$

Where $h= (\text{serine-}NH_2 - \beta)/\alpha$ meqv / g

α, β and h_{tot} constants are1.039,0.383 and 8.2 respectively

ISBN: 978-93-85822-77-3

Protein content by Lowry method

Trichloroacetic acid precipitates formed from 15 min proteolysis activity determination hydrolysis reaction, were further solublised in 2ml of 0.1N NaOH and used to determine protein content by Lowry *et al.*, 1951 in terms of bovine serum albumin equivalents.

Statistical analysis:

Experiments were performed in triplicates and the data expressed as mean value with standard error. Analysis of variance was performed using IBM SPSS Statistics software V21.0 to establish relationship between proteolytic activity, degree of hydrolysis and protein content as affected by concentration of substrate and type of enzyme using significant difference of 0.05. Means and standard errors were calculated, when *F*-values were significant ($P < 0.05$), mean differences were separated by the least significant difference procedure.

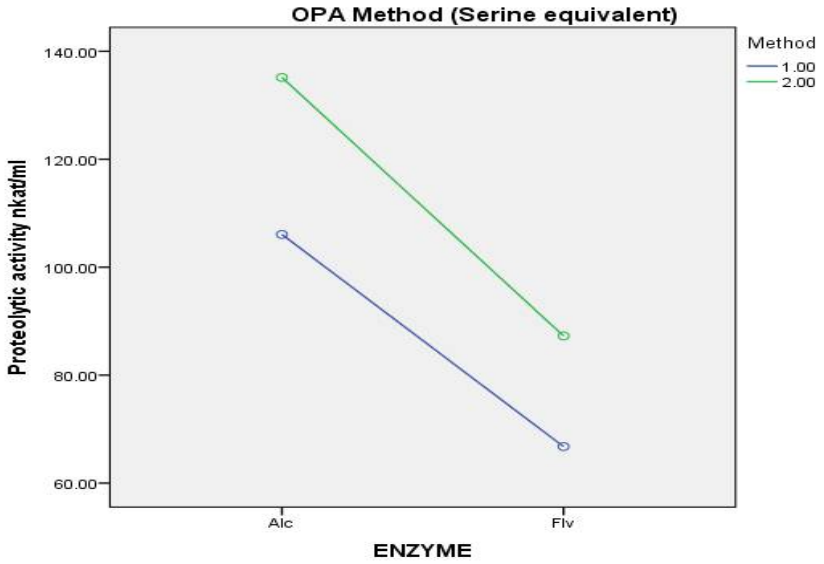
3. RESULTS AND DISCUSSION

Proteolytic activity of commercial proteases i.e., alcalase and flavourzyme was assessed using buffalo sodium caseinate (1% w/v protein) at pH 7.5 and incubating at 50^0C for 15 min. The free serine equivalents express the amount of released amino acids during hydrolysis, using OPA and tryptone equivalents express the amount of released amino acids Lowry method.

During enzyme hydrolysis, For alcalase, proteolytic activity was 135.149±1.89 nkat/ml, 33.883±0.737 nkat/ml and for flavourzyme 87.264±1.89 nkat/ml, 22.226±0.737 nkat/ml using OPA and Lowry method respectively**.** The effect was dependent on type of enzyme, processing temperature, hydrolysis time. The treatment of sodium caseinate with Alcalase showed the highest increase in free serine and tyrosine equivalents during 15 min of hydrolysis compared to flavourzyme as shown in figure 2. It can be seen in degree of hydrolysis and TCA precipitated protein content of both the samples using alcalase and flavourzyme. Degree of hydrolysis was also more for alcalase as compaired to flavourzyme after 15 min of hydrolysis. Protein content estimated after hydrolysis showed 7.775±0.189 mg/ml concentation for flavourzyme and 0.615±0.189 mg/ml concentration for alcalase after 15 min of hydrolysis which clearly indicates that alcalase hydrolysis was more as compared to flavourzyme. As expected from exopeptidase, alcalase alone showed high proteolytic activity within short interval of hydrolysis, on the other hand endopeptidase, flavourzyme alone showed low production of seine and tryptone equivalents. As flavourzyme plays the role of both exopeptidase as well as endopeptidase, but in present experiment we have seen the exopeptidase phase of flavourzyme after 15 min of hydrolysis and does not provide the time for endopeptidase phase to work. If we compare both the enzymes for their behavior as exopertidase on buffalo casein hydrolysates, alcalase effect was more significant, starts working fast as compared to flavourzyme. Combination of both the enzymes be a better approach for buffalo casein hydrolysates generation.

4. CONCLUSION

Proteolytic activity (PA) of the enzymes in both the methods is highly significant for alcalase as exopeptidase. Further research is needed for optimization of conditions for flavourzyme hydrolysis time to check its effectiveness as exopeptidase and endopeptidase.



