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Nutritional characterisation of hydrolysed and unhydrolysed whey protein -iron complex#

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Abstract

The present investigation aimed at the development of a whey protein concentrate-iron (WPC-Fe) complex using hydrolysed and unhydrolysed whey protein as supplements in iron-fortified foods. Differential hydrolysis of whey protein was achieved using trypsin and a combination of trypsin and chymotrypsin in 3:1 ratio. WPC-Fe complexes with added vitamin C were also prepared to study the effect of bioavailability enhancers. Seven different types of freeze-dried whey protein-iron complexes were prepared, such as WPC-Fe, trypsin hydrolysed WPC -iron, WPC -iron complex from WPC hydrolysed by a combination of enzymes at two different degrees of hydrolysis, and all hydrolysate iron complexes with added ascorbic acid. All the treatments were nutritionally characterised for their total protein, soluble protein, total solids, total ash and total iron using optimised analytical procedures. In general, the protein solubility of pure hydrolysates was higher than complexed hydrolysates by 20-40 per cent. However, for the unhydrolysed protein-iron complex, protein solubility was significantly lower. It was found that the protein-iron complex prepared using trypsin hydrolysate at five per cent degree of hydrolysis (DH) with added ascorbic acid was the best with a protein solubility of 76.8 per cent followed by the complex prepared using hydrolysate of enzyme combination at five per cent degree of hydrolysis (DH) with added ascorbic acid. Thus, the study enlightens the possibility of developing an ideal vehicle for carrying iron as a fortificant in the presence of hydrolysed protein and ascorbic acid.

Keywords: *Hydrolysates, fortification, whey protein concentrate, degree of hydrolysis*

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Iron is an essential mineral that controls vital biochemical processes in the body by functioning as an activator, gene regulator, and transmitter, modulating cell growth and differentiation. It is an essential nutrient for maintaining health and is required for oxygen storage and transport, energy transduction, and enzyme activities (Waldvogel *et al*., 2014; Sun *et al*., 2020). The most common type of micronutrient deficiency identified worldwide is iron deficiency anaemia (IDA), affecting 30 per cent of the global population (Wu *et al*., 2019). Iron deficiency anaemia poses women and infants at greater risk during the prenatal period, reduces the physical work capacity of manual workers, and delays the mental and physical development of children (Ren *et al*., 2011; Chang *et al*., 2013). Along with iron deficiency, cases of infection and inflammation further lead to anaemia. The onset of iron deficiency anaemia is one of the many consequences of iron deficiency and the prevalence of anaemia is higher in low- and middle-income countries (Safiri *et al*., 2021).

Iron fortification in food is an appropriate method of supplying this element to our body at the required levels. Even in developed countries, national fortification programs based on food fortification have an impressive history of public health success (Hurrell, 2002). The incidence of IDA can be reduced only by improving iron intake through foods frequently consumed in the regular diet of people and is an appropriate public health strategy as agreed by all researchers worldwide. Direct addition of iron in metal form will react with the food matrix and the environment to produce off-flavours, unacceptable colours, and metallic taste. Metallic or free iron can also cause an upset stomach, constipation, nausea, abdominal pain, vomiting, and diarrhoea (Jackson and Lee, 1991). Gera *et al*. (2012) reported that with iron-fortified foods, there were no such adverse effects as in the case of oral iron supplements. The body completely absorbs organic or bound iron, but inorganic iron absorption depends on the form of iron. From a nutritional point of view, iron and total iron are both significant in fortified foods, as the different forms of iron markedly differ in their absorption and bioavailability (Niedzielski *et al*., 2014). Several dietary factors also influence the absorption of iron, viz., inhibitors and enhancers. Ascorbate and citrate increase iron uptake in the duodenum by solubilising them through mild chelating action

The use of a suitable protein in the diet can increase the absorption of dietary iron as it is reported that amino acids in the intestine increase the rate of iron absorption (Nakano *et al*., 2007; Moustaraf and Daley, 2022). Beyond normal and adequate nutrition provided by proteins from both animal and plant sources in humans, many peptides released from them during digestion have regulatory and bioactive functions. The peptides from hydrolysates of milk (both caseinophosphopeptides and peptides from whey), sunflower protein, and blood plasma protein of pig and fish protein are found to possess mineral binding activity (Ren *et al*., 2011). Among milk proteins, whey proteins are considered superior due to their higher levels of essential amino acids (EAA) and functional properties. It is reported that whey protein hydrogels released most of their iron during the intestinal phase of simulated digestion and hence whey protein hydrogels are superior in iron absorption in the Caco-2 system (Nakano *et al*., 2007).

