

## Study of Bovine Whey Hydrolyzate to Enhance It's Antihypertensive and Antibacterial

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**Abstract:** Major aim of this work is to explore and enhance the nutraceutical properties of Bovine whey using enzymes to hydrolyze the whey proteins. There are established studies on the whey hydrolyzates obtained using digestive enzymes stating their nutraceutical benefits and thus the process of obtaining hydrolyzates and studying their benefits using plant and microbial enzymes, remained as a vast area to be explored. Hence the Fungal protease and Papain are utilized in the current study which involves preparation of hydrolyzates by two methods ie, by hydrolyzing the whey individual enzymes and by sequential addition of both at certain processing conditions (Double enzyme hydrolysis) and studying the hydrolyzates for antihypertensive and Antibacterial activity. Antihypertensive activity of the double enzyme hydrolyzate was having the lowest IC<sub>50</sub> of 0.24 mg/ml. Also the study on antibacterial property of whey hydrolyzate was relatively new and thus explored in this study. It is found that the Staphylococcus aureus was inhibited to 35.5% at a concentration of 20mg/ml using same hydrolyzate.

**Key words:** Bovine Whey, antihypertensive activity, antibacterial activity

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### INTRODUCTION

The whey has long been considered as a waste product in Cheese manufacturing Industry. However whey must be seen as by-product with valuable nutrients, whose manufacture or proper utilization has very high economic importance. Use of whey has the following advantages (Edgar, 2005), like complete and efficient utilization of the raw material milk, the manufacture of high-quality milk components for wide application in the food and pharmaceutical industry as well as for cattle feeding and the reduction of waste water load (BOD for whey is 30000-60000 mg oxygen per litre of waste water). The most valued component of whey is protein and their peptides which are highly regarded for their nutritional properties like  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin. As defined by Stephen Defelice, "A nutraceutical can be defined as any substance that may be considered a food or a part of a food and provides medical or health benefits including prevention and treatment of disease".

#### *Antihypertensive and Antibacterial activity of Whey:*

The antihypertensive effect of several peptides has been related to the inhibition of the angiotensin converting enzyme (ACE). ACE activity results in blood pressure increase via conversion of angiotensin I to angiotensin II, which is a vasoconstrictive peptide, and via degradation of bradykinin, which is a vasodilative peptide. Inhibition of ACE, e.g. by peptides, results in blood pressure decrease (Misel, 1996. Clare, 2000). Regulatory peptides can be released by enzymatic proteolysis of food proteins and may act as potential physiological modulators of metabolism during the intestinal digestion of the diet. The major whey protein components  $\alpha$ -Lactalbumin and  $\beta$ -Lactoglobulin contains bioactive sequences obtained by proteolysis of different digestive enzymes having ACE-I-converting enzyme inhibitory activity (Leppala, A.P. 2001). The unhydrolysed substrates have given very low ACE inhibitory indices, i.e. less than 10%. Hydrolysis of the whey proteins by pepsin, trypsin, chymotrypsin and the commercially available enzyme preparations, Corolase PP and PTN 3.OS, has resulted in high ACE inhibition indices, i.e. 73-90%. Hydrolyzates generated with elastase displayed relatively low ACE inhibitory activity. Also the order of trypsin and pepsin addition during

