Title: Designing Medical Foods for Inherited Metabolic Disorders: Why Intact Protein is Superior to Amino Acids

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Abstract

Phenylketonuria and tyrosinemia are inherited metabolic disorders characterized by high blood levels of phenylalanine (Phe) or tyrosine (Tyr), due to mutations in genes affecting Phe and Tyr metabolism, respectively. The primary management is a lifelong diet restricted in protein from natural foods in combination with medical foods comprised of mixtures of synthetic amino acids, vitamins and minerals. Compliance is often poor after childhood leading to neuropsychological sequelae. Glycomacropeptide, an intact 64 amino acid glycoposphopeptide isolated from cheese whey, provides a new paradigm for the management of phenylketonuria and tyrosinemia because glycomacropeptide contains no Phe and Tyr in its pure form, and is also a prebiotic. Medical foods made from glycomacropeptide have been used successfully for the management of phenylketonuria and tyrosinemia. Preclinical and clinical studies demonstrate that intact protein from glycomacropeptide provides a more acceptable and physiologic source of defined protein compared to amino acids in medical foods. For example, harmful gut bacteria were reduced, beneficial short chain fatty acids increased, renal workload decreased, protein utilization increased, and bone fragility decreased using intact protein versus amino acids. Advances in biotechnology will propel the transition from synthetic amino acids to intact proteins for the management of inherited metabolic disorders.

Introduction

Inherited metabolic disorders (IMDs) are genetic disorders that are often identified based on excessive amounts of specific intermediary metabolites that accumulate due to enzyme deficiency. Detection of IMD in newborn screening programs has expanded in the last decade because of improved technology using tandem mass spectroscopy and a greater capacity for genotyping (1). The major categories of IMD detected by newborn screening include disorders affecting the metabolism of amino acids, organic acids and the oxidation of fatty acids. The treatment of individuals diagnosed with these disorders often involves nutritional management with medical foods comprised of synthetic mixtures of amino acids (AAs); approximately 75 IMD require management with AA medical foods. A medical food is defined by the United States Food and Drug Administration as a “food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation” (United States Food and Drug Administration;

URL:<http://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/>).

Phenylketonuria (PKU, OMIM 261600), caused by a deficiency of hepatic phenylalanine hydroxylase (PAH EC 1.14.16.1) activity that catalyzes the conversion of Phe to Tyr, is the first human genetic disease to have effective programs for newborn screening and treatment (2). A low-Phe diet restricted in protein from natural foods and

supplemented with AA medical food, and since 2010 glycomacropeptide (GMP) medical foods, is the cornerstone of treatment to prevent profound cognitive impairment in individuals with PKU (3, 4). Lifelong adherence to the low-phe PKU diet in combination with AA medical foods is poor and new options are needed. GMP medical foods comprised of primarily intact protein provide a safe and acceptable option for the nutritional management of PKU with fewer side effects and improved acceptability compared with AA medical foods (5)**.

This review highlights evidence supporting the advantages of using intact dietary protein for IMD, with emphasis on the use of GMP medical foods in the nutritional management of PKU. Considerations in the biotechnology needed to attain intact proteins with specific amino acid profiles suitable for manufacture of medical foods for IMD are discussed.

Intact Protein Enables Production of a Variety of Palatable Foods

Medical foods made with intact dietary protein are superior to formulations of synthetic AAs because of improved odor and taste as well as functional properties suitable for the production of a variety of foods. Moreover, nutritionally-complete GMP medical foods made with the intact protein GMP supplemented with limiting AAs provide a more physiologic source of low-Phe protein than AA medical foods for the nutritional management of PKU with respect to gastrointestinal health, satiety, protein metabolism and bone development.

