Analysis of bound amino acids in the plasma of fed rats: a new preparation procedure

Isabelle Galibois, France Pitre, Guy Parent, and Laurent Savoie

Département de nutrition humaine et de consommation, Université Laval, Québec, Canada

Oligopeptides have been proposed as an important form of transport of amino acids in circulation, but current preparation and analysis methods do not allow detection of peptides in plasma samples. In order to measure the concentration and composition of bound amino acids in comparison with those under free form in plasma, blood samples were collected in portal vein and aorta of rats fed a 20% rapeseed protein diet. Samples were deproteinized with sulfosalicylic acid, and centrifuged in 3000 molecular weight cut-off filters. Analyses were performed by ion-exchange chromatography before and after hydrolysis of filtrates in 6N HCl, to assess by difference the amount of bound amino acids in plasma. Hydrolyzed samples from portal vein and aorta contained, respectively, 8.2% and 10.3% more total amino acids than nonhydrolyzed samples ($P < 0.05$). In portal vein, this difference was mostly due to proline (31%), arginine (17%), and threonine (10%). In aorta, proline (40%), glycine (18%), and threonine (11%) accounted for most of the difference. In both vessels, no difference was noted before and after hydrolysis for serine, valine, tyrosine, and histidine, indicating that these amino acids were found exclusively under free form. These results show that in the fed animal, conjugates may account for around 10% of amino acid transport in portal and systemic plasma, and do not contain all amino acids in equal proportions. The dietary or endogenous origin of these bound amino acids has not been established yet.

Keywords: peptides; amino acids; plasma; deproteinization

Introduction

Over the last decades, the development of rapid and accurate methods to measure even minute amounts of amino acids has made the analysis of plasma amino acids (PAA) a routine procedure in a wide variety of nutritional and physiological studies. Variations in PAA levels have been used mainly to estimate the appearance of protein digestion products in the portal bloodstream,[1-3] and also to study interorganal protein and amino acid metabolism.[4-6]

Ion-exchange chromatography is currently the most widespread technique for PAA analysis.[7,8] Standard elution methods allow for the measurement of all free (but not peptide-bound) amino acids in deproteinized plasma samples. The inability to detect the possible presence of small peptides has been criticized by some authors, who suggested that peptides could be an important form of portal absorption and transport of amino acids in plasma, under physiological conditions.[9,10] However, this hypothesis remains controversial, the most accepted view being that dietary peptides taken up by the intestine are almost totally hydrolyzed within the enterocyte before their appearance in circulation.[11,12]

In the present work, a new sample preparation procedure for PAA analysis is proposed, allowing for the assessment of the amount and composition of bound amino acids in plasma. These bound forms would comprise oligopeptides, as well as other amino acid conjugates possibly present in the circulation. The procedure involved first a chemical precipitation of plasma proteins with sulfosalicylic acid, as in usual methods,[7,8] followed by a filtration on a low molecular weight cut-off membrane. Amino acids were analyzed in samples before and after acid hydrolysis. Difference in concentrations between the two analyses was attributed to bound amino acids.

This method was tried out in samples collected in

Address reprint requests to Dr. Isabelle Galibois, Département de nutrition humaine et de consommation, FSAA, Pavillon Paul-Comtois, Université Laval, Québec, PQ G1K, 7P4, Canada.
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the portal vein and aorta of rats fed a rapeseed protein diet. In previous studies,[13,14] rats fed rapeseed often exhibited low PAA levels, compared to animals fed other protein sources, suggesting that some part of rapeseed digestion products might enter the bloodstream as peptides rather than as free amino acids.

Methods and materials

Animals and diets

Sixteen male rats of the Sprague-Dawley strain (Charles River, St. Constant, Qc), weighing between 200 and 220 g, were housed individually in suspended wire cages in a temperature-controlled room (22°C) and kept under a dephased 12:12-hour light:dark cycle, with the lights on at 21:30 hours. They were fed ad libitum a 20% rapeseed protein diet (*Table 1*) for eight days. Weight gain and food intake were recorded every other day. On day 8, at 17:00 hours, the animals were put under halothane anesthesia (4% halothane in oxygen). The abdominal cavity was opened and 2-mL blood samples were drawn from the portal vein and the abdominal aorta with heparinized syringes, as previously described.[14]

Plasma preparation

Immediately after sampling, blood was centrifuged at 1500*g* for 15 minutes. For each sample, 900 μL of plasma were then mixed with 100 μL of a solution containing 1.25 μmol of S-amino-ethyl-cysteine (SAEC, Sigma Chemical Co., St. Louis, MO), used as an internal marker of amino acid concentration in whole plasma. Deproteinization was performed immediately by placing these 1-mL samples in 15-mL conical tubes in which 1 mL of 5% sulfosalicylic acid (Fisher Scientific, Montreal, Qc.) in 95% ethanol had been evaporated. After mixing, the solutions were kept at 277°K for 16 hours and then centrifuged at 1500*g* for 15 minutes. Supernatants were collected and frozen until further analysis.

