Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXI, No. 5

PRODUCTION AND PURIFICATION OF MIXED ^{14}C-LABELLED PEPTIDES DERIVED FROM PLANT BIOMASS

John R. Ling, P. Bronwen Cooper, Stephen J. Parker and Ian P. Armstead

Department of Biochemistry, School of Life Sciences,
The University College of Wales, Aberystwyth SY23 3DD, Wales, U.K.

SUMMARY

Procedures are described for the production and purification of ^{14}C-labelled peptides of mixed composition, derived from phytomass. Barley seeds (*Hordeum vulgare*) were germinated and grown in the dark for 6 days. On day 7, the seedlings were exposed to light in a $^{14}CO_2$ atmosphere for 24 h. The plant leaves were harvested and their water-soluble ^{14}C-labelled proteins extracted. These ^{14}C-proteins were partially digested by sequential incubation with pepsin, α-chymotrypsin and trypsin. The resulting ^{14}C-labelled peptides were separated from contaminating amino acids by elution from columns of copper-Chelex resin, and finally fractionated by gel-filtration chromatography and assigned to groups according to molecular size. The purified ^{14}C-peptides ranged in relative molecular mass up to approximately 5,000, possessed a purity in excess of 97%, and were radiolabelled in all amino acid residues with an average specific radioactivity of 450 Bq/μmol. The methods described can be readily adapted to produce not only mixed ^{14}C-labelled peptides of any required attribute, such as molecular size or ionic charge, but also mixed ^{14}C-proteins or ^{14}C-amino acids.

Key words: biosynthesis; ^{14}C-proteins; proteolysis; gel-filtration; ^{14}C-peptides; ^{14}C-amino acids.

INTRODUCTION

There is an increasing interest in the biochemical and physiological functions of peptides. This interest has been paralleled by an increase in the number of commercially available peptides, including some that are radiolabelled. However, these radiolabelled peptides are, almost exclusively radiolabelled with either ^{3}H or ^{125}I (such as, [*tyrosyl*-3,5-^{3}H]vasopressin or (3-[^{125}I]*iodotyrosyl*)glucagon); commonly restricted to the radiolabel appearing on only one amino acid residue (such as, [*tyrosyl*-2,6-^{3}H]oxytocin); often combined with non-protein amino acids (such as, [*glycine*-1-^{14}C]hippuryl-L-histidyl-L-leucine); usually modified, for example as methyl or formyl derivatives (such as, [^{14}C]methylated insulin or

0362–4803/92/050417–10$05.00

Received 14 January, 1992
Revised 3 February, 1992

N-[*formyl*-^{14}C]-methionyl-leucyl-phenylalanine); generally of specific bioactivity (such as, (3-[^{125}I]*iodotyrosyl*)vasoactive intestinal polypeptide and several of the above examples); and invariably expensive. There are no mixtures of simple ^{14}C-peptides commercially available.

For many experimental approaches, a wide range of radiolabelled peptides is desirable. Our studies of peptide metabolism by anaerobic microorganisms demanded a supply of peptides, radiolabelled in each amino acid residue, ideally derived from plant material, of different compositions, but grouped according to molecular size. This paper describes procedures that are both uncomplicated and inexpensive that can produce such mixtures of ^{14}C-labelled peptides. These procedures can also be adapted to yield mixed ^{14}C-proteins or ^{14}C-amino acids.

MATERIALS AND METHODS

Growth and ^{14}C-labelling of Plants

Barley seeds (*Hordeum vulgare* cv. Kym or Gerbel) were sown in sterilized soil and incubated at 25° C in the absence of light. The soil was watered daily.

On the seventh day after sowing, about 100 etiolated seedlings were transferred to a glass culture vessel (height 330 mm x 180 mm i.d.; Quickfit) fitted with a double socket lid. One socket contained a thermometer, the other was sealed with a rubber stopper. A 50 ml glass beaker, lined with shredded filter paper, was suspended from the latter socket. Inside the beaker was a plastic vial containing 18.5 MBq of solid barium [^{14}C]carbonate (specific activity, 11 MBq/mg; 2.18 GBq/mmol; Amersham International). These procedures were carried out in a low illumination laboratory.

The jar was sealed and 3 ml 2 M lactic acid was injected onto the barium [^{14}C]carbonate via the rubber stopper. Immediately after this, the seedlings were exposed to artificial light. Heat filters of circulating water were placed between the jar and the light sources to maintain an internal temperature of between 25 and 30° C. The photosynthetically active radiance inside the jar was 300 μeinsteins m^{-2} sec^{-1} (Macam Q101 lightmeter; Macam Photometrics, Livingstone, Scotland).

