Effect of Hydrolysis on Allergenicity and Sensory Quality of Whey Protein Concentrate

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A B S T R A C T

The extent of allergenicity of whey protein was revealed by inhibition ELISA using blood serum obtained from milk allergic infants. The effect of in vitro proteolysis on allergenicity and development of bitterness was studied using enzymes individually and in combination. Selective proteolysis of WPC was done to 3 and 5 per cent DH. A combination of enzymes was effective in reducing allergenicity at a lower DH. Sensory evaluation of hydrolysates was done to give bitterness scores. A hydrolysate with minimum bitterness and maximum reduction in allergenicity could be used to develop an ingredient for hypoallergenic cow milk formula.

Introduction

Although breast feeding has absolute priority in the nutrition of the newborn, infant formulae will continue to be needed to supplement or substitute for human milk as and when condition demands.

The modification of cow’s milk for this purpose is accomplished by the addition of whey and whey proteins such as β-lactoglobulin, α-lactalbumin or others become frequent allergens (Jost et al., 1987).

The incidence of food induced allergic disease in children has been estimated to be between 0.3- 7.5 %. Immunologic mechanisms particularly IgE antibodies are responsible for the development of these food allergies in infancy and childhood (Harris et al., 1989; Robert and Zeiger, 1990). Cow’s milk allergy is a hypersensitivity reaction to bovine milk proteins caused by immunological reaction (Bahna, 1980).

Among the whey proteins β-lactoglobulin is considered as the most potent allergen because
it is absent in human milk and gives highest 
rate(60%) of positive oral challenges in 
children with milk allergy (Asselin et al.,1989). Baldo et al., (1984) observed 
that the specificity of IgE antibodies in sera from 
infants with milk intolerance revealed 
decreasing hypersensitivity reactions to the 
individual proteins which were in the order of 
β-lactoglobulin α-lactalburnin, caseins and 
bovine serum albumin.

The effects of enzymatic digestion of milk 
product were assessed by many researchers. 
Peptic and peptic-tryptic hydrolysis yielded 
breakdown products recognized by IgE 
antibodies by many researchers. (Haddad et al., 1989; Pahud et al., 
1985) indicated that trypsin hydrolyzed whey 
protein (4 h) remained inactive in inducing 
oral sensitization in guinea pigs. However, 
such an extensive hydrolysis could result in 
production of bitter peptides (Clegg, 1977), 
Jost et al., (1987) indicated that combining 
selective hydrolysis by specific proteases with 
proceeding of subsequent heat treatment was 
promising in developing a hypoallergenic 
infant formula. Lahl and Grindstaff (1989) 
noticed that with an optimal enzyme mixture, 
any source protein can be hydrolyzed or 
‘attacked’ to obtain the optimal nutritional 
profile attainable from the substrate.

Lahl and Braun (1994) did hydrolysis under 
controlled condition to account for taste, 
solubility and certain physical properties of 
the hydrolysates. Enzymatic protein 
hydrolysates are usually characterized by a 
bitterness associated with terminal 
hydrophobic amino acids attached to peptides 
liberated during hydrolysis. Because of 
bitterness leading to poor palatability and 
increased cost, extensively hydrolyzed whey 
protein formulae are not suitable for routine 
use in a large population of allergic infants. 
Many companies have produced 
hypoallergenic formulae (Willerns et al., 
1993) in which whey proteins were subjected 
for tryptic digestion with less amino acid 
degradation. Petrich et al., (1972) stated that 
the enzyme specificity has some influence on 
bitterness of hydrolysates. However, they 
concluded that this field needs further 
investigations.

Materials and Methods

Enzymatic hydrolysis was performed on 
commercial whey protein concentrate (70 % 
protein) obtained from Mahaan proteins, New 
Delhi. The following products were purchased 
for the experiment. Enzymes, viz., trypsin 
(1:250 S.D. Fine Chemicals, Ltd), 
chymotryosin (40-60 units/mg protein, 
HIMEDIA). Sera of milk allergic infants 
(positive serum) and healthy infants (negative 
serum) was collected from Bowring hospital, 
Bangalore. All immune reagents were 
obtained from Genei, Bangalore.

Enzymatic hydrolysis of whey protein 
concentrates

Hydrolysis was performed on 4 per cent (on 
protein basis) solution of whey protein 
concentrate at 40°C and pH 8.0 at an E: S ratio 
of 1:100 using enzymes individually and in 
combination. The hydrolysis was performed in 
triplicate for each reaction and time required 
to attain 3 and 5 per cent DH was noted in 
each case. Hydrolysis was arrested by heat 
treatment (80°C/15 min), cooled and were 
freeze dried.