Table 1 Composition of the experimental diet[1]

Ingredient	Proportion (g/100 g)
Rapeseed concentrate[2]	37.3
Corn oil[3]	10.0
Cellulose[4]	5.0
Minerals[5]	3.5
Vitamins[6]	1.0
Corn starch[7]	43.2

[1] Energy content of the diet, kcal/g (kJ/g): 4.533 (18.966).
[2] Low-glucosinolate protein concentrate, prepared by the FRI-71 process, Food Research Institute, Ottawa, Ont. % protein (N × 6.25): 53.6%, as determined on a Kjel-Foss apparatus (Foss Co, Denmark).
[3] Mazola corn oil, Best Foods, Canada Starch, Montreal.
[4] Alphacel non nutritive bulk, ICN Nutritional Biochemicals, Cleveland, OH.
[5] AIN mineral mixture 76, ICN.
[6] Vitamin mix, Teklad Test Diets, Madison, WI, supplying (mg.kg diet): retinyl palmitate, 39.7 (19850 IU); ergocalciferol, 4.4 (2200 IU); a-tocopheryl acetate, 485 (121 IU); ascorbic acid, 987; i-inositol, 110.2; choline dihydrogen citrate, 3715; menadione, 49.6; p-aminobenzoic acid, 110.2; niacin, 99.2; riboflavin, 22; pyridoxine HCl, 22; thiamin HCl, 22; calcium pantothenate, 66.1; biotin, 0.44; folic acid, 1.98; vitamin B-12, 29.8.
[7] ICN.

Amino acid analyses

Samples were thawed, pooled (2 rats/pool), and mixed with an equal volume of sample dilution buffer (Li-S, system 6300, Beckman Instruments, Palo Alto, CA). As in the usual procedure, an aliquot (900 μL) of this solution was filtered through a 0.22 μm Millipore filter (Millipore, Bedford, MA), and termed sample F.

Another aliquot (900 μL) of the solution was centrifuged at 2700*g* for 3 hours in a Centricon-3 microconcentrator of 3000 MW cut-off (Amicon Canada, Oakville, Ont.). After centrifugation, the retentate was discarded, and the filtrate, termed C, was divided into two 400-μL parts, one for immediate analysis, the other for acid hydrolysis. This was done under vacuum in 1.0 mL 6N HCl, at 373°K for 24 hours. The hydrolysate was then evaporated and suspended in 1.0 mL of sample dilution buffer. This fraction was termed sample H.

Amino acid analyses of fractions F, C, and H, and of a standard mixture of amino acids (Beckman), were performed by ion-exchange chromatography on the physiological column of model 6300 Beckman Auto-Analyser (Beckman Instruments, Palo Alto, CA). Before analysis, samples and standards were added known amounts of aminoadipic acid (Sigma Chemical Co., St. Louis, MO), used as an internal marker of injection.

In order to establish whether the filtration of bound amino acids in the microconcentrator was quantitative, a control experiment was carried out using a mixture of the following peptides: Lys-Lys, Gly-Lys, Gly-Gly-Leu, and Gly-Gly-Gly-Gly-Gly-Gly. Centrifugation was performed in duplicates at three concentrations (0.1 mg/mL, 0.25 mg/mL, and 0.5 mg/mL), at 2700*g* for 3 hours. Alpha-amino nitrogen was assayed in filtrates by reaction with O-phtaldialdehyde,[15] using the peptide mixture as the standard. Mean recuperation was 92.6% (range: 91–96%).

Calculations and statistical analysis

The concentrations of free amino acids and internal markers in deproteinized plasma were calculated for fractions F, C, and H. For each amino acid, values were then converted to whole plasma concentrations, using the following formula:

$$CWP = CDP \times (138.9/SAEC) \quad (1)$$

where

CWP = amino acid concentration in whole plasma, in μmol/L
CDP = amino acid concentration in deproteinized plasma, in μmol/L

138.9 = concentration of SAEC in whole plasma, in μmol/L (125 nmol/900 μL)
SAEC = measured concentration of SAEC in deproteinized plasma, in μmol/L

The statistical significance of differences between the fractions was assessed by means of an analysis of variance, followed by a Duncan's Multiple Range test or a Student's paired *t*-test. All calculations were performed with the Statistical Analysis System (SAS Institute, Cary, NC).