After 24 h of such exposure, 5 ml 2 M NaOH was injected through the rubber stopper onto the shredded filter paper. The seedlings were illuminated for a further 24 h. Plant material was then harvested by cutting the stems at the point of emergence of the first leaves.

Isolation of Water-soluble ^{14}C-labelled Plant Proteins

All the subsequent extraction procedures were carried out at 4° C unless stated otherwise. The harvested plant leaves (typically 10 g fresh material) were homogenised in a Waring blender (3 x 60 sec at full speed) with buffer (5 ml/g fresh tissue) composed of 0.05 M Tris; 0.01 M EDTA; 0.01 M dithiothreitol and 0.03 M sodium dithionite, pH 8.0.

The homogenate was strained through several layers of cheesecloth and the filtrate was brought to 35% saturation with solid ammonium sulphate. The mixture was stirred for 30 min and then centrifuged at 27,000*g* for 15 min. The supernatant fraction was brought to 60% saturation with ammonium sulphate, again stirred for 30 min and centrifuged at 27,000*g* for 15 min. The resulting pellet was dissolved in 15 ml 0.1 M ammonium bicarbonate containing 450 mg sodium dodecyl sulphate (SDS) and boiled for 10 min. The solution was then cooled and ice-cold trichloroacetic acid (TCA) was added to give a final concentration of 20% (w/v). After being left at 0° C for 1 h, the sample was centrifuged at 27,000*g* for 15 min and the pellet was washed twice with 20% (w/v) TCA. The pellet was redissolved by the careful addition of a minimal volume of 2 M NaOH at room temperature. The entire sample was loaded onto a column (400 mm x 25 mm i.d.) of Sephadex gel (G-25 fine) and eluted with distilled water. The column effluent was monitored at 280 nm and the fractions corresponding to the first major peak (at approximately the void volume of the column) were collected, pooled and concentrated using a stirred ultrafiltration cell (Amicon; membrane PM10), which also removed compounds of relative molecular mass of less than approximately 10,000. The remaining solution (5 ml), which contained mixed, water-soluble, ^{14}C-labelled proteins, was freeze-dried.

Production, Purification and Fractionation of ^{14}C-labelled Peptides

These freeze-dried, ^{14}C-labelled proteins were digested by methods similar to those of Allen (1); they were dissolved (50 mg/ml) in 90% formic acid and then a

19-fold volume of 0.1 mM HCl containing 1% (w/v) pepsin (lyophilized powder from porcine stomach mucosa; Sigma Chemical Co. Ltd., Poole, UK) was added slowly. This mixture was incubated for 2 h at 25° C. The reaction was stopped by freezing and freeze-drying the digest. This was then redissolved (5 mg/ml) in 0.1 M ammonium bicarbonate (pH 8.0) containing 0.1 mM calcium chloride. α-Chymotrypsin (Type II from bovine pancreas; Sigma) was added at 1% (w/v) and the mixture was incubated at 37° C for 2 h. After this time, trypsin (type III from bovine pancreas; Sigma) was added at 1% (w/v) and the mixture incubated for a further 2 h at 37° C. The reaction was stopped by freezing at -20° C and the sample was freeze-dried.

Contaminating free amino acids, produced during the proteolytic digestions, were removed by the chromatographic separation method 2, as described by Armstead and Ling (2). A sample (10 mg ^{14}C-labelled enzymic digest) was dissolved in 2 ml 0.1 M acetic acid and loaded onto a column of copper-Chelex resin (125 mm x 25 mm i.d.). Fractions (8 ml) eluted from this column were collected and either assayed for radioactivity or pooled and repeatedly washed with distilled water and dried under reduced pressure by rotary evaporation. Residual copper was removed from pooled samples by dissolving them in 5 ml distilled water and eluting them from a column (60 mm x 7.5 mm i.d.) of Chelex 100 with 0.01 M ammonia solution; the latter was removed by rotary evaporation.

Samples (up to 5 ml) of these purified ^{14}C-labelled peptide preparations were fractionated by loading them onto a gel-filtration (Sephadex G-25 or G-15 fine) column (230 mm x 25 mm i.d.) that had been calibrated with known relative molecular mass standards of peptides, amino acids, cytochrome C and vitamin B_{12}. The column was eluted with distilled water and fractions (each 4 ml) of the radiolabelled peptides were pooled, according to the range of molecular size required, filter-sterilised (0.22 μm pore size) and stored at -20° C.

