Development of Technologies for Separation and Functional Improvement of Individual Milk Protein Fractions

(Advances in The Use of Milk Protein Fractions)

Armis No. 4216

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Summary and Conclusions

Milk proteins can be hydrolysed (i.e. fragmented) using proteolytic enzymes to give enhanced functional and nutritional properties. There is an increasing demand for hydrolysed protein ingredients with specific properties for nutrition of individuals with specialised dietary requirements including infants, the critically ill, the immuno-compromised and athletes. Such hydrolysed proteins can be specifically designed to provide distinctive tailor-made solutions to meet customer needs in these areas. This project explored the technologies for the production of two types of hydrolysates i.e. acid-soluble and glutamine-rich. Acid-soluble protein hydrolysates have potential in the fortification of acidic beverages, including soft drinks. Glutamine-rich hydrolysates are suggested as an optimal glutamine source for administration during periods of stress, such as recovery from strenuous exercise, or from surgery. Casein was selected as the protein for development of acid-soluble product and cereal protein for the glutamine-rich product.

The main conclusions were as follows:

- A number of protein hydrolysate products with value added properties and the processes required for their manufacture have been developed and are available for uptake by the food industry.
- Laboratory investigations identified conditions for the generation of two casein hydrolysates with desirable functional properties.
- Scale-up conditions for the manufacture of these hydrolysates in the pilot plant were successfully developed.
- Both hydrolystates were 100% soluble at pH 4.6, exhibited clarity in solution at low pH in clear soft drinks and in caramelised beverages and were stable in solution over a wide temperature range (from 4 to 30ºC) for extended periods.

- Solutions containing these hydrolysates exhibited no foaming properties and had acceptable sensory properties, being considered as weakly bitter compared to unsupplemented solutions. These performance characteristics make the acid-soluble hydrolysates useful supplements for caramelised beverages, such as colas, and clear soft drinks.
- Six glutamine-enriched peptide products were produced at laboratory scale using two commercially available enzyme preparations. These products had desirable characteristics such as increased levels of peptide bound glutamine, low free amino acid and free pyroglutamate levels.
- Pilot plant processes were developed for manufacture of the two glutamine-rich hydrolysates with most suitable compositional properties and these were fully characterised chemically. The manufacturing process was modified to enable industrial scale batches (5,000 litres) to be produced.

Commercial Impacts

- A patent application entitled "Glutamine-enriched peptide products" was filed in July 1997.
- A Food Ingredients company is currently assessing one of these glutamine peptide products for suitability for incorporation into health food drinks and supplements aimed at the American market.

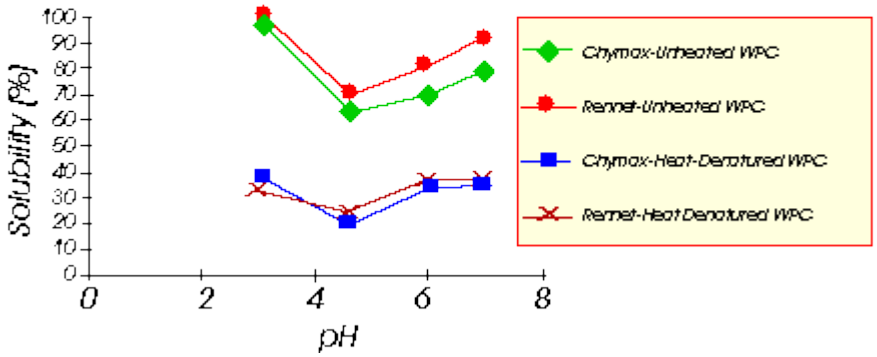
Research and Results

a) Acid-Soluble Milk Protein Hydrolysate

Hydrolysis of proteins is undertaken for a variety of reasons, including to improve the nutritional characteristics, retard deterioration, impart texture, increase or decrease solubility, add foaming or coagulation properties, add emulsifying capacity, prevent undesired interactions, remove off-flavours or odours, and/or to remove toxic or inhibitory ingredients. Hydrolysis can be accomplished by enzymes, acids or alkali, but protein hydrolysates resulting from enzymatic hydrolysis are far more desirable for nutritional purposes than those resulting from strictly chemical methods.