Results

Total amino acids in the three fractions

Figure 1 shows the sums of amino acids measured in the three fractions. For the purpose of comparison, amino acids known to be severely affecterd by hydrolysis (asp, asn, glu, gln, cys, and met) were not taken into account.

The sums of the remaining amino acids were very similar in fraction F and fraction C, both in arterial and venous samples. However, in the hydrolyzed fractions, the concentrations of free amino acids were significantly higher ($P < 0.05$). In portal samples, the difference between fraction C and fraction H was 8.8%, and this difference reached 11.2% in the aortic samples.

Regardless of the preparation method, amino acid concentrations in portal plasma were largely higher than those in arterial plasma. In all cases, about 60% more amino acids were found in portal fractions than in arterial ones.

Individual amino acid concentrations

Tables 2 and *3* report the individual amino acid concentrations measured in fractions C and H, in aortic and portal vein samples. Individual concentrations in fraction F are not shown, as they were very similar to those in fraction C.

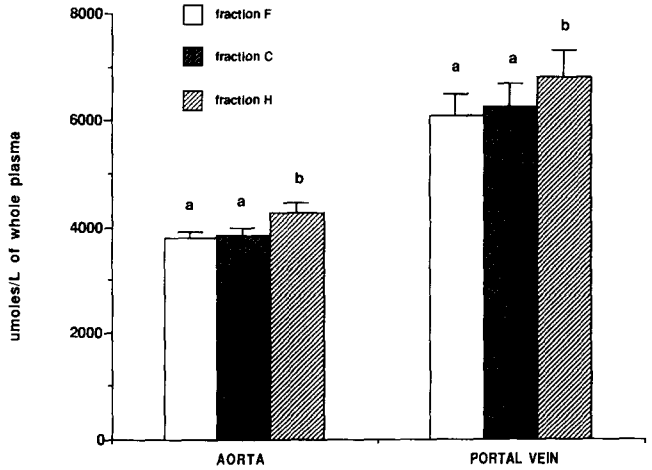

Figure 1 Sum of amino acids (excluding asp, asn, glu, gln, cys, and met) measured in each fraction. Each value is the mean + SD of 8 samples. Within a vessel, values bearing a different letter are significantly different ($P < 0.05$).

Table 2 Amino acid concentrations in whole plasma, aortic samples

AA	Fraction C[1]	Fraction H[2]
	μmol/L	
PSER	6.6±0.4[3]	19.0±4.7
TAU	119.5±10.6	138.8±7.1
PETH	11.9±0.6	28.3±0.8
ASP	9.0±0.5	97.5±1.6
THR	553.0±20.3	616.5±18.5
SER	437.0±8.3	439.3±10.6
GLU	92.4±3.5	923.8±9.5
GLN	719.8±11.6	—
PRO	286.1±8.7	401.8±9.3
GLY	390.6±12.0	459.4±11.3
ALA	566.1±8.4	584.4±6.9
CITR	89.2±3.3	62.2±7.7
VAL	212.2±10.3	233.8±8.9
CYS	55.2±1.5	27.1±2.8
MET	67.0±0.5	51.8±1.0
ILE	83.8±2.7	89.7±3.1
LEU	135.8±4.6	149.2±6.0
TYR	83.8±2.9	92.3±3.5
PHE	61.9±2.1	67.4±1.9
NH3	200.9±23.8	2126.5±140.7
ORN	64.0±1.9	108.2±6.6
LYS	436.4±10.7	448.6±10.5
HIS	81.5±2.2	91.3±3.3
ARG	220.6±5.1	241.1±7.5

[1] Deproteinized plasma filtered on microconcentrator.
[2] Deproteinized plasma filtered on microconcentrator and hydrolyzed.
[3] Mean ± SE of 8 samples.