Prebiotic Properties of Glycomacropeptide Support Gastrointestinal Health

Glycomacropeptide or caseinomacropeptide is a bioactive 64-amino acid glycoposphopeptide derived from κ -casein in bovine milk that is produced during the manufacture of cheese, when rennet (chymosin) cleaves the C-terminal of κ -casein between Phe 105 – Met 106 releasing GMP into the cheese whey and precipitating para- κ -casein to form cheese (6), **Figure 1**. The unique AA profile of GMP with an absence of aromatic amino acids (Phe, Tyr and Trp) enables the formulation of medical foods for PKU and tyrosinemia. GMP is an acidic, highly polar peptide (isoelectric point below 4) that is hydrophilic and heat stable, with good functional properties for formulation of a variety of foods including beverages, baked goods and gelled products such as puddings (7). GMP has a high degree of glycosylation with mucin-type carbohydrate chains resulting in prebiotic properties in mice (8)*. Positive prebiotic effects include changes in the intestinal microbiota to reduce harmful sulfate-reducing bacteria and synthesis of short-chain fatty acids (SCFA) that lower colonic pH and prevent colonization with infectious bacteria (8, 9).

The human gut contains trillions of commensal bacteria referred to as the intestinal microbiota. Diet and other environmental factors are known to affect the composition of the intestinal microbiota, potentially resulting in dysbiosis as a mediator of chronic diseases such as Type 2 diabetes mellitus and inflammatory bowel disease (9). Children with PKU were noted to have distinct taxonomic groups within the intestinal microbiota as determined by 16S rRNA sequencing compared to children without PKU which may reflect PKU itself or the low-phe diet and AA medical foods used to manage the disease (10)**, **Figure 2A**. Consistent with the glycosylation

moiety of GMP being a prebiotic that is fermented in the colon, PKU mice fed a GMP diet show increased concentrations of the SCFAs acetate, propionate and especially butyrate, as well as reduced indices of inflammation, compared to PKU mice fed an AA diet (8), **Figure 2B**. Prediction of microbial function by metagenome analysis suggests fewer genes involved in starch and glucose metabolism with lower production of SCFA by the intestinal microbiota of PKU children consuming AA medical foods (10)**.

Production of SCFA, known to have beneficial effects on colon health including improved barrier function and reduced inflammation, are a characteristic of foods with prebiotic properties (9, 11) . Taken together, these preclinical and clinical data suggest that the prebiotic effects of GMP may induce a more beneficial intestinal microbiota in human PKU resulting in improved gastrointestinal health compared with AAs.

Consistent with this concept, PKU subjects participating in our recent clinical trial reported fewer gastrointestinal side effects, including heartburn, nausea, abdominal pain and altered bowel habits, with GMP medical foods compared with AA medical foods (5)**. This may reflect the prebiotic effects of GMP as well as the lower osmolality and renal solute load provided by GMP medical foods (350-650 mOsm/kg) compared with AA medical foods (1000-2500 mOsm/kg).

Intact Protein from GMP Improves Protein Utilization, Reduces Renal Workload and Promotes Satiety compared with Amino Acids

Protein synthesis and N retention are known to be greater with ingestion of intact protein compared with AAs, an effect associated with slower digestion and absorption of AAs from intact protein compared with single AAs in humans (12, 13). The common practice in human PKU to consume AA formula in big gulps of one or two servings per

day further exacerbates the lower efficiency of AAs to support protein synthesis because this stimulates hepatic degradation of AAs to produce urea coupled with renal excretion of the urea. The net effect of gulping AA formula is increased hepatic and renal workload and a net loss of AAs to support protein synthesis. Indeed, recommendations for protein intake from AAs for IMD are suggested to be 120-140% of known requirements to account for reduced efficiency of dietary amino acids to support protein turnover (4). More frequent, and smaller ingestions of GMP medical foods throughout the day, as noted in our recent clinical trial (5), supports greater efficiency of protein utilization and may normalize protein needs to be more consistent with the lower requirements for the general population.

