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Original article

^{15}N-Labelling and preparation of milk, casein and whey proteins

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Summary — One of the main problems of *in vivo* protein digestion studies is the contribution of endogenous protein secretion. Nutritional studies have shown that the use of stable isotopes in this context is an appropriate technique to perform certain metabolic experiments with proteins. Thus the purpose of this work was to determine the optimum conditions for the production of stable isotope ^{15}N-labelled milk and for the subsequent partition of two crude fractions of milk proteins: casein and whey proteins. ^{15}N-labelled milk was prepared with milk from two lactating cows: one received daily 25 g $(^{15}NH_4)_2SO_4$ and the second received 50 g. Native phosphocaseinate (NPPC) and whey protein concentrate (WPC) were separated from raw pooled ^{15}N-milk (RPM) by membrane microfiltration and then purified through water diafiltration. The ^{15}N-enrichment of milk reached 0.4213 atom-% excess (APE) and 0.5177 APE for the cows receiving 25 g and 50 g $(^{15}NH_4)_2SO_4$, respectively. The microfiltration technique used allowed to obtain from 47 kg RPM both WPC (1.3 kg) and NPPC (9.76 kg) with yields of 34.4% and 82.5%, respectively. NPPC was 0.5070 APE ^{15}N-enriched and consisted of 99.9% proteic nitrogen. WPC was 0.4999 APE ^{15}N-enriched and consisted of 96.8% proteic nitrogen. The ^{15}N enrichments of skim milk, NPPC and WPC were not significantly different ($P < 0.05$) and were high enough to perform *in vivo* metabolic experiments.

milk protein / ^{15}N / stable isotope / microfiltration

Résumé — Enrichissement en ^{15}N-azote et préparation de lait, caséine et protéines du lactosérum. *Un problème majeur rencontré lors de l'étude de la digestion des protéines est la contribution des protéines endogènes. Des études nutritionnelles ont montré que l'utilisation des isotopes stables est une technique appropriée pour réaliser des études métaboliques avec des protéines. Le but de ce travail est de déterminer les conditions optimales d'une production de lait de vache marqué à l'azote-15 suivie d'une séparation des fractions caséine et protéines du lactosérum. Le ^{15}N-lait est préparé à partir de 2 vaches en lactation : une vache reçoit chaque jour 25 g $(^{15}NH_4)_2SO_4$ et la seconde reçoit 50 g. Le phosphocaséinate natif (NPPC) et les protéines du lactosérum concentrées (WPC) sont séparés par microfiltration et diafiltration à partir d'un mélange de ^{15}N-lait (RPM). L'enrichissement en*

^{15}N du lait atteint 0,4213 atom-% excess (APE) pour la vache recevant 25 g ($^{15}NH_4)_2SO_4$ et 0,5177 APE pour la vache en recevant 50 g. La technique de microfiltration utilisée permet d'obtenir, de 47 kg de RPM, 1,3 kg de WPC et 9,76 kg de NPPC avec un rendement respectif de 34,4% et 82,5%. Le NPPC est enrichi en ^{15}N à 0,5070 APE et est constitué à 99,9% d'azote protéique. Le WPC est enrichi en ^{15}N à 0,4999 APE et est constitué à 96,8% d'azote protéique. Les enrichissements en ^{15}N du lait écrémé, du NPPC et de WPC ne diffèrent pas significativement ($P < 0,05$) et sont suffisamment élevés pour permettre des études métaboliques in vivo.

protéine de lait / azote-15 / isotope stable / microfiltration

INTRODUCTION

The labelling of proteins by introducing non-abundant isotope-labelled atoms, either radioisotopes (^{14}C, ^{3}H, ^{125}I) or stable isotopes (^{13}C, ^{2}H, ^{15}N), is often necessary to analyse their digestive and metabolic fate precisely. Compounds labelled with non-abundant stable isotopes are used more and more in various biological and medical disciplines, including clinical pharmacology, gastroenterology and nutrition (Wolfe, 1992).

Nitrogen present in the lumen of the intestinal tract is both of exogenous and endogenous origin. A conventional way to estimate the endogenous nitrogen contribution to the total nitrogen in the intestine is the use of stable isotope labelling techniques. The ^{15}N techniques have been proved to be of great interest for investigating the dynamics of nitrogen and protein metabolism in both healthy and sick humans (Matthews *et al*, 1979; Dietz *et al*, 1982). During the last years, a number of essential studies on protein metabolism in man using amino acids labelled with stable isotopes have been performed. The most commonly used ^{15}N-labelled compounds are ^{15}N-glycine and leucine (Waterlow, 1981; Tessari *et al*, 1985; Yu *et al*, 1990). ^{15}N-labelled proteins might also be used beneficially in the study of protein absorption in the gut (Mahé *et al*, 1994). In addition, ^{15}N-labelled glycine, leucine, egg and yeast protein have been used to measure the nitrogen turnover rate in man (Wutzke *et al*, 1983; Plath *et al*, 1987).