Table 3 Amino acid concentrations in whole plasma, portal vein samples

AA	Fraction C[1]	Fraction H[2]
	μmol/L	
PSER	24.9±2.4[3]	21.2±2.3
TAU	122.5±13.6	164.1±12.9
PETH	13.3±0.9	34.4±2.7
ASP	20.0±0.8	204.1±5.5
THR	675.5±28.4	744.3±22.3
SER	564.9±10.6	571.9±11.6
GLU	149.5±5.2	844.9±26.2
GLN	564.8±13.4	—
PRO	479.9±16.8	628.2±29.5
GLY	753.8±18.2	819.6±19.6
ALA	1354.0±37.6	1399.7±36.7
CITR	175.2±5.6	131.1±14.1
VAL	333.2±14.1	357.3±11.3
CYS	74.1±1.9	46.2±5.4
MET	88.5±0.9	70.6±2.4
ILE	164.9±4.7	177.8±5.7
LEU	265.5±7.2	288.9±11.2
TYR	111.0±3.8	111.2±11.6
PHE	107.4±3.5	115.1±4.0
NH3	286.9±36.7	2225.8±139.3
ORN	94.5±2.1	149.9±9.0
LYS	585.2±16.3	610.6±15.2
HIS	138.3±2.9	142.6±3.7
ARG	293.7±10.0	342.5±7.8

[1] Deproteinized plasma filtered on microconcentrator.
[2] Deproteinized plasma filtered on microconcentrator and hydrolyzed.
[3] Mean ± SE of 8 samples.

Prior to hydrolysis (fraction C), the most prevalent amino acids in aortic samples and portal vein samples were glutamine (*Table 2*) and alanine (*Table 3*), respectively. Hydrolysis brought about a complete loss of glutamine, with an increase in glutamic acid and ammonia. Glutamic acid concentration in fraction H was even larger than the sum of glutamine and glutamic acid concentrations in fraction C. Aspartic acid concentration was also much enhanced by hydrolysis, most likely due to the loss of asparagine (values for this amino acid could not be precisely measured and are not reported in the tables).

Decreases in concentration following hydrolysis were observed for cystine, methionine, and citrulline. Concentrations of taurine, phosphoethanolamine, and ornithine were increased, as were those of the 13 remaining amino acids.

Statistical significance of the differences in concentration

As these thirteen amino acids were susceptible to being found in peptides, in contrast with those that only exist in the free form (e.g., ornithine), the importance of their difference of concentration between fraction C and fraction H was subjected to a statistical analysis. Results are shown in *Table 4*.

In aortic samples, differences were found to be significant for seven amino acids, four of them essential (thr, ile, leu, and lys), and three nonessential (pro, gly, and ala). In portal vein samples, significant differences were also found for the same seven amino acids, and also for two other essential amino acids, phenylalanine and arginine.

The largest increase in concentration after hydrolysis was observed for proline: In portal as well as in aortic plasma, a difference larger than 30% was noted. Free glycine concentration was also enhanced by hydrolysis, but not to the same extent in the two vessels, the largest difference being found in aortic samples. The contrary was found for arginine, with a significant proportion (17%) in bound form in portal vein plasma, and a nonsignificant one in aorta. Regarding threonine, alanine, isoleucine, leucine, and lysine, the proportion in the bound form was similar in portal plasma and aortic plasma, and averaged 10% or less.

In both vessels, serine, valine, tyrosine, and histidine were seemingly present only in the free form, as no significant differences in their concentration were observed when comparing fraction H to fraction C.

Table 4 Magnitude of differences in plasma amino acid concentrations between fraction C[1] and fraction H[2]

	Aorta		Portal vein	
AA	P[3]	Difference[4]	P	Difference
THR	< 0.001	11%	< 0.01	10%
SER	NS[5]		NS	
PRO	< 0.001	40%	< 0.001	31%
GLY	< 0.001	18%	< 0.01	9%
ALA	< 0.01	3%	< 0.01	3%
VAL	NS		NS	
ILE	< 0.001	7%	< 0.01	8%
LEU	< 0.01	10%	< 0.01	9%
TYR	NS		NS	
PHE	NS		< 0.001	7%
LYS	< 0.001	3%	< 0.001	4%
HIS	NS		NS	
ARG	NS		< 0.001	17%

[1] Deproteinized plasma filtered on microconcentrator.
[2] Deproteinized plasma filtered on microconcentrator and hydrolyzed.
[3] Level of significance, as assessed by a Student's paired t test.
[4] $\frac{(\text{Concentration in fraction H}) - (\text{Concentration in fraction C})}{(\text{Concentration in fraction H})} \times 100$
[5] Nonsignificant at the $P = 0.01$ level.

Discussion

The present findings suggest that although the major part of amino acids in plasma are in the free form, a significant portion of some of them are also present in oligopeptides, or in other conjugates. This is, however, indirect evidence: As the current chromatography methods do not allow for the detection of free and bound amino acids at once, an approach by difference had to be taken.