Evidence from an inpatient metabolic study demonstrates improved protein utilization and reduced renal workload with ingestion of GMP medical foods. Ingestion of a low-Phe meal comprised primarily of intact protein from GMP compared with AA medical foods in PKU subjects produced higher postprandial concentrations of AAs in plasma, consistent with a slower rate of digestion and absorption of AAs, and lower blood urea nitrogen concentration, consistent with reduced hepatic ureagenesis from amino acid degradation and decreased renal workload (14), **Figure 3**. The impact of chronic ingestion of AA medical foods on the kidney is of potential concern. Mice fed an AA diet demonstrate significant 15-30% increases in renal mass and urine volume, and an acidic urine pH of less than 5.5 compared with mice fed a GMP diet (15). Moreover, Hennermann et al has reported chronic kidney disease in adolescents and young adults with PKU treated since infancy with AA medical foods (16).

The slower digestion and absorption of GMP compared to AA medical foods promotes satiety and may modulate control of postprandial blood glucose levels (17). A meal comprised of GMP compared to AA medical foods in PKU subjects induced the normal physiologic pattern of reduced postprandial concentration of the orexigenic hormone ghrelin along with greater subjective indices of fullness (18). This is important given that many individuals with IMD have an “always hungry” feeling when consuming a diet with AA medical foods. Whey-derived proteins such as GMP have been demonstrated to modulate plasma glucose, insulin and AA responses to a glucose load in a dose-dependent manner in healthy subjects (17). The mechanisms underlying these responses are unknown but may reflect the action of AAs and/or milk-protein derived bioactive peptides acting locally within the gastrointestinal tract to increase incretin secretion and/or entering the bloodstream to exert systemic effects on insulin secretion or uptake of glucose into muscle. In humans fed milk or yogurt, GMP was detected in the intestine and in plasma for up to 8 hour after feeding (19). Further research is needed to understand the bioavailability of milk-derived proteins such as GMP and their potential to modulate glycemic control and metabolism.

GMP Improves Bone Size and Strength Compared to Amino Acids in Murine PKU

Skeletal fragility characterized by low bone mineral density and a greater risk for fractures has emerged as a poorly understood chronic complication of PKU, and potentially other IMD, treated with AA medical foods. The incidence, etiology, implications and risk factors for skeletal fragility in the PKU population are unclear. Elevated blood Phe concentrations often associated with poor adherence to the low-Phe diet show no association with low bone mineral density in the majority of studies

(20). PKU mice have a bone phenotype of reduced bone mineral density with a deficiency in biomechanical properties resulting in femora that are both weak and brittle

(21). The AA diet worsens the PKU bone phenotype compared with the casein and GMP diets, resulting in smaller and weaker femora in both wild type and PKU mice,

Figure 4. Bone size is a major determinant of bone strength, so failure to achieve normal bone size with an AA diet increases the risk of subsequent fracture. Conversely, consumption of intact protein from casein or GMP results in greater bone size and strength with reduced risk for fracture in mice.

There are several possible explanations for the beneficial effects on bone of a diet containing GMP compared with AAs. The higher potential renal acid load provided by AA medical foods with its higher content of methionine and cysteine compared with GMP medical foods is one explanation (22). In humans, chronic consumption of a high dietary acid load adversely affects skeletal health due to bone buffering of H^+ ions, evoking increased bone resorption and renal calcium excretion with lower bone mineral density (23-26). Impaired biosynthesis of the bone matrix protein collagen due to poor retention and subtle deficiencies of AAs required for collagen synthesis, such as glycine, proline and lysine, may occur, and/or collagen cross-link formation and maturation may be altered, thus reducing the tensile strength of the bone matrix (27). Lastly, calcium absorption may be increased with ingestion of the prebiotic GMP as noted in adolescents fed the prebiotics found in inulin-type fructans (28) or soluble corn fiber (29)*. Longitudinal studies are needed to determine the pathogenesis of skeletal fragility in human PKU to permit clinicians to recommend appropriate nutritional management to support bone health.

