GLOBAL JOURNAL OF PURE AND APPLIED SCIENCES VOL. 7, NO. 2, MARCH 2001:213-217
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ISOLATION OF MILK GRP IMMUNOREACTIVE PEPTIDE

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(Received 29 February 2000; Revision accepted 12 November 2000)

ABSTRACT

Gastrin Releasing Peptide (GRP) has been isolated from bovine milk and the molecular mass and partial amino acid sequence elucidated. GRP immunoreactivity was detected by radioimmunoassay and the immunoreactive peptide was purified- to homogeneity using reverse phase High Pressure Liquid Chromatography (rp-HPLC). ^{252} Cf-plasma de sorption mass spectroscopy of the purified peptide indicated a molecular mass of 2810Da. Gas phase sequencing established the sequence of 15 of the first 20 residues. This structure indicated homology with a region of bovine alpha-sl casein, residues 101-120. Examination of alpha- s1 casein structure established the tentative sequence of milk GRP as: LKKYKVPQLEIVPNSAEER (LHSM). This 23 residue peptide has a similar molecular mass to that derived by mass spectroscopy when phosphorylation of ser-15 (Ser 115 in alpha casein) is taken into account. Generation of this peptide could be achieved by single site cleavage of peptide at cleavage sites Arg and Lys, with removal of the Lys residue from the C-terminus. The isolation and purification of a GRP immunoreactive peptide from bovine alpha casein is further proof of the large reservoir of peptides in bovine milk proteins.

Key words: Isolation, Purification, Milk, GRP-immunoreactive Peptide.

INTRODUCTION

Since the discovery of bombesin in 1970 (Erspamer et al., 1970), the primary structure of its mammalian counterpart, Gastrin Releasing Peptide (GRP) has been elucidated (McDonald et al, 1979). Two GRP-like decapeptides have been isolated from porcine spinal cord, designated 'Neuromedin B and Neuromedin C (Minamino et al., 1983, Minamino et al., 1984). Jahnke and Lazarus described a bombesin-immunoreactive peptide in bovine milk (Jahnke and Lazarus, 1984). Previously, the presence of GRP like-immunoreactivity had been reported in milk and many milk products (Frank- Peterside, 2000). GRP Like-IR has also been detected in low levels in bovine plasma. Lower level of GRP-IR was detected in rat mammary tissue. An immunocytochemical study of rat mammary tissues for GRP was negative. One possible explanation for the above observation is that milk GRP like-IR could be due to a GRP-sub family peptide not yet identified. It is well recognized that many hormones fall into groups or families, members of which are related to each other by sequence homology and by similarities of conformation. Similarities have also been reported for several polypeptide hormones of apparently diverse structure and function (Epand, 1983). However, molecular heterogeneity is a feature of all animal proteins and polypeptides. Such heterogeneity results from post-translational proteolysis, modification of translation products by derivatization of individual amino acid residues and cleavage of peptide bonds. In addition, different types of cells may express genes encoding the same regulatory peptide or slightly different genes originally duplicated from a single gene and subsequently modified by varying degrees of mutation. The basic principle of most immunologic reactions is the ability of an antibody to combine with a particular amino acid sequence. This sequence may be present in a family or sub family of peptides. Hence, the necessity for isolation, purification and amino acid sequencing of peptides of unknown primary structure.

MATERIALS AND METHODS

Milk Extraction

One liter of skimmed bovine (Dunnes Stores Brand milk was purchased from a local supermarket before the expiry date. The pH was reduced from 6.3 to 3.8 using glacial acetic acid. At a pH below 4, many milk proteins precipitate. The milk sample was centrifuged at 3,500 x g for 30min and the supernatant was decanted and extracted with an equal volume of acidified ethanol for 24hr. The sample was centrifuged at 3,500 x 9 for 30min. The supernatant was decanted and rotary evaporated to remove the alcohol. After centrifugation, the supernatant was freeze-dried and later reconstituted with 120ml of 2m acetic acid. Peptides in the sample were concentrated using a Sep-pak C-18 cartridge (Millipore). The cartridges were eluted with 1.3ml of 0.1% (v/v) trifluoroacetic acid (TFA) in acetronitrile. The sample was injected onto 1.6 x 90cm column of sephadex G-50. Table 1 is a summary of the milk extraction procedure.

Gel permeation chromatography

The concentrated milk extract was chromatographed on a Sephadex G-50 column, equilibrated with 2M acetic acid. The column was eluted at 11.2mlh^{-1} and 90 fractions of 2.8ml were collected. A 100μl aliquot of each fraction was diluted to 1000μl with assay buffer. Each fraction was assayed for GRP-IR.