Completely marked highly enriched ^{15}N-labelled milk protein represents an interesting and easily available source of ^{15}N-labelled protein prepared by ruminal administration of ^{15}N-labelled ammonium salt in cows. The purpose of this work was to determine the optimum conditions for both the production of stable isotope ^{15}N-labelled milk and the subsequent partition of casein and whey proteins fractions by membrane microfiltration and ultrafiltration techniques.

MATERIALS AND METHODS

^{15}N-milk labelling procedure

^{15}N-labelled milk was prepared with milk from two lactating cows. The first cow (cow A) underwent three consecutive 7-day test periods as follows: during the first 5 days it received 25 g ($^{15}NH_4)_2SO_4$ (10 atom % isotope enrichment, Euriso-Top, CEA, Saint-Aubin, France) daily into the rumen *via* a permanent rumen fistula, followed by 2 days off. The second cow (cow B) received 50 g ($^{15}NH_4)_2SO_4$ daily into the rumen *via* a permanent rumen fistula during one 8-day test period. Both cows had normal diets during the experiment. The milk was collected twice a day, early in the morning and at the end of the afternoon and then frozen at –20°C.

Casein and whey protein partition

The raw pooled ^{15}N-milk (RPM) of cow B was kept frozen at –18°C until use, at which time 47 kg of the milk was thawed, skimmed and microfiltrated according to the technique used by Pierre *et al* (1992). After two successive centrifugations

at 35°C (Bernard E16, France), fat was removed from the RPM. The defatted RPM was then microfiltrated at 35°C on a 2S37 M14 1.6 m^2 Carbosep membrane (Techsep, Miribel, France) giving the retentate and the microfiltrate. The retentate was diafiltrated with sterile distilled water and native phosphocaseinate (NPPC) as well as diafiltrate were obtained. Microfiltrate and diafiltrate were then concentrated on UF P10-2 m^2 IRIS 3038-Rhône Poulenc 20 kDa membrane and gave both permeate and whey proteins (WP). The whey proteins were diafiltered and concentrated on DC 10LA-0.9 m^2 Amicon 3 kDa membrane to obtain a whey protein concentrate (WPC).

Analytical methods

The pH of the samples was measured after homogenization. Aliquots were used to measure the total nitrogen (N_{total}) using the Kjeldahl method, as well as the non-protein nitrogen (NPN), the non-casein nitrogen (NCN) and the minerals. The calcium, magnesium, sodium and potassium concentrations were determined by atomic absorption spectrophotometry using the method developed by Brulé *et al* (1974). The total flora and coliform were enumerated using PCA medium (30°C, 72 h) and VRBA medium (30°C, 24 h), respectively.

The isotopic ratio of ^{15}N/^{14}N was determined by isotope ratio mass spectrometry (IRMS) as previously described (Mahé *et al*, 1994). An aliquot of the freeze-dried samples was burned in the presence of purified oxygen in the combustion unit of an elemental analyzer (NA 1500, Fisons, UK) at 950°C. The combustion unit was coupled with an isotope ratio mass spectrometer (Optima, Fisons, UK). The N_2 isotope ratio was measured in reference to a secondary laboratory standard. For this purpose, different amounts of acetanilide with the same ^{15}N/^{14}N isotope ratio were compared with each set of samples. The N_2-pressure originating from the acetanilide samples was recorded. The ^{15}N/^{14}N isotope ratio of the acetanilide, which depends on the N_2 pressure, allowed to calculate the ^{15}N/^{14}N isotope ratio of the samples. The values were then recalculated in atom-% relative to the atmospheric nitrogen.

Statistical analysis

The results were expressed as mean ± standard deviations. Statistical analysis was performed using 1-way variance analysis (ANOVA, SAS/STAT™ 6.03, 1990, SAS Institute Inc, Cary, NC, USA).