Using Biotechnology to Attain Intact Proteins with Defined Amino Acid Profiles

Creating intact proteins missing the amino acids Phe and Tyr can take several routes. Some examples are: (1) peptide synthesis, (2) recombinant protein expression, and (3) isolation from nature. Because peptide synthesis technology has evolved rapidly in the last decade (30), now peptides of ≥ 50 amino acids can be made, approaching the length of GMP at 64 amino acids. However, the longer the peptide chain, the lower the yield, and synthesis of glycoproteins remains one of the grand challenges of peptide synthesis. Additionally, the use of non-food grade chemicals is problematic in the manufacture of food proteins. Furthermore, the cost of manufacture for 1 mg of crude peptide is about \$2 per amino acid for up to 30 amino acids. The cost rises by about ten-fold to attain >98 % purity (Bachem AG, Bubendorf, Switzerland). This makes peptide synthesis about ten-million-fold more expensive than the cost of purified GMP (\$120/kg), and not realistic.

An example of the second approach is the expression of recombinant proteins in cell culture or the milk of livestock. Transgenic animals are one-tenth the cost of cell culture systems in the production of recombinant proteins (31)*. Yet, the cost of manufacture of recombinant proteins in transgenic animals of \$6/g (31) is about fifty-fold more than the cost of purified GMP. Furthermore, the use of genetically modified organisms in foods faces stiff consumer resistance.

The third approach is to find a protein in nature that has no Phe or Tyr, and use protein purification technology to eliminate all the other proteins that naturally are present in that source that do contain Phe and Tyr. Very few such proteins are known. A striking example is GMP, which is found in cheese whey, an inexpensive and widely

available source. An alternative to finding a protein in nature that has no Phe or Tyr is to create one by protein hydrolysis. For example, three dairy proteins other than GMP that have long sections of the peptide chain having no Phe are: (1) alpha-s2 casein between Lys 1 and Thr 38, (2) alpha-s1 casein between Gly 33 and Tyr 144, and (3) beta-lactoglobulin between Leu 1 and Val 81 (32). Selectively cleaving the Thr-Phe bond in alpha-s2 casein or the Val-Phe bond in beta-lactoglobulin would free the Phe-free peptide from the protein chain much like chymosin frees GMP from kappa-casein during cheese manufacture. Highly selective proteases are an active area of research that includes directed evolution (33), and metagenomics and bioinformatics (34). Using biotechnology to engineer a protease to fit the need is now possible using advances in protein engineering.

It is important to realize that purification costs are often the largest cost in the production of purified proteins, and that purification costs are encountered in all three approaches mentioned above. Recombinant protein expression produces crude mixtures of proteins, containing the Phe-free target protein and many other proteins that are not Phe-free. Removing nearly all of the other Phe-containing proteins from the Phe-free target protein is the largest expense. Finding a natural source of the Phe-free protein essentially eliminates the cost of synthesis, leaving only the cost of purification, and provides a significant cost advantage over peptide synthesis or recombinant protein expression.

Cheese whey contains about 1.5 g/L GMP and 5.5 g/L other whey proteins. The other whey proteins contain about 36 mg Phe/g protein, whereas GMP naturally has none. Working backwards, to obtain 2 mg Phe/g protein in the final product, the 1.5 g

GMP can have no more than 0.088 g of residual other whey protein. In that case, the final product contains 1.588 g protein and 3.18 mg Phe contributed solely by the residual other whey protein. This requires removal of 98.4% of the other whey protein from the GMP (5.5 g protein down to 0.088 g protein). The final GMP protein purity is about 94.5% (1.5 g GMP in 1.588 g protein). Increasing the purity of GMP from about 20% of the protein in the cheese whey to about 94.5% of the protein in the final GMP product requires several steps, each with an inherent cost of manufacture and a cost due to yield loss. Purification steps traditionally used in GMP manufacture are membrane filtration and ion exchange chromatography (35). Newer technologies such as charged ultrafiltration membranes may lower the cost of manufacture and increase purity of GMP by integration of membrane filtration and ion exchange chromatography into a single step (36)*.