Methods of Analysis

Spectral scans were produced using a Pye Unicam SP8-150 spectrophotometer. SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Weber and Osborn (3); the gels were stained with Coomassie Brilliant Blue G-250. Protein concentrations were measured by the biuret method and the

presence of peptide bonds was detected by the Folin-Ciocalteu procedure (4). Free amino groups were assayed with ninhydrin reagent. Carbohydrates were hydrolysed and the monomers converted to alditol acetates, which were then separated and quantified by GLC (5). Total nucleic acid concentrations were estimated by a fluorimetric procedure (6); DNA was measured by the method of Labarca and Pangen (7) and the RNA concentration was calculated by difference. The volatile fatty acid content of samples was quantified by a GLC method (8). Long-chain fatty acids were detected and quantified as their derivatised methyl esters by GLC (9). Protein-containing samples were deproteinised with ice-cold picric acid (10). Acid hydrolysis was performed by refluxing samples with 6 M HCl for 22 h. Amino acid contents of samples were determined by cation-exchange chromatography and ninhydrin detection with an amino acid analyser (Model 5; Locarte Co., London) fitted with a Roseate data management system (Drew Scientific, London) as described by Ling and Buttery (11). Radioactivity was measured with a liquid scintillant (Ecoscint; National Diagnostics, Manville, NJ) and a scintillation counter (Model SL 30; Intertechnique SA; Plaisir, France). Quenching was, where appropriate, corrected by the external-standard channel-ratio facility.

RESULTS AND DISCUSSION

The incorporation of $^{14}CO_2$ into plant biomass was the chosen approach for producing ^{14}C-labelled peptides. Plants have distinct advantages over the use of either microbial or animal material; they are cheap, easy to cultivate and their nutritional requirements are simple and non-fastidious.

Initially, maize (*Zea mays*) was the selected plant species. However, brown material, probably due to the presence of large amounts of phenols and quinones, interfered with the protein extraction procedures. Furthermore, the specific radioactivities of the isolated water-soluble ^{14}C-proteins from this source were always unacceptably low. The use of barley (*Hordeum vulgare*) overcame both of these problems.

Isolation of Water-soluble ^{14}C-labelled Plant Proteins

Protocols that are specific for extracting one particular radiolabelled plant protein, such as [^{14}C]ribulose-1,5-bisphosphate carboxylase, already exist (12).

But the primary intention of the extraction procedures developed and reported here was to produce as high a purity and yield as possible of the many water-soluble ^{14}C-proteins that were present in the plant biomass.

Throughout the development of these procedures, extracts were monitored for non-protein compounds. Extraction methods were modified until the assays for RNA, DNA, carbohydrates and fatty acids indicated that these impurities were minimal. Nucleic acid contamination, which on some occasions was as high as 10% of the protein content, was found to be a particular problem. Incubation of extracts with RNAse and DNAse followed by dialysis had little effect. Eventually, the inclusion of the SDS step reduced the nucleic acid contamination to 3.5 μg RNA/mg ^{14}C-protein, while DNA was undetectable. Concentrations of carbohydrates in the final ^{14}C-protein mixture were reduced to less than 3.0 μg/mg protein and fatty acids were not detectable. Spectrophotometric scans (240-360 nm) of this radiolabelled extraction product also indicated that it was essentially protein. Furthermore, SDS-polyacrylamide gel electrophoresis showed that it was composed of six major protein bands that ranged in relative molecular mass from 560,000 to 53,200.

These reported extraction procedures yielded approximately 3.5 mg purified ^{14}C-protein/g fresh weight plant material with a specific radioactivity of approximately 2,350 Bq/mg protein.

Acid hydrolysis and subsequent analyses of the ^{14}C-protein mixture showed that sixteen amino acids were present (cysteine and tryptophan were not assayed) and all were radiolabelled (see, Table 1). Their average specific radioactivity was 347 ± 75.1 Bq/μmol (mean ± SE for 16 observations), with a range from 1,250 Bq/μmol for phenylalanine to 76 Bq/μmol for lysine.

Production, Purification and Fractionation of ^{14}C-labelled Peptides

In preliminary trials, trypsin was used to produce ^{14}C-labelled peptides from these ^{14}C-labelled proteins. This procedure resulted in high proportions of both large ^{14}C-peptides (>5,000 relative molecular mass) and ^{14}C-amino acids, but an insignificant portion of intermediate-sized ^{14}C-peptides. Additional trials showed that sequential digestion with pepsin, α-chymotrypsin and trypsin produced the most satisfactory range of ^{14}C-peptide sizes. This is shown in

Fig. 1 which represents a typical elution profile from a gel-filtration column of the ^{14}C-peptides and ^{14}C-amino acids derived from this digestion procedure.