RESULTS

^{15}N-labelling of milk

The ^{15}N enrichment of the milk was measured in each cow as a function of time (fig 1). The natural ^{15}N enrichment of milk, determined before $(^{15}NH_4)_2SO_4$ administration was 0.3679 atom-% excess (APE). The ^{15}N enrichment in cow A, which received 25 g $(^{15}NH_4)_2SO_4$ daily, reached 0.4213 APE during the 5 days, immediately dropping once the administration had stopped, and then rising again when the

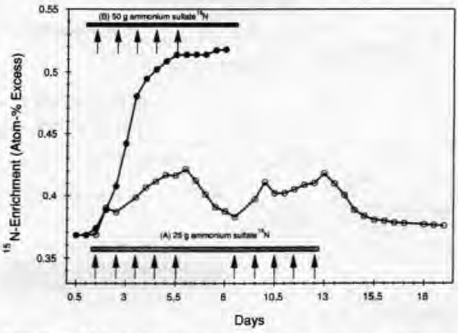

Fig 1. ^{15}N-labelling pattern of milk prepared from lactating cows receiving $(^{15}NH_4)_2SO_4$ (10 atom% isotope enrichment). Cow A received 25 g/day of $(^{15}NH_4)_2SO_4$ into the rumen *via* a permanent rumen fistula during 22 days, 5 consecutive days followed by 2 days off. Cow B received 50 g of $(^{15}NH_4)_2SO_4$ per day into the rumen *via* a permanent rumen fistula during 8 days. The arrows represent the days where $(^{15}NH_4)_2SO_4$ was administered.

Profil d'enrichissement en ^{15}N-azote du lait de vaches en lactation recevant du $(^{15}NH_4)_2SO_4$ (enrichi à 10%). La vache A recevait 25 g/j de $(^{15}NH_4)_2SO_4$ pendant 22 j, 5 j consécutifs précédant 2 j d'arrêt. La vache B recevait 50 g de $(^{15}NH_4)_2SO_4$ par jour pendant 8 j. Les flèches représentent les jours d'administration de $(^{15}NH_4)_2SO_4$.

$(^{15}NH_4)_2SO_4$ administration was repeated. In comparison, the ^{15}N enrichment of the milk in cow B, which received 50 g $(^{15}NH_4)_2SO_4$ daily, increased rapidly after the beginning of administration and reached a plateau of 0.5177 APE within 5 days.

Casein and whey protein partition

Fourty-seven kilograms of raw pooled ^{15}N-labelled milk (RPM) obtained from cow B were used to purify milk proteins (fig 2). After centrifugating the RPM, 3 kg of fat were removed and 41.16 kg of skim RPM were subjected to microfiltration which produced both a retentate and a microfiltrate (20.56 kg). From the retentate, 9.76 kg of native phosphocaseinate (NPPC) were obtained as well as 89.06 kg of diafiltrate. Both microfiltrate and diafiltrate were pooled and concentrated to obtain 7.70 kg of whey proteins (WP) which were further diafiltrated and concentrated to 1.30 kg of whey protein concentrate (WPC).

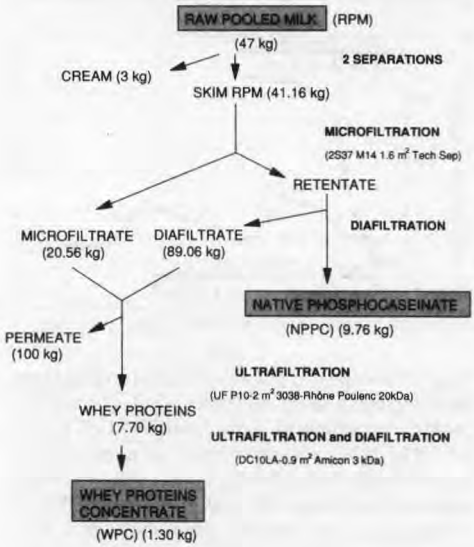

Fig 2. Preparation of 9.76 kg native micellar phosphocaseinate (NPPC) from 47 kg raw pooled milk (RPM) by the microfiltration technique.
Schéma de préparation de 9,76 kg de phosphocaséinate natif (NPPC) obtenu par la technique de microfiltration de 47 kg d'un mélange de lait cru (RPM).