the hydrolysis of  $\alpha$ -Lactalbumin and  $\beta$ -Lactoglobulin has not appeared to affect the ACE inhibitory activity of the resulting hydrolyzate. The ACE IC<sub>50</sub> inhibition values obtained for ultrafiltered tryptic digests of  $\beta$ -Lg and WPC ranges between 13-201 mg / L (Mullally, M.M., 1997). Antibacterial activity is due to Lactoferrin (LF). LF is a bilobate iron-binding and therefore may be purified from whey glycoprotein and exists in most biological fluids of mammals, such as saliva, tears and mucous secretions, as well as in milk (Jenness, 1982). This protein is active against a wide range of pathogenic bacteria (Batish, v.k., 1988; Todhunter, D.A., 1990; Dionysius, D.A., 1993) and it is thought to contribute to the protection of infants from infectious disease. During milk processing (e.g. cheese making), most of the LF is retained in the cheese whey fraction, i.e., about 30–100 mg/ L (Jenness, R., 1982; Reiter, B., 1985; Mitchell, I.R., 1993). Bioactivities of several milk proteins including whey proteins are either inactive or incomplete in the native protein and the nutraceutical properties are enhanced by the proteolytic digestion of protein. Although plant as well as animal proteins have potential bioactive sequences, whey proteins are becoming the main source as they are abundant with whey derived hydrolyzates. There are several studies on whey hydrolyzates obtained by proteolysis of digestive enzymes and their bioactives, and however the study on hydrolyzates obtained using microbial and plant based enzymes are yet to be established. Thus there exists a vast area that had to be explored for the antihypertensive and antibacterial capacity of whey hydrolyzates obtained using fungal protease and Papain either singly or through sequential addition to enhance their action. The findings of the study can be exploited to develop functional foods with special health claims using the Whey which is a by product of cheese manufacturing industry.

## MATERIALS AND METHODS

### ***Manufacture of Spray Dried Bovine Whey:***

The raw milk is taken and acid precipitated at pH 4.6 using 6M citric acid and left for 3h. Then it is filtered and whey and casein are separated using muslin cloth. The centrifugation is done at 12500 rpm to remove casein particles and the centrifuged whey at 13°B is concentrated to 21°B using concentrator and spray dried at a flow rate of 70 ml/min, inlet temperature of 140°C and outlet temperature of 95°C to get spray dried bovine whey.

### ***Protein estimation:***

Protein is estimated according to micro Kjeldahl method described in AOAC 2000.

Digestion mixture: 100g of K<sub>2</sub>SO<sub>4</sub>, 20g of CuSO<sub>4</sub>.5H<sub>2</sub>O and 2.5g SeO<sub>2</sub> is weighed and mixed uniformly.

Mixed indicator: 0.1% Bromocresol green and 0.1% methyl red indicator in 95% alcohol are prepared separately. 10ml of Bromocresol green is mixed with 2ml of methyl red solution in a bottle provided with a dropper which will deliver about 0.05ml per four drops.

### ***Procedure:***

0.5g of sample is weighed into a Kjeldahl flask. 1-2 gram of the catalyst is added and digested with 20ml of concentrated H<sub>2</sub>SO<sub>4</sub> until all the organic matter is oxidized and uniform greenish – blue digest is obtained. The digest is cooled and made upto 50ml with distilled water. An aliquot of 5ml is taken for steam distillation in Gerhardt distillation unit with excess of 40% NaOH solution (10ml). The liberated ammonia is absorbed in 10ml of boric acid containing a few drops of mixed indicator. This is titrated against N/70 HCl. From the nitrogen content of the sample the protein content is calculated by multiplying by a factor of 6.38

### ***Hydrolysis of spray dried whey:***

#### ***Hydrolysis by Fungal protease:***

Materials : Enzyme Fungal protease from Amanopro, Japan The spray dried whey powder is mixed with water in the ratio of 1:10 (W/V) to obtain slurry. The slurry is mixed thoroughly using a magnetic stirrer for 30 minutes. The pH is adjusted to 7.6 using 1N NaOH and then kept in an incubator to reach 45°C, the fungal protease enzyme is added at E : S ratio of 1 : 100 where E, the enzyme and S, the protein content in sample and the sample is hydrolysed and samples of hydrolysis of 10ml were with drawn at 0, 5, 10, 20, 30, 40, 50 and 60min respectively and are inactivated by keeping in boiling water bath for 10min and are stored in refrigerator for studying the angiotensin converting enzyme inhibition activity.

#### ***Hydrolysis by Papain:***

Materials : Enzyme papain from Enzochem, India The spray dried whey powder is mixed with water in the ratio of 1:10 (W/V) to obtain slurry. The slurry is mixed thoroughly using a magnetic stirrer for 30

minutes. The pH is adjusted to 6.2 using 1N NaOH and then kept in an incubator to reach 55°C, the fungal protease enzyme is added at E : S ratio of 1 : 100 where E, the enzyme and S, the protein content in sample and the sample is hydrolysed and samples of hydrolysis of 10ml are with drawn at 0, 5, 10, 20, 30, 40, 50 and 60min respectively and are inactivated by keeping in boiling water bath for 10min and were stored in refrigerator for studying the angiotensin converting enzyme inhibition activity.