Initially, a variety of whey protein hydrolysates were produced from whey protein concentrate (WPC) for protein enrichment of soft drinks. A range of hydrolysates were produced from undenatured and heat-denatured WPC by food-grade protease enzymes which exhibited optimal activity at acid pH. The hydrolysates were most soluble at the extremes of the pH curve, i.e. pH 3 and 7 *(Fig. 1)*. Hydrolysates produced by the acid proteases Chymax and rennet from undenatured WPC exhibited greater solubility than the heat-denatured counterparts. At pH 3, both whey protein hydrolysates derived from undenatured WPC exhibited 100% solubility, while those derived from hydrolysis of heat-denatured WPC exhibited only 27-33% solubility. However, the clarity of all whey protein hydrolysates in acidic solutions was poor, and they all exhibited cloudiness. The cloudiness was not due to the fat content of WPC, because when a clarified defatted WPC (Provon, Avonmore) was used as the substrate for hydrolysis, the cloudiness remained when the resultant hydrolysates were incorporated into clear soft drinks.

Fig. 1. Nitrogen Solubility of Whey Protein Hydrolysates as a Function of pH.



Sensory analyses of clear soft drinks supplemented with the whey protein hydrolysates revealed that they could be detected and had an 'acidic' aftertaste. Addition of the whey protein hydrolysate (produced from undenatured WPC using the enzyme Chymax) to colas, which are acidic caramelised beverages, caused a colour change from the characteristic colour to a 'murky' brown, which could not be removed by filtration and which remained unchanged after several days at room temperature. When the pH of the colas was first neutralised, then supplementation with the whey protein hydrolysate caused no change in appearance of the solution. Therefore, the whey protein hydrolysates produced may be useful supplements for neutral, but not acidic beverages.

As an alternative to WPC for the production of acid-soluble hydrolysate, acid casein was subsequently used as the substrate for enzymes with pH optima in the range 7 to 8. Enzyme combinations used in these experiments included Alcalase, mainly composed of endoprotease activity (Subtilisin Carlsberg) from Bacilluslicheniformis, Flavourzyme, which is a fungal protease/peptidase complex produced from Aspergillusoryzae, containing both endo and exopeptidase activities, DBP50 which is an exopeptidase preparation, specifically designed to reduce bitterness in hydrolysates and has pH optimum of 7.0 and temperature optimum of 50ºC and Corolase PP, which is a proteinase preparation from pig glands rich in trypsin and chymotrypsin activities.

Two acid-soluble casein hydrolysates were manufactured successfully at lab-scale and pilot-scale, using two different sets of enzymes. During the course of this project, however, the enzyme Corolase PP, which was used in the production of one of the casein hydrolysates was withdrawn from the market, so the information below pertains to that casein hydrolysate which was manufactured using the second enzyme combination. The hydrolysates were characterised chemically and functionally. All peptides were less than 1500 D *(Fig. 2)* indicating that the hydrolysate was extensively hydrolysed and contained peptides no longer than 11 residues (based on average Mw/residue of 125 D). The protein content of the spray-dried product was 78-80%, lactose was <0.2%, fat was <1%, moisture was 6%, ash was 16% and pH was 4.6. The hydrolysate was easily dispersible, exhibiting clarity, solubility and stability in solution at low pH, being 100% soluble at pH 4.6 with no quantifiable foaming properties and the hydrolysate formed low viscosity solutions at high protein concentrations. When compared to an existing commercially available acid-soluble casein hydrolysate (Peptigen 901), the performance of the pilot-scale hydrolysate was superior. In this regard, the pilot-scale hydrolysate was a useful supplement for caramelised beverages, such as colas, in addition to clear soft drinks, while the commercial hydrolysate was only useful in clear soft drinks. Upon addition of the commercial casein hydrolysate to a cola-type drink, a colour change similar to that observed upon addition of the whey protein hydrolysate was obtained. In addition, the pilot-scale hydrolysate was considered only weakly bitter by a

trained sensory panel and compared favourably with the commercial hydrolysate in terms of sensory criteria.

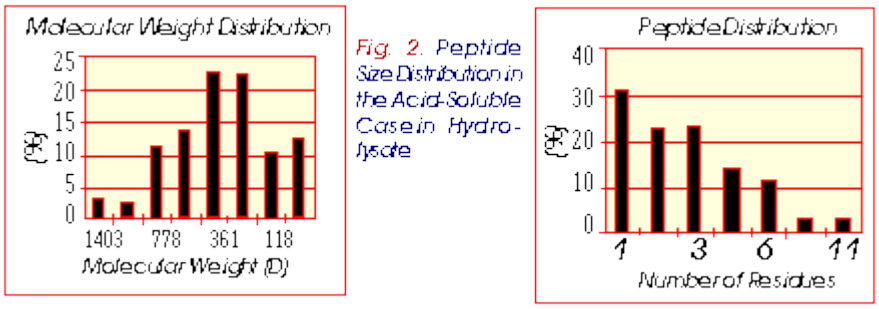


Fig. 2. Peptide Size Distribution in the Acid-Soluble Casein Hydro-lysate

This extensively hydrolysed protein product possessed the desired functional and physicochemical properties for fortification of such products as acidic beverages, both clear and caramelised, in addition to health, energy and sports drinks. The nutritional benefits and amino acid profile of the protein in the casein hydrolysate was similar to casein from which it was derived. Therefore, because of the high biological value of the protein in the hydrolysate, it may be used as the sole protein source, or in combination with other proteins, depending on the application. Furthermore, the hydrolysate in solution showed stability upon heat treatment, thereby allowing formulations based on the product to be autoclaved.