The choice of iron fortification vehicle for national programs until now is based on the consumption pattern of the general population and, to some extent, the degree of industrialization of the respective vehicle selected. Hurrell (2018) reported that dried milk powder has been the preferred food vehicle to provide additional iron to infants and children. Hence whey protein concentrate and its hydrolysate were selected as the most suitable medium for fortification in this study.

In the present study, iron-fortified whey protein concentrate (WPC) and iron-fortified whey protein concentrate hydrolysates (WPCH) were prepared and examined for their physicochemical properties, iron content, protein solubility and effect of ascorbic acid on nutritional quality.

Materials and methods

Whey Protein Concentrate (WPC 80) was procured from Mahaan Proteins, New

Delhi. Chymotrypsin (350 units/mg protein), and Ferrous sulphate ($FeSO_4$. $7H_2O$) were procured from Sigma Aldrich, St. Louis, Mo., USA. Trypsin (1:250), analytical grade sodium hydroxide, boric acid, sulphuric acid and L-Ascorbic acid, were procured from Sisco Research Laboratories, Mumbai. Deionised water was used to avoid iron contamination. All hydrolysates and fortified samples were dried in a freeze dryer (OPERON -70 °C, Korea) and stored under refrigeration. A water bath Shaker (ROTEK, Ernakulam, Kerala) was used for sample incubation after the incorporation of an iron source. Acid-washed glassware was used throughout the experiment.

Enzymatic hydrolysis of whey protein concentrate and complexing with iron

Enzymatic hydrolysis of four per cent WPC solution was performed at 40 °C and pH 8.0 at an Enzyme: Substrate (E:S) ratio of 1:25 using trypsin and a combination of trypsin and chymotrypsin (T: C) in a 3:1 ratio. The hydrolysis was performed in triplicate for each reaction to attain three and five per cent hydrolysis (Lukose *et al*., 2018). The desired degree of hydrolysis (DH)was attained and confirmed by pH stat technique, further reaction was arrested by heating at 85 °C for 15 minutes, followed by rapid cooling to 40 °C, and then pH was reduced to 7.0. Ferrous sulphate was added as an iron source at a ratio of 40:1 (protein: iron) and was incubated in a shaking water bath at 40 °C for two hours followed by freeze-drying. Whey protein concentrate hydrolysate-iron (WPCH-Fe) complexes in the presence of ascorbic acid was prepared by adding ascorbic acid along with iron to the hydrolysed protein at a ratio of 1:0.5 (iron: ascorbic acid) (Venkatasubramanian *et al*., 2014).

Analysis of fortified whey protein concentrate - iron complexes

Seven different treatments of fortified whey protein concentrate-iron complexes (T1-T7) and a control sample (T0) were analysed for physicochemical properties, protein solubility and total iron. For protein solubility studies, the solubility of all fortified treatments was compared with that of pure protein hydrolysates H1, H2 and H3. Table 1 shows the treatment and the content of each treatment.