**Hydrolysis by double enzyme (sequential addition of fungal protease and Papain):**

The spray dried whey powder is mixed with water in the ratio of 1:10 (W/V) to obtain slurry. The slurry is mixed thoroughly using a magnetic stirrer for 30 minutes. The pH was adjusted to 7.6 using 1N NaOH and then kept in an incubator to reach 45°C, the fungal protease enzyme is added at E : S ratio of 1 : 100 where E, the enzyme and S, the protein content in sample and the sample is hydrolysed for 30 minutes at this optimum condition of fungal protease and inactivated by keeping in boiling water bath for 10min and then pH is again adjusted to 6.2 by adding 1N HCl and incubated at 55°C, the Papain enzyme is added at E : S ratio of 1 : 100 where E, the enzyme and S, the protein content in sample and the sample is hydrolyzed for 1 hour and inactivated by keeping in boiling water bath for 10min and stored in refrigerator for studying the anti bacterial and antihypertensive activity.

**Angiotensin Converting Enzyme Inhibition Activity:**

In this method (Blanea, H.I., 2003) the Angiotensin converting enzyme is inhibited by the compounds having antihypertensive activity. The standard is ACE inhibitor and the samples which showed antioxidant activity with lowest IC<sub>50</sub> value are used for finding out the antihypertensive activity, thus the samples hydrolysed by fungal protease for 10 min, and for papain 20 min and with double enzyme are used and thus the others were not of any importance.

**Materials:**

- Angiotensin converting enzyme – I, sigma chemicals, USA
- Angiotensin converting enzyme – I inhibitor, sigma chemicals, USA
- Hippuryl-His-Leu, sigma chemicals, USA
- Ethyl acetate

**Assay Method for Anti-hypertensive Activity:**

- HLL concentration: 5 to 40Mm, approximately 10mM.
- ACE: 6mU in 25ml corresponding to 40 mU/mL of the reaction medium.
- Buffers: potassium phosphate of 0.2M and pH 8.3.
- Incubation time: 80min

Hippuryl-His-Leu (HLL) is used as the substrate for Angiotensin converting enzyme – I (ACE). ACE catalyses the hydrolysis of the substrate to yield hippuric acid and His-Leu. The substrate of 110ml is dissolved in a pH 8.3 buffer with 0.3 M NaCl and 25ml of ACE dissolved in glycerol at 50% are added to 15ml of distilled water. In case of determining the sample's activity the 15ml of distilled water is replaced with the sample. For the reaction blank 1N HCl of 110ml is added before adding the enzyme. The reaction solution is incubated at 37°C for 80min. ACE activity is stopped by a decrease in pH by addition of 110ml of 1N HCl. The hippuric acid formed in the enzymatic process is extracted with 1ml of ethyl acetate, shaken and later centrifuged at 3000g for 10min.

750ml is taken out of the organic layer and dried at 95°C for 10min. The residue is redissolved in 1ml of distilled water. The absorbance measured at 228 nm.

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**Antibacterial activity:**

In this method (Negi, P.S. 1999) whey hydrolysed by double enzyme is taken for studying its antibacterial activity. The bacterial cultures taken are the *Staphylococcus aureus* and *Yersinia*. 1 loop full of test bacteria are taken from the slants kept at 4°C and inoculated in brain heart infusion agar broth of 9ml and incubated at 37°C for 24h. After 24h 1ml of culture broth is transferred to the 9ml of brain heart infusion broth and thus sub cultured. After 24h the culture broth is centrifuged at 5000g for 10min and the pellet is taken and

suspended in saline of 10ml, this will have 8 log micro organism growth. This is then serially diluted and the 10<sup>-3</sup> dilution is taken for further studies. The nutrient agar of 20ml is prepared in conical flask and autoclaved. Then to this 5ml of the sample is added and 100ml of the culture added and poured into petriplates and kept for incubation at 37°C for 24h and the colonies are counted. The inhibition % of the colonies are calculated by the formula,

$$\% \text{ Inhibition} = \frac{\text{colony forming unit in control} - \text{colony forming unit in sample}}{\text{colony forming unit in control}}$$

## RESULTS AND DISCUSSION

### **Estimation of Protein:**

The protein content of the samples are estimated by Kjeldahl and the results are tabulated in the Table1.