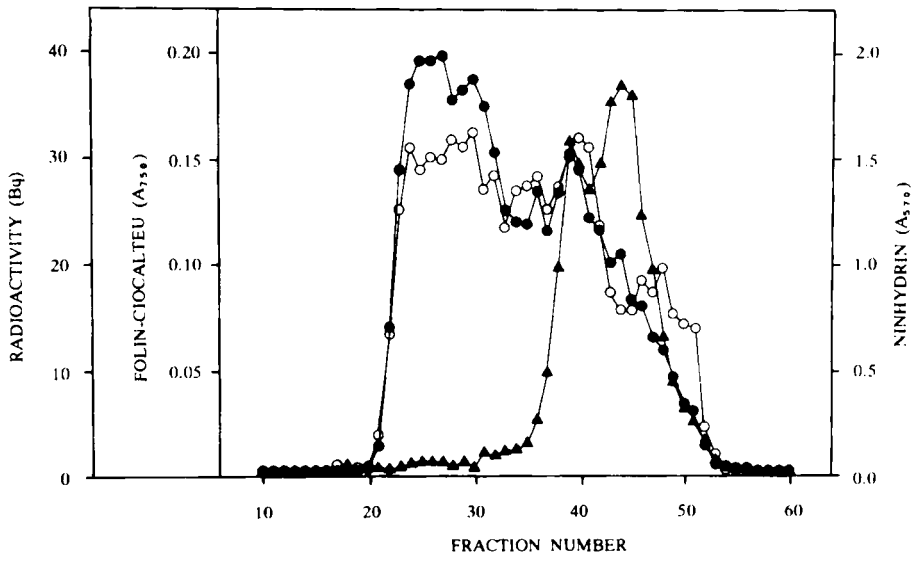

Fig. 1. Fractionation pattern of a ^{14}C-labelled peptide preparation eluted from a gel-filtration column (230 mm x 25 mm i.d.; Sephadex G-25 fine) and assayed for radioactivity (●), and by the Folin-Ciocalteu (○) and ninhydrin (▲) reactions. The column was eluted with distilled water and the fraction volumes were 4 ml each. The recorded values refer to assays performed on 100 μl fraction subsamples. The ^{14}C-peptides were prepared as described under Materials and Methods.

The rapid increase of both radioactivity values and Folin-Ciocalteu absorbances from about fraction 20 is indicative of the elution of ^{14}C-labelled peptides with a relative molecular mass of the order of 2,500. These values for radioactivity and peptidyl material were approximately parallel during the elution of the remaining smaller ^{14}C-peptides; this suggests that the peptides produced were of similar specific radioactivity. The rise in ninhydrin absorbance from fraction 35 onwards was caused mainly by the elution of free amino acids, but also some relatively small peptides.

Table 1 shows that the constituent amino acid contents of the produced mixed ^{14}C-peptides were generally similar to those of the ^{14}C-labelled proteins, though methionine was too low to be measured accurately. On the other hand,

Table 1. Concentration and radioactivity of constituent amino acids in ^{14}C-labelled protein and ^{14}C-labelled peptide preparations.

Amino acid	^{14}C-labelled proteins		^{14}C-labelled peptides	
	μmol/mg protein	Bq/mg protein	μmol/mg peptide	Bq/mg peptide
Asp	0.80	318.0	0.84	471.3
Ser	0.37	72.3	0.44	112.6
Thr	0.24	70.5	0.31	107.9
Glu	0.88	274.9	0.95	376.0
Pro	0.53	53.1	0.81	77.1
Gly	0.76	175.9	0.80	210.9
Ala	0.80	338.5	0.89	400.6
Val	0.68	98.0	0.68	135.9
Met	0.01	5.2	*	*
Ile	0.36	52.3	0.40	57.8
Leu	0.65	142.8	0.79	229.2
Tyr	0.16	122.2	0.05	60.8
Phe	0.33	413.7	0.27	532.4
His	0.18	23.2	0.39	63.1
Lys	0.71	53.8	0.40	58.8
Arg	0.41	144.0	0.34	143.8

*, not measured.

their specific radioactivities were consistently higher with a mean value of 450.5 ± 128.6 Bq/μmol (mean ± SE for 15 observations).

To further characterise the produced ^{14}C-peptides, they were fractionated by gel-filtration and divided into groups, designated A - D, by pooling several elution fractions. Typical results are shown in Table 2. Plots of K_{av} versus log relative molecular mass for the calibration standards were approximately linear. Nevertheless, separation of compounds by gel-filtration often deviates from an ideal elution profile, so the allocation of a relative molecular mass range to a particular group is recognised to be an approximate guide only.