b) Glutamine-enriched peptide hydrolysates

Glutamine has a range of physiological effects. For example, glutamine is involved in the rapid restoration of muscle glycogen levels following periods of vigorous exercise and is therefore required in the rapid recovery from exercise. It stimulates the immune system and is therefore required for rapid recovery from infections or sepsis and in wound repair as pertains in the situation of post operative patients. There is a considerable body of evidence to show that glutamine plays a role in the maintenance/restoration of nitrogen balance. Glutamine is also important in oral rehydration and has a role in preservation of mucosal structure following periods of parenteral nutrition. Glutamine is considered a non essential amino acid as it can be synthesised in the body. However, during periods of metabolic stress, glutamine cannot be synthesised as quickly as it is required and as a consequence plasma glutamine levels are severely depleted. Glutamine is therefore considered as a conditionally essential amino acid.

It is evident from the above that glutamine can be considered as an ingredient in the formulation of "physiologically functional foods". In the free form, glutamine is insoluble in solution, and is unstable at high temperatures, as it cyclises to form pyroglutamate which is toxic. In contrast, glutamine in peptide bound form has improved solubility, is absorbed faster into the bloodstream and is more stable at high temperatures, thus markedly improving its suitability for food supplementation. Glutamine peptide enriched hydrolysate products should ideally have a number of characteristics. These include, high peptide bound glutamine contents (i.e. > 25% peptide bound glutamine), low levels of free amino acids and low free glutamine levels in order to minimise pyroglutamate formation. Furthermore, in order to facilitate efficient absorption, glutamine peptide enriched products should have low levels of high molecular mass material and high levels of low molecular mass material. The objective of this section of the project was therefore to develop glutamine peptide enriched products having all or most of the above characteristics.

Table 1: Comparative characteristics of large scale glutamine-enriched hydrolysate products

	DPCGP6001	DPCGP6002	Commercial product 1	Commercial product 2
Protein % (w/w)	74.44	77.78	73.61	82.31
%Free amino acids	3.21	3.05	3.92	0.39
%Free glutamine	0.035	0.015	0.020	0.008
%Peptide bound glutamine	28.60	29.07	19.70	32.39
%Pyroglutamate	0.42	1.23	2.21	0.01
Molecular mass distribution profiles (%)*				
>10kDa	1.21	1.04	0.07	26.67
<3 kDa	94.44	97.12	98.1	43.65
<1 kDa	77.87	84.70	86.39	23.85
Solubility	>99% pH 2-8	> 98% pH 2-8	>98% ph2-3, 5-7 94% pH4	>97.5% pH 2-8

Clarity	>99% pH 2-8	>98% pH 2-8	> 98% pH 2-8	>98% pH 2-3 < 98% pH 4-8
Acid stability	No turbidity	No turbidity	No turbidity	ND
Heat stability (80ºCx10mins)	No reduction of glutamine	No reduction of glutamine	No reduction of glutamine	1.51g/100g reduction of glutamine
Osmolality (mOsm/kg)	20.00	18.00	34.00	28.00

**Values expressed as % total area for a gel permeation profile obtained at 214 nm*

ND = Not determined

Initially, analytical methods were developed to assay hydrolysates for peptide bound glutamine, free glutamine and free pyroglutamate contents. Milk and cereal proteins were then investigated as potential sources of glutamine-enriched peptides. Cereal proteins are naturally rich in glutamic acid and as such were selected as good substrates for the preparation of glutamine-enriched peptide hydrolysates. An extensive screening programme was carried out and two commercial enzyme preparations were identified at laboratory-scale which generated glutamine-enriched peptide preparations having high levels of peptide bound glutamine. Using the selected enzyme preparations, pilot-scale trials (200 1) were carried out which yielded kilogram quantities of glutamine-enriched prototype products. These prototype products (DPCGP6001 and DPCGP6002) were extensively characterised and were shown to have characteristics superior to some commercially available products *(Table 1)*. When compared to commercially available product, the DPC products were shown to have increased levels of peptide-bound glutamine while retaining other desirable characteristics such as low free amino acid, low free glutamine and low free pyroglutamate levels. *Table 1* shows that commercially available product 2 has a high peptide bound glutamine content (> 30%), and low free amino acid, free glutamine and free pyroglutamate levels. However, the molecular mass distribution profile for this product shows a high percentage of material greater than 10 kDa. Commercially available product 2 does not display the same clarity in solution as DPC 1 and 2. DPCGP6001 and DPCGP6002 have lower osmolarity values than both commercially available products, this is favourable in terms of rapid absorption.