Physico-chemical properties of Whey protein concentrate -iron complexes

The total solids, moisture content and ash content in the fortified supplements were

Table 1. Treatments and composition

Sl. No.	Treatment	Composition
1	T0	Whey protein concentrate
2	T1	Whey protein concentrate complexed with iron
3	T2	Whey protein concentrate hydrolysed with trypsin to 5% DH and complexed with iron
4	T3	Whey protein concentrate hydrolysed with a combination of T: C (3:1) to 3% DH and complexed with iron
5	T4	Whey protein concentrate hydrolysed with a combination of T: C (3:1) to 5% DH and complexed with iron
6	T5	Whey protein concentrate hydrolysed with trypsin to 5% DH and complexed with iron and ascorbic acid
7	T6	Whey protein concentrate hydrolysed with a combination of T:C (3:1) 3% DH and complexed with iron and ascorbic acid
8	T7	Whey protein concentrate hydrolysed with a combination of T: C (3:1) 5% DH and complexed with iron and ascorbic acid
9	H1	Whey protein concentrate hydrolysed with trypsin to 5% DH
10	H2	Whey protein concentrate hydrolysed with a combination of T: C (3:1) to 3% DH
11	H3	Whey protein concentrate hydrolysed with a combination of T: C (3:1) 5% DH

determined by the analytical method given by the Association of Official Agricultural Chemists (AOAC, 2016).

The crude protein in the fortified supplements was determined by the Micro Kjeldahl method (AOAC, 2016). The protein content of supernatants prepared from fortified supplements was also determined for protein solubility studies.

The protein solubility of fortified hydrolysates and unfortified hydrolysates was evaluated as per the procedure of Alahmad *et al. (*2022) with slight changes. Sample solutions were prepared by dissolving 100 mg of freeze-dried protein-iron complex in 10 mL of deionised water. Solutions were incubated at 30^{0}C with continuous stirring for 30 min at 150 rpm. After incubation, the samples were centrifuged at 8000g for 20 min. Protein content in the supernatant and freeze-dried samples was determined using the Kjeldahl protein estimation process. Solubility was calculated in percentage from the following equation

Solubility % = (supernatant protein/ total protein in freeze-dried sample) x 100

The total ash content in the hydrolysates and fortified supplements was determined by the method described by AOAC (2016). A 10 per cent solution of samples was analysed for the pH using a pH meter.

The total iron was determined by the atomic absorption spectroscopy**.** The method suggested by Niedzielski *et al*. (2014) was followed with some modifications for the preparation of the sample for analysis. The freeze-dried sample was subjected to extraction with 2 M HCl by diluting one gram in 20 mL and was kept at 37^{0}C under cover for one hour. After filtration using ashless Whatman No. 42 filter paper earlier rinsed with deionised water, the filtrate was made up to 20 mL and diluted to 200 mL before analysis. For the determination of total iron by AAS, five mL of diluted sample was subjected to digestion. The total iron content in the fortified treatments was analyzed by atomic absorption spectrometry with air-acetylene flame atomisation (Perkin Elmer, USA).

Statistical analysis

Statistical analysis was done using SPSS version 24. Comparison between more than two groups was done by using one-way ANOVA followed by the Duncan multiple range test (DMRT) to find out which of the treatment groups are homogenous. The comparison between the two groups was done using an independent t-test.

Results and discussion

For most protein hydrolysates, better functional properties were exhibited at a lower degree of hydrolysis of three to five per cent

Table 2. Protein solubility of hydrolysed and unhydrolysed samples and their iron complexes

Treatment	Protein (%)	Protein in supernatant (%)	Protein solubility (%)
T0	78.15 ± 0.015^{a}	70.26 ± 0.004^{c}	89.99
T1	73.42 ± 0.022^{e}	8.93 ± 0.009^{h}	12.17
H1	76.86 ± 0.013^{b}	71.47 ± 0.118^{ab}	95.21
H2	77.06 ± 0.017^{b}	70.92 ± 0.663^{bc}	91.16
H3	75.05 ± 0.029^{c}	72.04 ± 0.612^{a}	95.21
T2	69.17 ± 0.081^{i}	43.22 ± 0.171^{f}	62.45
T3	71.28 ± 0.212^{f}	35.13 ± 0.031^{g}	49.31
T4	70.53 ± 0.005^{g}	44.65 ± 0.032^{e}	63.31
T5	69.76 ± 0.116^{h}	53.59 ± 0.151^{d}	76.8
T6	73.89 ± 0.127^{d}	43.89 ± 0.17^{ef}	59.46
T7	69.18 ± 0.021^{i}	44.66 ± 0.024^{e}	64.56
F-value	1468.43**	4644.28**	-
-value	<0.001	<0.001	-