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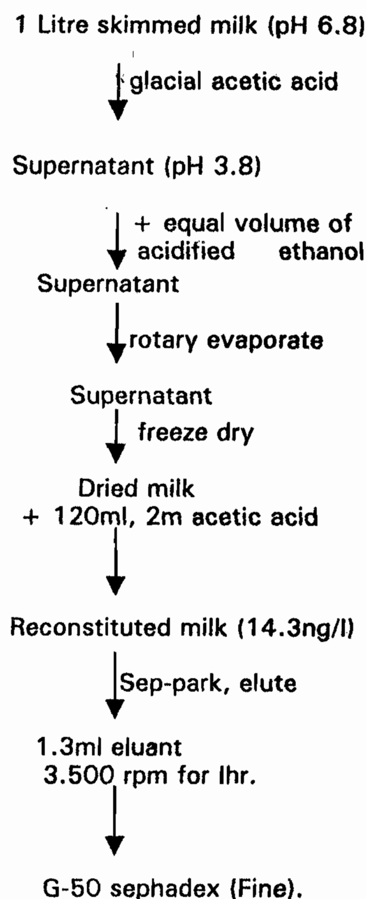
10^{6}pgml^{-1} of synthetic Neuromedin C (NMC) standard was also chromatographed on the same column.

Table 1: Milk extraction procedure



Reverse - phase HPLC

Fractions 52-64 above, with GRP-IR were pooled and chromatographed using a waters Z module system fitted with different columns as indicated in Table 2. The columns, in each min, were equilibrated with 0.1% TFA/H_2O prior to sample injection. 0.1% TFA/CH_3CN (v/v) was used for the elution of the sample. All columns were calibrated to run at a flow rate of 1.5ml min^{-1} with gradients as indicated in Table 2. Seventy fractions were collected into polypropylene tubes, in each run. 10μl of each fraction was diluted to 100μ1 with assay buffer and assayed directly for GRP-immunoreactivity; as described by Shaw et al; 1987.

Bombesin Radioimmunoassay

Bombesin (GRP) radioimmunoassay was carried out according to the method of Shaw et al; 1987 with antiserum R-354(4). This antiserum was raised in a rabbit immunized with a conjugate prepared by coupling porcine GRP to bovine serum albumen using glutaraldehyde. It exhibits full molar cross-reactivity with bombesin and GRP-IR but exhibits no cross. reactivity towards structurally unrelated peptides. All samples were assayed in duplicate serial dilutions. All data were calculated relative to a porcine GRP standard (Shaw et al., 1987).

Amino Acid Sequencing

The 7th rp- HPLC run resolved two peaks of immunoreactivity .These two peaks were pooled and subjected to amino acid sequencing. 30pmol of purified milk GRP was subjected to ^{252}Cf-plasma desorption mass spectroscopy using a Biolon 20K time-of flight instrument. The sample was dissolved in 0.1% (v/v) aqueous TFA and applied to a nitrocellulose-covered target which was spin-dried and micro-rinsed. The peptide was subjected to automated Edman degradation using an Applied Biosystems 470A gas-phase sequencer. The limit for detection of PTH-amino acids was 0.5pmol.

RESULTS

Gel Permeation Chromatography

The chromatographic profile of milk GRP-immunoreactivity is shown in Figure I. Milk GRP-IR coeluted with synthetic NMC.

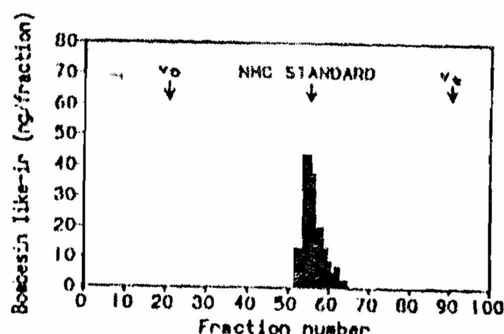


Figure 1: Gel permeation chromatogram (Sephadex 0-50) of 1 Litre of bovine milk. Fraction coinciding with the void volume (Vo) and the total volume (Vt) of the column are indicated.

Reverse Phase HPLC (rp-HPLC)

GRP like- IR from the G-50 column, fractions 54-58, were pooled and injected onto the rp-HPLC system. Figures 2 and 3 are profiles of the 6th and 7th rp-HPLC runs.

Peptide Isolation

Plasma desorption mass spectroscopy of milk GRP confirmed its purity and determined the molecular mass as 2810Da. Automated Edman degradation established a partial primary structure as stated below:

X- LYS-X- TYR- LYS- VAL- PRO- GLN- LEU-GLU- ILE- VAL- PRO-ASN-X-ALA-X-GLU-LEU (X-no identified amino acids of these cycles).