ISBN: 978-93-85822-77-3

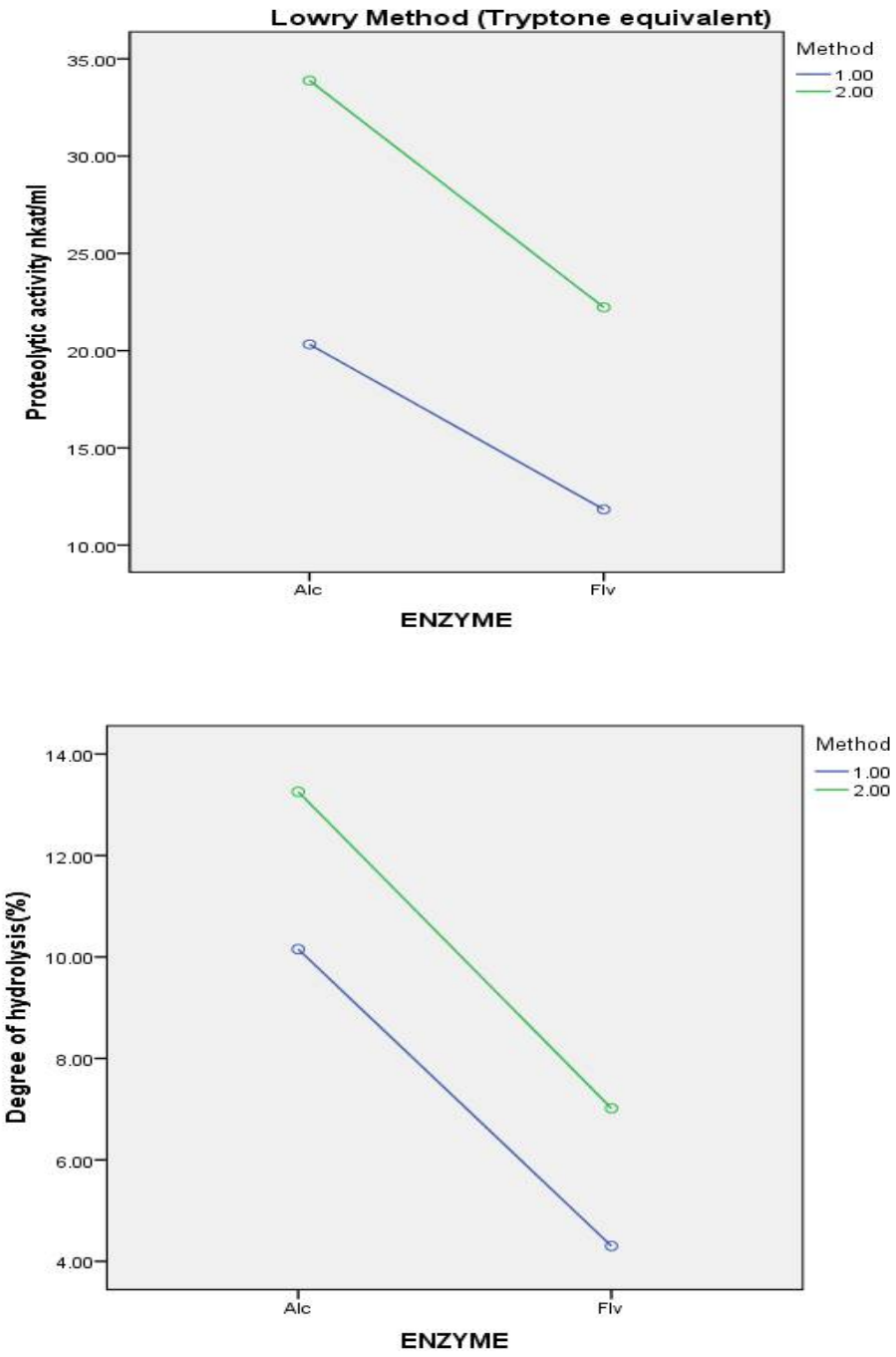


Fig. 2: Effect of alcalase and flavourzyme on Proteolytic activity (OPA Method & Lowry method) and Degree of hydrolysis of buffalo casein hydrolysates

ISBN: 978-93-85822-77-3

REFERENCES

[1] Kristinsson, H. G. and Rasco, B. A. (2000). Biochemical and functional properties of atlantic salmon *Salmo salar* muscle hydrolyzed with various alkaline proteases. *J. Agric. Food Chem*., 48: 657-666

[2] Liu, T. X., & Zhao, M. M. (2010). Thermal pretreatment and chemical modifications as a means to alter hydrolytic characteristics and prevent bitterness in hydrolysates of fishery by-catch (Decapterus maruadsi) protein. International Journal of Food Science & Technology, 45, 1852-1861.

[3] Lowry, O. H., Rosebrough, N.F., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., 193: 265-275.

[4] HrckovaM., Rusnakova M., Zemanovic J. (2000): Enzymatic hydrolysis of defatted soy flour by three different proteases and their effect on the functional properties of resulting protein hydrolysates. Czech J.Food Sci., 20: 7-14

[5] Parmar, A, Jaiswal, A, Rinku, Bajaj, R.K and Mann. B. (2015). Effect of single and sequential treatment of alcalase and flavourzyme on antioxidant activity of buffalo casein hydrolysates. Indian J. Dairy Sci. 68(6).

[6] Stressler, T., Eisele, T., Schlayer, M., Lutz-Wahl, S. and Fischer, L. (2013) Characterization of the recombinant exopeptidases PepX and PepN from *Lactobacillus helveticus* ATCC 12046 PLOS *ONE* .**8(7)**: e70055. doi:10.1371/journal.pone.0070055

[7] Qian, Z. J., Je, J. Y., & Kim, S. K. (2007). Antihypertensive effect of angiotensin I converting enzyme-inhibitory peptide from hydrolysates of bigeye tuna dark muscle, Thunnus obesus. Journal of Agricultural and Food Chemistry, 55, 8398–8403.

ISBN: 978-93-85822-77-3