Characterization of whey protein 
hydrolysates

The degree of hydrolysis (DH) is defined as 
the percentage of cleaved peptide bonds as 
assessed by tht pH stat technique described by 
Adler-Nissen (1986). In this method the 
volume of standard NaOH required to keep 
ph constant was directly converted to DH 
using the following formula
DH=B x Nₐ x 1/α x 1/MP x 1/htot x 100%

Where b= Base consumption in ml (NaOH); Nₐ = Normality of the base (0.1 N); α= average degree of dissociation of the α-NH₂ group; MP= Mass of protein in g; htot= total number of peptide bonds in the protein substrate (meq/g protein).

**Allergenic analysis of hydrolysates**

Briefly, whey protein coated plates were first incubated with a pool of serum from patients allergic to milk and also from healthy infants; Optical density (OD) reading was noted to establish the allergenicity of WPC. Plates were coated with different concentration of whey to determine the concentration capable on inhibiting the reaction. This concentration (1mg/ml) was used when hydrolyzed proteins were tested as inhibitors on subsequent experiments. The allergenity of various hydrolysates were analyzed by their ability to inhibit the binding of human serum specific IgE antibodies to whey protein coated and results were expressed as the percentage reduction in allergenicity/ percentage inhibition for each hydrolysates using the following formula

(Blank-control) - (Blank-Sp. Inhibition X 100)
Reduction in allergenicity (%) = ---------------------
(Balnk- Control)

**Sensory analysis**

2 per cent solution of WPC hydrolysates was prepared (Adler-Nissen, 1986) by hydrolyzing with 2 different enzymes and their combination were evaluated from bitterness on five points hedonic scale by five trained judges. A score from 1 to 5 was assigned to bitterness for each sample. The scores were 5 (no bitterness), 5-4 (slightly bitter), 4-3 (moderately bitter), 3-2 (strongly bitter), 2-1 (intensive bitterness).

**Results and Discussion**

The allergenicity of WPC was determined by measuring absorbance of sera for various dilutions and the optical density values are given in Table 1. The absorbance value is almost double when positive serum is used and this establishes the fact that whey proteins being allergenic forms antigen-antibody complex. The optimum antigen- antibody reactivity was observed at 1 mg/ml of protein concentration and at a serum of 1:50. The antigenicity may be due to sequential or surface epitopes (Speurgen et al., 1996). Conformational epitopes depend on the tertiary structure of β- lactoglobulin is known (Burova et al., 1998) and the allergenic sites are in peptide presents between 25-107 and 108-145 amino acid regions (Otani et al., 1985). The poor digestibility of whey protein is also considered to be one of the reasons for their allergenicity (Boza et al., 1995).

Time required to obtain hydrolysates with 3 and 5 per cent DH is given in Table 2. Chymotrypsin is known to cleave bonds formed by involving amino acids with hydrophobic side chains of phenylalanine, tyrosine and tryptophan (Fersht, 1997).

Trypsin had specificity of bonds associated with the hydrophobid side chains of the amino acids lysine and arginine. From the table it is evident that chymotrypsin is efficient in hydrolyzing WPC and as the concentration of chymotrypsin is more in the combination efficiency is also improved. Pelissier (1984) stated that because of its high specificity and expected hydrolysate composition, chymotrypsin was chosen for enzymatic hydrolysis of whey proteins.

Table 3 reveals the reaction in allergenicity of WPC on hydrolysis. At 5 per cent DH trypsin was found to reduce allergenicity to the maximum extend of 77.74%.
Table 1: The allergenicity of WPC as determined by measuring absorbance of sera for various dilutions

| WPC Concentration (mg/ml) | Sera          | Absorbance values (405 nm) | Serum dilutions |
|---------------------------|---------------|----------------------------|-----------------|
|                           |               |                            | 1:25 | 1:50 | 1:100 |
| 0.1                       | Positive      | 0.152                      | 0.245 | 0.265 |
|                           | Negative      | 0.342                      | 1.361 | 0.626 |
|                           | Difference in Absorbance | 1.181                  | 0.386 | 0.361 |
| 1.0                       | Positive      | 0.165                      | 0.257 | 0.247 |
|                           | Negative      | 0.803                      | 1.460 | 1.001 |
|                           | Difference in Absorbance | 0.638                  | 1.203 | 0.754 |

Table 2: Enzymatic hydrolysis of WPC using enzymes and their combination

| Enzyme/ Enzyme Combination | Time required (min) |
|----------------------------|---------------------|
|                            | 3 % DH  | 5 % DH  |
| Trypsin:Chymotrypsin       | 60      | 170     |
| Trypsin:Chymotrypsin Ratio | 10      | 23      |
| 75:25                      | 30      | 105     |
| 50:50                      | 13      | 45      |
| 25:75                      | 10      | 30      |