Table 2: Chromatographic Procedure (B = 0.1% TF A/acetonitile)

Procedure	Column type	Fraction number	Gradient
Gel permeation	G-50 Sephadex		20mlh^{-1}
1st rp-HPLC	Partisil 10-ODS	54-58	1 %/min B 70min.
2nd rp-HPLC	Vydac C8	38-41	0-15%B5min. 15-45%B60min.
3rd rp-HPLC	Vydac C4	25-30	0-15%B5min. 15-35%B65mins.
4th rp-HPLC	Supelcosil LC-308	30-32	0-10%B5min 10-30%B60min
5th rp-HPLC	Supelcosil LC-18 DB	38-40	0-15%B5min. 15-35%B60min.
6th rp-HPLC	Vydac C4	41-43	0-10%B 5min. 10-30%B 60min.
7th rp-HPLC	Supelcosil LC-308	45-46	0-10%B 5min. 10-30%B 60min.
Sequencing		32,37	

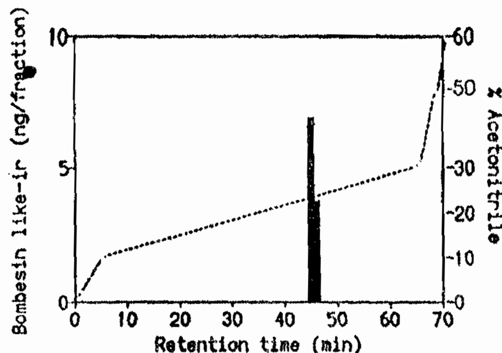


Figure 2: rp-HPLC profile (sixth run) of GRP immunoreactivity in gel permeation chromatographic fractions of bovine milk.

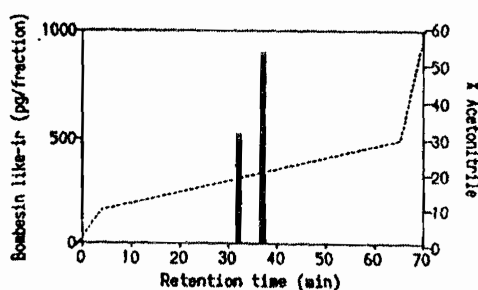


Figure .3: rp-HPLC profile (seventh run) of GRP immunoreactivity in gel permeation chromatographic fractions of bovine milk.

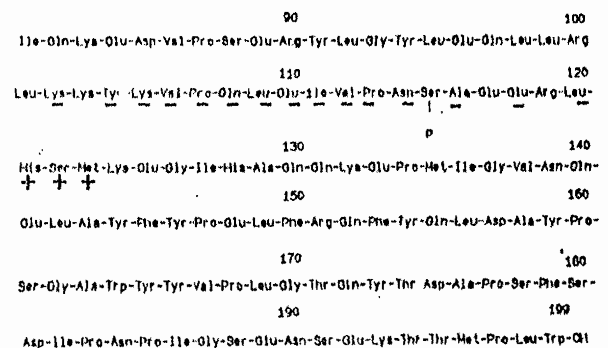


Figure 4: Representation of bovine alpha-s1 casein (81-199) Indicating Isolated milk GRP-IR peptide (101-120, -) and the tentative structure (101-123, +),

Table 3 is the amino acid sequence of the milk GRP-IR peptide.

DISCUSSION

The recovery of the GRP- like peptide from milk was low due to the complex nature of milk. This complex nature, made extraction difficult. There was a great deal of interference from other milk components. However, acidification of milk to pH 3.8, precipitated most of the milk proteins reducing the non-specific interferences. The series of extraction steps shown in Table 1 successfully concentrated milk GRP immunoreactivity to approximately 14.3ngl^{-1}. This was followed by a series of chromatographic runs as shown in Table 2, which resolved two peaks of GRP-IR, the first peak probably representing an oxidized form. Mass spectroscopy has indicated the milk GRP immunoreactive peptide to be of molecular mass, 2810Da. The presence of milk bombesin immunoreactive peptide was first reported by Jahnke and Lazarus in 1984 (Jahnke and Lazarus,1984). Mass spectroscopy is a considerably more accurate method of molecular mass determination than gel filtration chromatography (personal communication). Gas phase sequencing established the sequence of

Table 3: Amino acid sequence on milk GRP Immunoreactive Peptide

Cycle No.	PTH-a.a	Yield (pmol)
1.	-	-
2.	Lys (K)	18
3.	-	-
4.	Tyr (Y)	6
5.	Lys (K)	7
6.	Val (V)	27
7.	Pro (P)	27
8.	Gln (O)	23
9.	Leu (L)	21
10.	Glu (E)	16
11.	Ile (I)	18
12.	Val (V)	18
13.	Pro (P)	15
14.	Asn (N)	10
15.	-	-
16.	Aia (A)	11
17.	-	-
18.	Glu (E)	12
19.	-	-
20.	Leu (L)	3