From the Table, 1 it is clear that at every step of processing of whey, the protein content has enhanced. The protein content of spray dried whey is 3 times the protein content of raw milk which is 3.2%. The protein content is estimated for the purpose of calculating the amount of the enzyme required to act on the substrate to prepare the hydrolyzate.

### **Angiotensin Converting Enzyme Inhibition of Bovine Whey Hydrolyzate:**

The antihypertensive activity is done using ACE inhibition technique using hippuryl histidine leucine as substrate and the samples are tested for the above. The sample which showed antioxidant activity with lowest IC<sub>50</sub> value are used for finding out the antihypertensive activity, thus the samples hydrolysed by fungal protease for 10 min, and for papain 20 min and with double enzyme are used. The following Tables 2, 3 and 4 shows the antihypertensive activity.

The graphs (Fig.1, Fig.2, Fig.3) are drawn for the Tables 2, 3 and 4 to find out the IC<sub>50</sub> concentration which inhibits the angiotensin converting enzyme activity.

From the figures 1, 2 and 3 the IC<sub>50</sub> value are obtained for the hydrolyzate of spray dried bovine whey using the enzyme Fungal protease at 10min is 0.3 mg/ml, Papain at 20 min of hydrolysis is 0.7 mg/ml and by double enzyme is 0.24 mg/ml.

**Table 1:** Protein content in different processing stages

Sl.No	Samples	Protein (%)
1	Whey at 13°B	0.70 ± 0.01
2	Whey at 21°B	1.39 ± 0.05
3	Spray dried whey	9.76 ± 0.28

**Table 2:** ACE Inhibition activity of whey hydrolysed by fungal protease for 10min

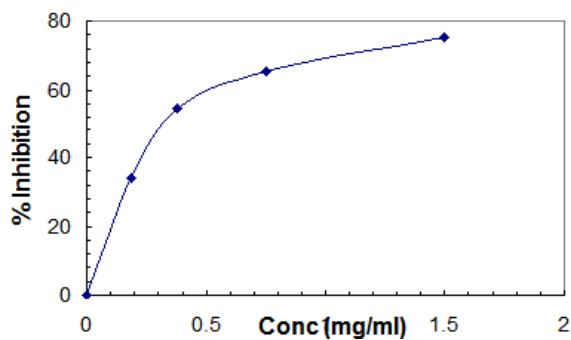
Conc (mg/ml)	% inhibition
0.1875	34.05
0.375	54.72
0.75	65.42
1.5	75.22

**Table 3:** ACE Inhibition activity of whey hydrolysed by papain for 20 min

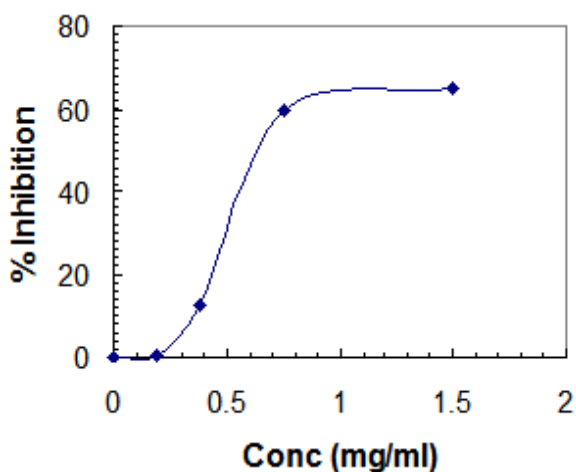
Conc (mg/ ml)	% inhibition
0.1875	0.5
0.375	12.3
0.75	59.89
1.5	64.88