Conclusions

Glycomacropeptide medical foods support human physiology and organ function better than amino acids for individuals with PKU and tyrosinemia. The glycosylation moieties of GMP act as a prebiotic, reducing harmful bacteria in the gut, and stimulating the synthesis of beneficial SCFA. Intact proteins stimulate muscle protein synthesis and reduce renal workload better than amino acid medical foods. Skeletal fragility is less, and bone size and strength greater using diets containing intact protein from GMP compared to amino acids. Intact proteins found in nature, such as GMP, are the preferred way to supply dietary proteins with defined AA profiles compared to synthetic proteins or proteins made using genetically modified organisms. In the future, advances

in biotechnology and the development of new protein purification technologies will speed the transition from AAs to intact proteins in designing medical foods for IMD.

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Figure Legends

Figure 1. Structure of glycomacropeptide (GMP), a bioactive glycosylated peptide released from κ -casein during cheesemaking. GMP is a unique peptide lacking aromatic amino acids and thus has been isolated from whey for use in medical foods for the management of phenylketonuria and tyrosinemia. Bovine GMP represents a heterogeneous group of 64 amino acid peptides due to genetic variance (variants A and B) and post-translational modification, including phosphorylation at serine residues and O-glycosylation at threonine residues. The primary structure of bovine variant A is shown; the 2 sites corresponding to mutational differences in the B variant are indicated. Glycosylated forms of GMP include five different mucin-type carbohydrate chains containing N-acetylneuraminic acid (sialic acid), N-acetylgalactosamine, or galactose. The majority of glycosylated GMP molecules include trisaccharide and tetrasaccharide chains as shown. Adapted from Sawin EA et al Am J Physiol 309:G590-G601, 2015.

Figure 2. Altered intestinal microbiota of PKU Children (A) and increased cecal butyrate concentration in PKU mice fed low-Phe glycomacropeptide (GMP) compared with amino acid (AA) diets (B). The average frequencies of the most abundant phyla are shown for PKU and control children; * $P < 0.05$. Adapted from de Oliveira FP et al PLoS

ONE 11(6):e0157513. Increased cecal concentration of the short chain fatty acid butyrate in PKU (*Pah^{enu2}*) mice fed GMP compared with AA diets from weaning through 8-9 wk of age, n=6-8. Adapted from Sawin EA et al Am J Physiol 309:G590-G601, 2015.

Figure 3. Changes in plasma concentrations of total amino acids and blood urea nitrogen in PKU subjects fed low-Phe diets supplemented with AA and GMP medical foods in an inpatient metabolic study. Plasma was obtained 2.5 hr after eating breakfast. Total plasma AAs indicate the sum of all AAs measured in plasma. Total plasma AAs increased and blood urea nitrogen decreased with ingestion of GMP when compared with AAs. Adapted from van Calcar SC et al Am J Clin Nutr 89:1068-77, 2009.

Figure 4. Bone size and strength are reduced with an AA diet in mice. Representative photographs of femoral cross-sectional geometry in PKU mice fed casein, AA and GMP diets (A). Provision of dietary protein from AAs decreases femoral cross sectional area (B) and strength shown as maximum load before fracture based on the 3-point bending test (C) in both WT and PKU mice compared with a GMP diet (C). Adapted from Solverson P et al PLoS ONE 7(9):e45165.

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In this book chapter, the authors present emerging methods for the purification of proteins, including glycomacropeptide, using charged membranes as an alternative to filtration membranes and ion-exchange chromatography.

Figure 1

Glycomacropeptide – 64 amino acid chain

Mucin – type carbohydrate chains

Figure 2

A

PKU Children, n=8

Control Children, n=10

B

Figure 3

Figure 4

Medical foods made with intact protein from glycomacropeptide (GMP) support human physiology and organ function better than synthetic amino acids (AA).