Table 4 C-terminal decapeptides of GRP related peptides

Peptides	*Amino acid sequence*
Porcine GRP	GNHWAVGHLM NH_2
Human GRP	GNHWAVGHLM NH_2
Neuromedin B	GNLWATGHFM NH_2
Neuromedin C	GNHWAVGHLM NH_2
Bombesin	GNQWAVGHLM NH_2
Bovine milk GRP	NSAEER (LHSM)

15 of the first 20 residues. This structure indicated homology with residues 101 to 120 of alpha-sl bovine casein. However, regulatory peptides with molecular mass of about 281ODa are usually about 25-27 amino acid residues in length suggesting that the sequence above is probably incomplete. Examination of the structure of bovine alpha-sl casein; established the tentative sequence of milk GRP as LKKYKVPQLEIVPNSAEER (LHSM). Figure 4 is a representation of bovine alpha-sl casein (81-199) indicating isolated milk GRP-immunoreactive peptide (101-120) and the tentative structure, 101-123. This 23 residue peptide has a similar molecular mass to that derived by mass spectroscopy when phosphorylation of Ser-15 (Ser 15 in alpha casein) is considered. Generation of this peptide is possible as cleavage could occur at two sites, Arg and Lys, with removal of the Lys residue from the C-terminus. Lazarus and co-workers in 1984, reported milk bombesin immunoreactive peptide to be trypsin sensitive (Lazarus et at., 1984). The presence of three lysine residues is evidence that such cleavage by trypsin, is possible. The C-terminus of the bombesin group of peptides have the following amino acid sequence, HIS-LEU- MET -NH_2 as shown in Table 4. These three amino acids are present in the C-terminal of bovine alpha- sl casein 101-123, His and Met being at identical positions and Leu being two amino acid residues away from GRP C-terminal LEU. It is therefore possible that R-354(4) is reacting with this region of the milk peptides. It is also possible that in the 3-dimensional structure of bovine alpha-sl casein, LEU, HIS and MET are aligned, so that the 3 amino acids mentioned above are in similar positions to the C-terminal GRP .The existence of endogenous opioid peptides in various mammalian tissues has been reported. These, range in size, from 5-31 amino acid residues with N-terminal sequence of TYR-GLY-GLY- PHE (Brantl et al., 1981). This sequence is presumed to be responsible for their opioid properties. More recently, the B-casomorphins have been discovered with N-terminal amino acid sequences of TYR – PRO - PHE- PRO. Different though these primary structures are, it has been reported that B-casomorphins -7, -6, -5 and -4 displayed opioid activity in an opiate receptor binding assay (Brantl et al.,1981). The C-terminal tetrapeptide amid fragment Trp-Met-Asp- Phe - NH_2, common to all gastrins is reported to display the full range of physiological actions of the natural hormone. About 500 derivatives/analogues of this tetrapeptide were prepared by Morley (1968), for structure/activity studies. He reported that within the tetrapeptide, changes may be made in the Trp, Met and Phe positions. The analogues thus formed still retained their gastric acid secretory activity. He concluded that these positions are concerned only with binding at the site of action. However, small changes of the aspartyl residue resulted in loss of activity pointing to a functional, rather than binding role, for this residue (Morley, 1968). This observation indicated that the requirement for bioactivity is a correct peptide conformation and at least a single amino acid residue for bioactivity. The N-terminus of the peptide may be necessary for the protection of the bioactive C-terminus from in vivo degradation. It may also be needed for effective transport of the active site along the complicated pathway between the site of release to the site of action. This phenomenon, though reported for gastrin, might apply to other peptides. In the extraction procedure carried out in this study, oxidation of milk GRP immunoreactive peptide was observed. Lazarus and co-workers in 1986 also reported the

oxidation of the milk GRP immunoreactive peptide (Lazarus et al., 1986), The presence of a MET residue at position 123 of bovine alpha-sl casein makes oxidation of this peptide a possibility. The biological activity of the milk GRP immunoreactive peptide has been reported (Jahnke and Lazarus, 1984; Lazarus et al., 1984, Lazarus et al., 1986). All studies reported milk GRP immunoreactive peptide to possess biological activities similar to bombesin/GRP. Hence the possibility of the binding of the milk GRP immunoreactive peptide to GRP receptors.

ACKNOWLEDGEMENT

This work was funded by a Ph.D. studentship from the Nutritional Consultative Panel of the UK Dairy Industry to N.U. Ekeke (now N.Frank-Peterside). Experiments were carried out in the laboratory of the Department of Medicine, The Queen's University of Belfast, Northern Ireland. I acknowledge that the amino acid sequencing of the milk GRP was performed by Dr. L. Thim, of Novo Nordisk *A/S,* Denmark.

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