**Table 4:** ACE Inhibition activity of whey hydrolysed by double enzyme

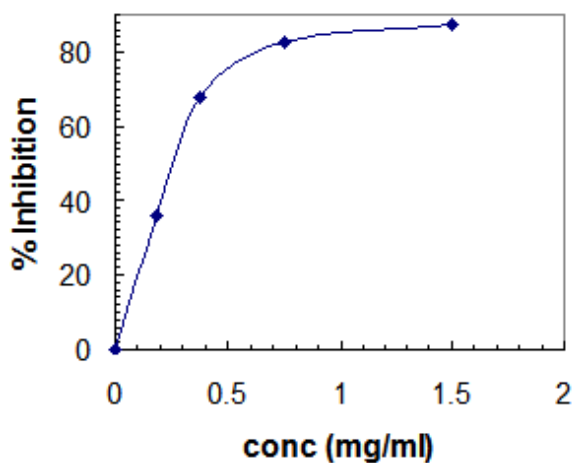
Conc (mg/ml)	% inhibition
0.1875	35.87
0.375	67.68
0.75	82.81
1.5	87.22



**Fig. 1:** ACE Inhibition activity of whey hydrolysed by fungal protease



**Fig. 2:** ACE Inhibition activity of whey hydrolysed by papain



**Fig. 3:** ACE Inhibition activity of whey hydrolysed by double enzyme

**Table 5:** The antibacterial activity of the whey hydrolyzate

Samples	Micro organism	Concentration mg/ml	% inhibition
Standard	<i>I. S. aureus</i>	0.003	50
(Chloramphenicol)	<i>2.Yersinia</i>	0.007	50
Fungal protease and Papain hydrolysed spray dried	<i>1.S. aureus</i>	20	35.5
bovine whey	<i>2.Yersinia</i>	20	No inhibition

**Antibacterial Activity of Bovine Whey Hydrolyzate:**

The IC<sub>50</sub> value of sample for antihypertensive activity is minimum for the double enzyme hydrolyzate and so this sample is selected for finding out the antibacterial activity. The cultures of *Staphylococcus aureus* and *Yersinia* of 10<sup>4</sup> numbers are grown in brain heart infusion agar together with the sample of known concentration and the controls is with distilled water instead sample and are compared. The inhibition in the number of micro organisms is calculated and results are in Table 5.

As the Table 5 shows, 35.5% of *Staphylococcus aureus* has been inhibited by sample of 20mg/ml concentration and it is high compared to the standard used, which shows 50 % inhibition at 0.003mg/ml concentration. There is no inhibition of *Yersinia* even at higher concentration (20mg/ml) of the sample but standard has shown 50% inhibition 0.007mg/ml.

**Antihypertensive activity:**

The whey hydrolysed with pepsin and trypsin has inhibition of 50% at 0.42 mg/ml (Leppala, A.P. 1998). The whey protein hydrolysed by a pancreatic mixture has given the IC<sub>50</sub> value for ACE inhibition at 0.16-0.84 mg/ml concentration (Ven. C.V.D 2002). Thus it is clear from the results in our present antihypertensive study, that the inhibition of ACE and is more when we use fungal protease for hydrolysis i.e., at 0.3 mg/ml than papain at 0.7 mg/ml. Also the inhibition of ACE is effective with double enzyme i.e., 0.24 mg/ml at lower concentration than both the papain and fungal protease hydrolysed sample.

**Antibacterial activity:**

It is clear from the current study that there is 35.5% inhibition of *Staphylococcus aureus* at 20 mg/ml concentration of double enzyme hydrolyzate while there is no inhibition of *Yersinia pestis*, which is a gram-negative bacteria. As a nutraceutical or as designer food the sample (Whey hydrolyzate made with double enzyme) can be eaten much more than this concentration of 20mg/ml and thus it will definitely have antibacterial activity to *Staphylococcus aureus*, a toxic pathogen which causes food poisoning. This antibacterial activity may be due to the lactoferrin, lactoperoxidase, lysozyme and immunoglobulins which are present in whey (Hutches, T.W. 1994; Viljoen, M. 1995). Also lysozyme is very active in inhibiting gram positive micro organisms and a few gram negative micro organisms and thus the *Staphylococcus aureus* which is been inhibited upto 35.5% which is also gram positive bacteria.

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