DH: Degree of Hydrolysis

Table 3: Influence of enzymatic hydrolysis on reduction in allergenicity

| Enzyme/ Enzyme combination | Absorbance values (405nm) | Reduction in Allergenicity (%) |
|----------------------------|---------------------------|-------------------------------|
|                            | Blank | Control | 3 % DH | 5 % DH | 3 % DH | 5 % DH |
| Trypsin                    | 0.722 | 0.340   | 0.469  | 0.637  | 33.76  | 77.74  |
| Chymotrypsin               | 0.439 | 0.497   | 0.412  | 0.461  | 25.90  | 41.10  |

Trypsin: chymotrypsin combination

|                       | 3 % DH | 5 % DH | 3 % DH | 5 % DH |
|-----------------------|--------|--------|--------|--------|
| 75:25                 | 0.23   | 11.5   |
| 50:50                 | 0.23   | 11.5   |
| 25:75                 | 0.23   | 11.5   |

DH: Degree of Hydrolysis
Table 4 Sensory evaluation of WPC hydrolysates prepared by using different enzymes and their combination

| Enzyme/ Enzyme Combination | Hedonic Scores | Relative bitterness |
|----------------------------|----------------|---------------------|
|                            | 3 % DH | 5 % DH | 3 % DH | 5 % DH |
| Trypsin                    | 4.50   | 3.97   | Slightly bitter | Moderate bitter |
| Chymotrypsin               | 1.70   | 1.11   | Intensely bitter | Intensely bitter |
| Trypsin: chymotrypsin      | 3.46   | 2.48   | Moderate bitter | Strongly bitter |

DH- Degree of Hydrolysis

Schymidt and Poll (1991) based on their findings reported that the major allergen β-lactoglobulin is a good trypsin substrate. Asselin *et al.*, (1989) stated that the allergenicity was reduced in hydrolysates compared to untreated proteins. Pahud *et al.*, (1985) concluded that the powerful allergens in whey proteins lost its sensitizing capacity when hydrolyzed with trypsin. Even though chymotrypsin is better with respect to rate of hydrolysis it is less efficient in reducing allergenicity. This may be attributed to the resistance of β-lactoglobulin to chymotryptic digestion as described by Reddy *et al.*, (1988). Similar results were obtained for Schmidt and pool (1991) who reported that α-lactalbumin was hydrolyzed easily and β-lactoglobulin more gradually by chymotrypsin. (At 3 per cent DH, combination was better with reduction in allergenicity of 63.6 per cent than the individual efficiency of enzymes. This is supported by Asselin *et al.*, (1989) who stated that the specificity of enzymes should be complementary to form an effective combination and reported that the combination of chymotrypsin and trypsin significantly reduced allergenicity.

Even though the rate of hydrolysis was more for chymotrypsin it is less efficient in reducing allergenicity. Based on their study Asselin *et al.*, (1989) also reported that degree of hydrolysis is not the factor affecting allergenicity and the susceptibility of peptide bonds involved in the allergenic sites β-lactoglobulin and α-lactalbumin depend on the specific activates of the enzyme. From the amino acid sequence of β- lactoglobulin it can be seen that the active site of the enzyme chymotrypsin in the region 25-107(antigenic site) is much lesser than that of trypsin.

The results of sensory evaluation of hydrolysates for bitterness and hence acceptability is given in Table 4.

Sensory evaluation studies proved that threshold for bitter taste decreased with higher degree of hydrolysis. The potential level of bitterness was different for hydrolysates of different enzymes (Petrichek *et al.*, 1972) and so can be related to the specificity of the enzyme and also depends on the degree of hydrolysis. This is supported by the findings of Ennis and Harper (1986) and other coworkers (Fox *et al.*, 1982; Cowan, 1983; Kinsella 1982) who suggested that amino acid residues having hydrophobic side chains are implicated as determinants of better taste. The hydrolysates of chymotrypsin are intensely bitter whereas in some cases i.e., 5 per cent hydolysate of trypsin exhibited moderate degree of bitterness.

The requirement demanded in the formation of hydrolysates is that they should not have a bitter taste, should be hypoallergenic, should be low in free amino acids (Ney, 1979; Grimble *et al.*, 1986; Otani *et al.*, 1990). It is possible to reduce allergenicity by extensive hydrolysis but it imparts bitterness. However
it is suggested that the moderate bitterness encountered in a partial hydrolysate can be masked if it is used in food formulation processes.

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How to cite this article:

Smitha J. Lukose, Narasimha Murthy and Anjum. 2018. Effect of Hydrolysis on Allergenicity and Sensory Quality of Whey Protein Concentrate. Int.J.Curr.Microbiol.App.Sci. 7(05): 2899-2905. doi: https://doi.org/10.20546/ijcmas.2018.705.338