Previous studies had proceeded similarly. For instance, before and after hydrolysis, McCormick and Webb[16] compared the amino acid concentrations in plasma samples deproteinized with sulfosalicylic acid, and attributed the difference to peptides. They reported that peptide fraction accounted for the greatest absolute amounts of amino acids in blood. This work was criticized on the grounds that chemical deproteinization might not have been completely efficient, and that amino acids released by hydrolysis were likely of protein rather than peptide origin.[12] Gardner[17] also proceeded by difference to detect the passage of intact peptides through isolated intestinal segments, but in his in vitro studies, samples were not collected in blood vessels and plasma proteins could not interfere with the analysis.

In the present work, after treatment with sulfosalicylic acid, samples were filtered by centrifugation through a 3000 molecular weight cut-off filter, to ensure that all proteins remaining in solution would be excluded from the material submitted to hydrolysis. This technique seemed adequate, since for many amino acids (e.g., serine, *Tables 2* and *3*), the concentrations before and after hydrolysis were nearly the same. This would not have been the case if proteins, which normally contain all amino acids, had remained in the samples.

Thus, filtration with the microconcentrator proved useful for deproteinization, and also for an adequate recovery of both free and bound amino acids in samples. When first considered, this method presented a drawback. After centrifugation, a certain volume of the solution is lost in the retentate, and the filtrate is concentrated to an unknown extent. This could have led to major errors in the estimations of amino acid concentrations, particularly when working with sam-

ples of very low volume. In this work, this problem was overcome by pooling samples (2 rats/pool) to increase volume, and by adding markers at two steps of the preparation procedure. The first one, S-aminoethyl-cysteine, was added to plasma prior to the treatment with sulfosalicylic acid. This allowed us to report molar concentrations in whole plasma rather than in deproteinized plasma, and thus to provide a basis for comparison of the different preparation methods. The second marker, aminoadipic acid, was added immediately before amino acid analysis to verify the reliability of the automatic injection, as some troubles were experienced previously with samples of various viscosity.

Final free amino acid concentrations found for fraction F (filtration on a 0.22 μm filter) and fraction C (microconcentrator) were almost identical (*Figure 1*), indicating that the calculation method based on markers was adequate. As the same equations were used for calculating concentrations in the hydrolysates, results obtained for fraction H are likely to be valid, too. Concentrations reported for bound amino acids may, however, be slightly underestimated, based on the control experiment on the recovery of peptides (92.6%) after centrifugation.

Some features of amino acid profiles in hydrolysates were expected. Glutamine and asparagine are known to be completely converted to glutamic acid and aspartic acid, respectively, during acid hydrolysis.[18] Cystine is also severely destroyed in the process,[19] and in the present work methionine was also affected. The gain in ornithine observed here could partly result from the hydrolysis of citrulline.

It has been reported that when SSA supernatants are analyzed with lithium buffers, as in the present study, early eluting amino acids present poor resolution that can be further affected by slight variations in pH.[20,21] This fact, coupled with the possible presence of cysteic acid that also elutes in the early period, could explain differences noted between fraction C and fraction H in phosphoserine, taurine, and phosphoethanolamine concentrations.

Previous studies had also indicated that serine and threonine are normally slightly degraded during acid hydrolysis.[18,19] This was not the case in this work, threonine concentration being even enhanced. Perhaps some degradation did take place, but it was compensated by a release of bound threonine and serine.

While significant increases after hydrolysis were noted for many amino acids, the largest one was found for proline. This observation is especially important, considering the growing interest developed in the last years for proline residues, due to their resistance to enzymatic hydrolysis and their role in the synthesis of a number of biologically active peptides.[22,23] While proline is not part of the N-terminal sequence of endorphins (Try-Gly-Gly-Phe), it is in that of β-casomorphins (Try-Pro-Phe-Pro) tht arise from casein hydrolysis and present opioid activities.[24] In this study, it was not possible to determine if bound proline was of dietary origin. In a previous in vitro work,[25] we observed that proline was almost exclusively found in peptides after digestion of rapeseed proteins with pancreatic enzymes. Moreover, Ganapathy et al.[26] showed that less than 10% of the dipeptide gly-L-pro was hydrolyzed during transport into rabbit highly purified brush border membrane vesicles. Thus it may be hypothesized that at least some part of bound proline in plasma is of dietary source. For the other amino acids found in excess in fraction H, and particularly the essential ones (ile, leu, phe, and lys), a relation with protein digestion products is harder to establish, as many of them are known to be preferential targets of enzymatic hydrolysis during digestion.

In summary, this work presented a new preparation procedure adequate to estimate the concentration of free and bound plasma amino acids. It showed that conjugates may account for around 10% of amino acid transport in plasma, and do not contain all amino acids in equal proportions. Additional research will be necessary to establish the endogenous or exogenous origin of these bound amino acids.

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