Purification and characterisation of a new hypothalamic satiety peptide, cocaine and amphetamine regulated transcript (CART), produced in yeast

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Abstract Cocaine and amphetamine regulated transcript (CART) is a newly discovered hypothalamic peptide with a potent appetite suppressing activity following intracerebroventricular administration. When the mature rat CART sequence encoding CART(1–102) was inserted in the yeast expression plasmid three CART peptides could be purified from the fermentation broth reflecting processing at dibasic sequences. None of these corresponded to the naturally occurring CART(55–102). In order to obtain CART(55–102) the precursor Glu-Glu-Ile-Asp-CART(55–102) has been produced and CART(55–102) was generated by digestion of the precursor with dipeptidylaminopeptidase-1. All four generated CART peptides have been characterised by N-terminal amino acid sequencing and mass spectrometry. The CART peptides contain six cysteine residues and using the yeast expressed CART(62–102) the disulphide bond configuration was found to be I–III, II–V and IV–VI. When the four CART peptides were intracerebroventricularly injected in fasted mice (0.1 to 2.0 μg) they all produced a dose dependent inhibition of food intake.

Key words: Brain; Appetite regulation; Disulfide bond; Mass spectrometry

1. Introduction

Cocaine and amphetamine regulated transcript (CART) was originally described as an mRNA induced in the brain after acute administration of cocaine to rats [1]. In the search for the factor that induces anorexia in rats implanted with certain tumour lines [2] we applied the method of subtraction cloning/hybridisation. By subtracting cDNA from a non-anorectic tumour cell line from that of an anorectic we found that the mRNA for CART was highly overexpressed in the latter. The CART cDNA encodes a peptide of either 129 or 116 amino acid residues in length [1]. The predicted leader sequence was 27 amino acid residues, thus resulting in a mature CART peptide consisting of either 102 (long form) or 89 (short form) amino acid residues [1]. In contrast to the rat, only the short form exists in humans [3]. The mature peptide contains several potential cleavage sites (mono- and dibasic sequences) and CART might be posttranslationally processed in vivo into several biologically active fragments. Posttranslational processing at a Lys-Arg sequence in the middle of the molecule would generate an N-terminal CART(1–52) and a C-terminal CART(55–102) fragment. The latter of these fragments has actually been isolated from ovine hypothalamic extracts [4] thus indicating that CART is processed at the Lys-53-Arg-54 sequence at least in the ovine brain. In a recent study several CART peptides have been shown to inhibit food intake when injected ICV in rats [5].

Only limited amounts of CART can be isolated from brain tissue [4], and due to the existence of three disulphide bonds in the C-terminal part of the molecule the preparation of CART by peptide synthesis is very difficult. In the present study we describe a yeast expression system suitable for the production of relatively large amounts of CART peptides, including the naturally occurring CART(55–102). The biological activity of the recombinant CART peptides was determined in an acute feeding assay after ICV injection, and we have elucidated the disulphide bond configuration in the CART molecule using recombinant CART(62–102).

2. Material and methods

2.1. General methods

Two different CART expression systems were constructed. In the first expression system the gene encoding the entire CART(1–102) was inserted in the expression plasmid. Several different CART peptides could be isolated after the fermentation of this construction, but none of these was full length CART(1–102) or CART(55–102) (see Section 3).

In the other system the gene encoding a Glu-Glu-Ile-Asp-CART(55–102) precursor was used. After fermentation the Glu-Glu-Ile-Asp-CART(55–102) peptide could be isolated from the fermentation broth and converted to CART(55–102) by digestion with dipeptidylaminopeptidase-1 (DAP-1).

2.2. Cloning of CART and plasmid construction

In the search for the factor that induces anorexia in rats implanted with certain tumour lines [2] we applied the method of subtraction cloning/hybridisation using the Subtractor kit (Invitrogen, CA) with minor modifications. By subtracting cDNA from a non-anorectic tumour cell line from that of an anorectic we found that the mRNA for CART [1] was highly overexpressed in the latter. In order to obtain the full length cDNA for CART we performed a PCR on the cDNA from the anorectic cell line using primers overlapping the translation start and stop sequences. For expression of either full length CART(1–102) or Glu-Glu-Ile-Asp-CART(55–102) we constructed the following plasmids for yeast expression using the α-leader sequence to facilitate secretion and the Kex2 endoprotease for maturation.