Biochemical and isotopic analysis

The compositions of the raw pooled ^{15}N-labelled milk (RPM) obtained from cow B as well as its derived fractions are reported in table I. The RPM fraction was 0.5103 APE ^{15}N-enriched and contained 5.47 g N/kg made up of 73.9% casein, 21.4% whey proteins (NCN) and 4.7% non-protein nitrogen (NPN). Both the ^{15}N enrichment and the nitrogen composition remained unaffected after fat separation (skim RPM). The native phosphocaseinate concentrate (NPPC) was 0.5070 APE ^{15}N-enriched and was made up of 99.9% proteic nitrogen (N_{total}-NPN) in which the native phosphocaseinate represented 94.9% (N_{total}-NPN-NCN). The whey protein concentrate (WPC) was 0.4999 APE ^{15}N-enriched and was 96.8% proteic nitrogen. The ^{15}N enrichment of the skim milk, NPPC and WPC did not vary significantly ($P < 0.05$). The ion concentration of NPPC showed a significant ($P < 0.05$) decrease of Na^+, K^+ and a significant ($P < 0.05$) increase of Ca^{2+} compared to RPM (table I). The ion concentration of WPC showed a significant ($P < 0.05$) decrease of Na^+, K^+, Ca^{2+} and Mg^{2+} compared to RPM. The bacteriological analysis showed the presence of $2.43\ 10^4$ total flora/ml in RPM, $2.31\ 10^5$ total flora/ml in NPPC and $8.70\ 10^3$ total flora/ml in WPC. No coliform bacteria were determined in any sample of NPPC but $3.56\ 10^2$ coliform CFU/ml were enumerated in WPC UF retentate.

Table I. Nitrogen composition, ^{15}N-enrichment, pH and ion concentration of the raw pooled milk (RPM) obtained from cow B as well as its derived fractions.
Composition en azote, enrichissement en ^{15}N, pH et concentration ionique d'un mélange de lait cru (RPM) obtenu chez la vache B et ses fractions dérivées.

	pH	*N_{total} g/kg*	*NPN g/kg*	*NCN g/kg*	*^{15}N-enrichment APE*	*Na^+*	*K^+*	*Ca^{2+} mg/kg*	*Mg^{2+}*
RPM	6.65	5.47	0.26	1.17	0.5103	327	1619	1275	104
Skim RPM	6.76	5.31	0.26	1.20	0.5090	—	—	—	—
NPPC	6.83	16.96	0.02	0.86	0.5070	20	98	3319	104
Microfiltrate	6.67	0.80	0.26	0.80	0.5246	—	—	—	—
Concentrated diafiltrate	6.72	0.67	0.25	0.66	0.5255	315	1529	375	73
WPC	6.96	15.06	0.08	14.58	0.4999	102	516	479	57

DISCUSSION

The purpose of this work was to determine the conditions for the preparation of stable isotope ^{15}N-labelled milk and purified milk protein fractions. The preparation of ^{15}N-labelled milk consists of introducing ^{15}N-enriched ammonium sulfate $(^{15}NH_4)_2SO_4$ into the rumen of a lactating cow. This source of nitrogen is used by the rumen bacterial flora which incorporates ^{15}N into their amino acids. These ^{15}N-labelled amino acids are used by cows to synthesize proteins, in particular milk proteins, which become ^{15}N-enriched. In this study we demonstrated the importance of the dose of $(^{15}NH_4)_2SO_4$: whereas 25 g/day of $(^{15}NH_4)_2SO_4$ induced an enrichment of 0.4213 APE after 5 days, 50 g/day produced an enrichment of 0.5177 APE after 5 days. These enrichments are high enough since an enrichment of 0.4200 APE is needed to perform digestibility experiments with ^{15}N-labelled exogenous proteins (Mahé *et al*, 1994).

The raw pooled ^{15}N-labelled milk (RPM) was thawed, skimmed and microfiltrated to purify the milk protein fractions. As previously described (Pierre *et al*, 1992), the membrane microfiltration on RPM leads to the separation of the two liquids, microfiltrate, containing the whey proteins (WP), and retentate, composed of native calcium phosphocaseinate (NPPC) thus explaining the high calcium concentration in this sample. The RPM protein composition consisted of 22.5% WP and 77.6% NPPC. The technique of microfiltration allowed to obtain, from 47 kg RPM, WP and NPPC with yields of 34.4% and 82.5%, respectively. The WP fraction was mostly made up of ß-lactoglobulin and α-lactalbumin which could be easily purified by low pH α-lactalbumin polymerization (Pearce, 1983; Pierre and Fauquant, 1986). The enrichment of the microfiltrated purified proteins demonstrated that all the nitrogen of the milk proteins was homogeneously ^{15}N-labelled since an identical enrichment was measured in RPM, WPC and NPPC. Further characterization has to be performed regarding where the

^{15}N are located in the protein fractions, *ie* all amino acids, main chain or side chains.

In conclusion, the present study describes a rapid and accurate method for the preparation of highly enriched ^{15}N-labelled milk proteins. The membrane microfiltration technique offers the opportunity to purify milk protein fractions, *ie* casein and whey proteins, with native physico-chemical characteristics and can be scaled up industrially . These proteins are of controlled and human consumption grade composition and represent an interesting model for investigating the dynamic of nitrogen, the dietetic requirements and protein metabolism in the healthy and sick humans.

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