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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 lgE 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 (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) stated 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. Petrichek *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 40^0C 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 (80^0C/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

$DH=B \times N_b \times 1/\alpha \times 1/MP \times 1/ htot \times 100\%$

Where b= Base consumption in ml (NaOH); N_b = Normality of the base (0.1 N); α=verage degree of dissociation of the α-NH_2 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 allerginity of various hydroysates 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

$$\text{Reduction in allergenicity (\%)} = \frac{\text{(Blank-control) - (Blank-Sp. Inhibition X 100)}}{\text{(Balnk- Control)}}$$

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 (Speurgin *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 chymotrysin is efficient in hydrolyzing WPC and as the concentration of chrymotrypsin 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 allergnicity 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	60	170
Chymotrypsin	10	23
Trypsin : Chymotrypsin Ratio		
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	25.90	41.10
Trypsin: chymotrypsin combination						
75:25	0.844	0.411	0.412	0.461	0.23	11.5
50:50	1.085	0.370	0.459	0.635	12.5	37.0
25:75	0.735	0.342	0.592	0.608	63.6	67.8

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 allerghenicity. 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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