*** Significant at 0.01 level*

Means having different superscripts differ significantly within a column

Table 3. Comparison of protein between two groups

Treatment	Protein (%)	Protein in supernatant (%)
T	71.92 ± 0.604	43.04 ± 3.366
H	76.32 ± 0.320	71.48 ± 0.309
t-value	4.438**	8.413**
P-value	<0.001	<0.001

*** Significant at 0.01 level*

as observed in a sample of vegetable protein with better whipping ability, solubility, emulsion capacity, and emulsion ability on enzymatic hydrolysis at 3.1 per cent DH (Liang *et al*., 2021). So, a maximum of five per cent DH was considered appropriate for the present study and the pH-stat technique was used to confirm the degree of hydrolysis. The results of protein solubility studies are shown in Table 2.

In Table 3, the comparison was done by using one-way ANOVA followed by DMRT to find which of the treatment groups are homogeneous.

These results show that all hydrolysed samples and fortified treatments had a higher solubility for protein as compared to the unhydrolysed WPC-iron complex. Solubility is a property that influences the application of a supplement in the food industry. Enzymatic hydrolysis cleaves larger polypeptides to low molecular weight peptides, thereby increasing the solubility of protein products. This result is consistent with the high solubility of protein hydrolysates at slightly acidic pH obtained in previous studies on fish hydrolysate (Alahmad *et al*., 2022). Similar results were reported by studies on hydrolysis by Alcalase and Papain (Noman *et al*., 2019; Li *et al*., 2012). The solubility of protein hydrolysates was found to influence protein and peptide functions. The extent of hydrolysis and fortification influenced the functional property and digestibility of a protein. Beena *et al*. (2023) reported that increased protein digestibility for a diet is one of the criteria for selection as a nutraceutical fortificant. There was a significant difference in the solubility of pure hydrolysates and complexed hydrolysates as revealed in Table 2. The per cent solubility is significantly higher than the control (89.99 %) for all pure hydrolysates and solubility increases with the degree of hydrolysis showing a maximum of 95.21 % solubility for both hydrolysates (H1 and H3) at five per cent degree of hydrolysis. Among complexed proteins least solubility of 12.17 per cent was

Table 4. Compositional analysis results and total iron content of hydrolysed and unhydrolysed protein iron complexes

Treatment	Total solids (%)Mean	Std. Error	Ash (%) Mean	Std. Error	Total Iron (ppm)	pH
T0	99.350	0.000	2.510^{e}	0.116	-	5.8
T1	97.571	0.379	6.762^{bcd}	0.293	13535.2	5.61
H1	97.530	2.270	6.158^{cd}	0.339	-	6.81
H2	97.615	0.385	5.023^{d}	0.140	-	6.48
H3	98.094	0.037	5.300^{d}	0.185	-	6.40
T2	98.735	0.075	8.812^{ab}	0.081	13568.8	5.6
T3	98.066	0.532	8.182^{abc}	0.536	10581.4	5.65
T4	98.219	0.887	8.912^{ab}	0.546	8980.96	5.57
T5	99.184	0.611	9.118^{a}	0.445	12972.23	5.47
T6	98.510	1.040	8.566^{ab}	0.875	12708.65	5.27
T7	98.594	0.734	10.191^{a}	1.704	12644.47	4.77
F-value = 0.549^{ns} P-value = 0.840			F-value = 10.438** P-value = <0.001		-	-

ns Non-Significant (P>0.05)
*** Significant at 0.01 level*
Means having different letter as superscript differ significantly

exhibited by the WPC-Fe (T2) complex which is unhydrolysed. In the case of hydrolysed whey protein iron complexes, a maximum solubility of 76.8 per cent was exhibited by five per cent hydrolysate of trypsin with added ascorbic acid (T5) followed by 64.56 per cent for five per cent hydrolysate prepared using enzyme combination with added ascorbic acid (T7). This established the fact that the hydrolysed protein iron complex has better functional properties and nutritional quality than the unhydrolysed protein iron complex, especially in the presence of ascorbic acid. Venkatasubramaniam *et al*. (2014) demonstrated that when ascorbic acid was added to the iron-fortified product in the ratio of 0.5:1 of ascorbic acid: iron, it enhances the availability of iron and is considered a bioavailability enhancer.