Table 2 shows that the average specific radioactivity of the peptide groups was 480 Bq/μmol amino acid present. Group D had a significantly higher specific radioactivity; this is because peptides containing a relatively large proportion of aromatic amino acids are typically retarded in their elution from columns of Sephadex, so such peptides would accumulate in group D, and because both tyrosine and phenylalanine possessed the highest specific radioactivities of all the amino acids present (see, Table 1).

Table 2. Characteristics of ^{14}C-labelled peptide groups fractionated by gel-filtration on a column of Sephadex G-15 fine.

Peptide group	Elution fraction numbers	Relative molecular mass[a]	Specific radioactivity (Bq/μmol)[b]
A	30 - 35	1000-2000	474
B	36 - 41	500-1000	459
C	42 - 47	200-500	449
D	48 - 53	<200	547

[a] determined by gel-filtration chromatography.
[b] determined as the average radioactivity/μmol of each amino acid present.

If a typical constituent peptide of group B is assumed to contain six amino acid residues, then its specific radioactivity can be calculated to be 2.8 kBq/μmol peptide. This is less than 3% of the value of a commercially available peptide derivative, such as [*glycine*-1-^{14}C]hippuryl-L-histidyl-L-leucine, nevertheless, it is still of sufficient magnitude for the purposes of many biological studies.

Contamination data for these fractionated peptide groups were even less than those for the ^{14}C-labelled protein mixture. They were 4.9 μg, 0.9 μg and 0.3 μg/mg peptide for RNA, DNA and carbohydrates respectively. Fatty acids were again undetected. The large contamination (as much as 20.3% w/w) caused by the presence of free amino acids was effectively reduced, by the method of Armstead and Ling (2), to 19 μg/mg peptide. Thus it may be stated that the purity of the ^{14}C-labelled peptides within the various fractionated groups was in excess of 97%.

If more precisely defined ^{14}C-peptide groups are required then other, more rigorous procedures, such as size exclusion high performance liquid chromatography, could be used. If different fractionation criteria, such as ionic charge or the presence of specific residues, are required, then techniques such as ion-exchange, or affinity chromatography could be employed. An additional advantage of the procedures described here is that the radiolabelled products are in aqueous solution and therefore particularly suitable as

substrates for incubation in biological systems, as already demonstrated (13, Armstead and Ling, unpublished work).

In conclusion, the preparative procedures reported here are capable of producing groups of ^{14}C-labelled peptides with a purity in excess of 97%, that are generally radiolabelled, of various molecular sizes and mixed composition. These ^{14}C-peptides are relatively cheap to produce and of sufficient specific radioactivity to be used as substrates for many experimental purposes. In addition, these procedures can be easily adapted to meet other particular requirements, such as the production of ^{14}C-proteins or ^{14}C-amino acids.

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support from the Ministry of Agriculture, Fisheries and Food, the Science and Engineering Research Council and the Agricultural and Food Research Council, U.K.

REFERENCES

1. Allen G. - Laboratory Techniques in Biochemistry and Molecular Biology; Sequencing of Proteins and Peptides (Work T.S. and Burdon R.H., Eds.), Vol. 9, pp. 73-159, Elsevier, Amsterdam, (1981)
2. Armstead I.P. and Ling J.R. - J. Chromatog. 586: 259-263 (1991)
3. Weber K. and Osborn M. - J. Biol. Chem. 244: 4406-4412 (1969)
4. Lowry O.H., Rosebrough, N.J., Farr, A.L. and Randall R.J. - J. Biol. Chem. 193: 265-275 (1951)
5. York W.S., Darvill, A.G., McNeil, M., Stevenson, T.T. and Albersheim P. - Methods of Enzymology (Weissbach A. and Weissbach H., Eds.), Vol. 118, pp. 3-40, Academic Press, Orlando, FL. (1986)
6. Le Pecq J-B. and Paoletti C. - Analyt. Bioch. 17: 100-107 (1966)
7. Labarca C. and Pangen K. - Analyt. Bioch. 102: 344-352 (1980)
8. Denholm A.M. and Ling J.R. - Arch. Anim. Nutr., Berlin 37: 327-334 (1987)
9. Draper S.R. - Biochemical Analysis in Crop Science, pp. 80-90, Oxford University Press, Oxford (1976)
10. Denholm A.M. and Ling J.R. - Appl. Environ. Microbiol. 55: 212-218 (1989)
11. Ling J.R. and Buttery P.J. - Brit. J. Nutr. 39: 165-179 (1978)
12. Thomas H. and Huffaker R.C. - Plant Sci. Lett. 20: 251-262 (1981)
13. Cooper P.B. and Ling J.R. - Proc. Nutr. Soc. 44: 144 (1985)