Table 2: Characteristics of glutamine-enriched hydrolysate produced at industrial scale

	DPCGP6001
Protein (g/100g powder)	83.14
Moisture (g/100g powder)	3.09
Ash (g/100g powder)	8.6
Fat (g/100g powder)	0.22
Free Amino Acids (g/100g powder)	
2.18	
Free Glutamine (g/100g powder)	0.031
Peptide Bound Glutamine (g/100g powder)	29.06
Pyroglutamate (g/100g powder)	0.33
Molecular Mass Distribution Profile *	
> 10kDa	
0.29	

< 3 kDa	96.44
< 1 kDa	79.12
Clarity	>98% pH 2-3, >94% pH 4-6, 92% pH 7.8
Appearance	White free flowing powder
Total Bacterial Count (per g of sample)	5900
Coliforms (per g of sample)	<10
pH of Reconstituted Sample	6.4

* values expressed as % total area for a gel permeation profile obtained at 214 nm

Industrial scale trials (5000 1) were performed following commercial interest in the products. As can be seen from *Table 2*, the glutamine-enriched product developed at industrial scale retained the favourable physicochemical characteristics of the hydrolysates produced at pilot scale. The industrial scale process also included a process modification which leads to an improved production process. Supplementation of orange juice and clear soft drinks with the glutamine-enriched peptide products yielded very acceptable drinks. An Irish food ingredients company is currently evaluating the American and European markets for the glutamine peptide products. A patent application was filed to protect each of the enriched glutamine peptide products prepared during the course of the project.

Pilot scale production of protein hydrolysis.

Publications

FitzGerald, R.J., Smith, M. McDonagh, D. and Slattery, H. (1996). Milk protein hydrolysates applications. *Proceedings of 3rd Food Ingredients Symposium* (Ed. Keogh, M.K.). 76-86. Silver Springs Hotel Conference Centre, Cork. 9-10 October.

Stanton, C. (1996). Hydrolysed milk protein. DPC Training Course-7 on Food Ingredients - Functional Properties, Applications and Formulation Technology. DPC, Moorepark, Fermoy, 25th January.

FitzGerald, R.J. (1996). Exploitation of milk protein variants as food ingredients. *DPC Training Course-7 on Food Ingredients - Functional Properties, Applications and Formulation Technology.* DPC, Moorepark, Fermoy, 25th January.

FitzGerald, R.J. and O'Connor, P.M. (1997). Glutamine-enriched peptide products. *Irish Patent Application No. S 970 570.*

Stanton, C. (1997). Development and application of milk protein hydrolysates. *DPC Training Course on Advances in Dairy Protein Applications.* DPC, Moorepark, Fermoy. 10th December.

Stanton, C. and O'Connor, P. (1997). Acid-soluble casein hydrolysates in soft drinks and glutamine-enriched peptide products. *DPC Training Course on Advances in Dairy Protein Applications.* DPC, Moorepark, Fermoy. 10th December.

Kenny, S., Wehrle, K., Stanton, C. and Arendt, E.K. (1998). Use of Dairy Powders as Functional Ingredients in Wheat Bread. *International Dairy Journal,*8 (5/6), 576.

Grau, H., Crowley, P., O'Connor, P., FitzGerald, R.J. and Arendt, E.K. (1998). Effect of Glutamine Peptide on Baking Characteristics of Bread using Experimental Design. Abstract. *Proceedings of the International Cereal Chemists Meeting.* Vienna, Austria.

Crowley, P., Grau, H., O'Connor, P., FitzGerald, R.J. and Arendt, E.K. (1999). Optimisation of a Production Process for Bread Containing Glutamine Peptide using Response Surface Methodology. *Irish Journal of Agricultural and Food Research,* Abstract. (In press).

Crowley, P., Grau, H., O'Connor, P., FitzGerald, R.J. and Arendt, E.K. (1999). Effect of Glutamine Peptide on Baking Characteristics of Bread Using Experimental Design. *Journal of Cereal Science.* (Submitted).

FitzGerald. R.J. and O'Connor, P. (1998). Glutamine-Enriched Peptide Products. *International Patent Application.*

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