The ash content of hydrolysates and its complexes showed a higher value than control. This may be due to the base added to maintain a specified pH of eight during the enzymatic hydrolysis of protein. For protein iron complexes, the ash content was further higher due to added iron for fortification. For treatments with added ascorbic acid the ash content was still higher. There was no significant difference in the total solids of pure hydrolysates and iron complexed treatments as given in Table 4. The pH was slightly lower due to added ascorbic acid in samples T5, T6 and T7. In general, all iron complexed samples had lower pH than corresponding hydrolysates due to an additional incubation of two hours at ambient temperature provided for the iron complexation. Among the different iron compounds identified as fortificants, ferrous sulfate is the preferred compound due to its high bioavailability but is most likely to cause unacceptable sensory changes to the food fortification vehicles or the foods they are fortified with (Hurrell, 2018). Shilpashree *et al*. (2016) demonstrated that unhydrolysed whey protein concentrate has remarkable iron binding ability and has good sensory properties when ferrous sulphate is added. Based on the maximum mineral binding ability of proteins, the method for the preparation of WPC-Fe complex was standardised to have a ratio of 40:1 for protein: iron which was followed in the present study and ferrous sulphate was selected as the fortificant. Shilpashree *et al*. (2016) also reported that the effect of a bound form of iron for fortification gave promising results with respect to flavour, colour and taste. Better results were reported by researchers for fortified protein iron complexes prepared using Alcalase hydrolysed WPC (Athira *et al*., 2021). Hence in the current study protein hydrolysates were used for fortification.

Solubility of iron is also important as far as absorption in the small intestine is involved. As documented by Hurrell *et al*. (2002), when iron concentration in the small intestine was high, it was absorbed by passive transport. Soluble iron in the small intestinal contents of rats fed the Fe-WPC diet was examined in a similar study and was found approximately twice as high as that fed on other hemin-based diets. This supports the view that bound iron enhances iron absorption in the intestine. The presence of ascorbic acid further enhances intestinal solubility (Hurrell *et al*., 2002). Thus, the hydrolysed protein-iron complexes have better nutritional quality due to protein solubility and bioavailability of iron.

Compared to the usual method for estimation of iron by AAS directly from the sample, the present procedure was modified to be done after incubation of the sample in a mildly acidic environment at 37^0C followed by filtration to interpret the results as in an actual biological system (Niedzielski *et al*., 2014). The method combines the determination of not only total iron but soluble forms of iron by a single analytical procedure which allows easy and quick assessment.

Complexed or bound iron assimilates best indicating that iron complexed proteins in hydrolysed forms are better in diet supplementation than pure mineral supplements which impart off flavour in the product. Bound iron gave almost no off taste when flavoured milk was prepared, whereas direct mineral supplementation gave an objectionable off-flavour (Banjare *et al*., 2019). Allergenicity studies proved a 77 per cent reduction in the allergic potential of WPC when hydrolysed with trypsin at five per cent DH (Lukose *et al*., 2018). So, a reduction in allergenicity is also expected in the case of hydrolysed protein-iron complex when compared to unhydrolysed WPC-iron complex but it needs to be established.

Conclusion

In the present study hydrolysed WPC-iron complex is found to be better than unhydrolysed WPC-iron complex in solubility and expected bioavailability of iron, allergy reduction and functional properties. Bioavailability studies also need to be done *in-vivo* along with iron species analysis to establish the correlation of protein solubility, iron forms and iron absorption. The present study ensures the suitability of iron complexed WPC hydrolysates (five per cent DH) with added ascorbic acid as an effective vehicle for mass fortification with better functional properties and competence to reduce the incidence of iron deficiency anaemia.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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