Plasmid pEA183, encoding CART(1–102), was constructed as follows: The DNA encoding mature CART was furnished with a 5′ *Nco*I site and a 3′ *Xba*I site allowing insertion into the yeast expression vector pAK405. The α-leader in pAK405 has been modified at positions 63 and 64 from Lys-Asp to Met-Ala introducing an *Nco*I site at this position in the DNA [6]. An expression vector encoding a fusion between α-leader and CART was constructed as follows:

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Briefly, a PCR reaction was performed on full length CART with primer CART1: 5′-ACGGAGGAGCCCATGGCTAAGAGA**CAGGAGGATGCCGAGCTGCAGC**-3′ and primer CART2: 5′-CTTAACGGCTTCTAGATCAC**AAGCACTTCAAGAGG**-3′ (letters in bold denote CART sequence). The PCR fragment was digested with *Nco*I and *Xba*I and ligated to the *Bst*XI/*Xba*I and *Bst*XI/*Nco*I fragments of pAK405. The sequence of the DNA encoding CART was confirmed using the dideoxy-chain termination method with the Sequenase enzyme (Amersham). The resulting plasmid was named pEA183.

Plasmid pSX637, encoding Glu-Glu-Ile-Asp-CART(55–102), was constructed by the use of the PCR technique 'Splicing by Overlap Extension' [7] and the product was inserted into pAK405 as mentioned above. The resulting expression plasmid is shown in Fig. 1. As can be seen from this figure a sequence of: Lys-Glu-Leu-Glu have been placed between the α-leader and Kex2 site in order to optimise processing [6].

2.3. Construction of CART secreting yeast strains

Plasmids pEA183 and pSX637 were transformed into *Saccharomyces cerevisiae* strains ME1487 (*MATα Δyap3::URA3 Δtpi::LEU2 pep4*-3 *Δura3 leu2*) and ME1719 (*MATα/MATa Δyap3::URA3/Δyap3::URA3 Δtpi::LEU2/Δtpi::LEU2 pep4*-3/*pep4*-3 *Δura3/Δura3 leu2/leu2*), respectively. Host cells were cultured in YPGGE medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) galactose, 2% (v/v) glycerol and 1% (v/v) ethanol) to OD_{600nm} of 0.2. Transformation was made by using a standard protoplast method. Transformants, YEA183 and YES1789, were obtained, which contained CART(1–102) and EEID-CART expressing plasmids, respectively, after transformants were selected on minimal plates containing glucose.

2.4. Fermentations

Fermentations of CART(1–102) (yeast strain: ME1487) and EEID-CART(55–102) (yeast strain: YES1789) were carried out in 6 l stainless steel fermenters from Chemap A/B, Schwitzerland, equipped with bottom stirrer and in situ steam sterilisation. The medium was composed essentially as previously described [8] and a starting volume of 4 l was chosen. Ammonia was added to adjust pH throughout the fermentation to 4.9 and the temperature was kept constant at 30°C with steam/cooling water. Dissolved oxygen was kept above 50% saturation by frequent adjustment of the stirrer speed. The inoculum was from a YPD (yeast-extract peptone dextrose) culture (2 days, 30°C). Glucose (1250 g) was dissolved in water to a volume of 2 l, sterilised separately in an autoclave (30 min, 121°C), and added with a constant rate of 30 g/h over the first 24 h. The rate was increased to 60 g/l over the next 24 h. After 48 h of cultivation the broth was harvested.

The CART(1–102) broth was adjusted to pH 4.0 with 1 N HCl and the cells separated by centrifugation in a Beckman J6 cooling centrifuge (at 4°C, 15 min, 4000 rpm). The EEID-CART(55–102) broth was adjusted to pH 11 with 3 N NaOH and kept at 25°C for 30 min before centrifugation as above. The supernatant was adjusted to pH 9.7 to protect against proteolyses before the purification was initiated. The dry biomass in the two fermentations was: 70.4 g/l (CART(1–102)) and 73.6 g/l (EEID-CART(55–102)), respectively. The weight of the total fermentation broth was 5466 g and 5706 g, respectively.

2.5. Purifications

The fermentation broth from yeast strain ME1487 encoding CART(1–102) was analysed by HPLC (Fig. 2). A series of expression products was seen in this analysis. Preliminary sequence analysis indicated that several of the peptides eluting at a retention time between 20 and 30 min were fragments of the mature full length CART(1–102) molecule.

The CART peptides from 4.25 l of fermentation supernatant were separated and purified using the following method: The fermentation broth (pH 4.6, Λ 8 mS) was adjusted to pH 5.0 and diluted with 25 l of water (resulting Λ 1.3 mS) and pumped (500 ml/h) onto an SP-Sepharose (Pharmacia) column (5×15 cm) previously equilibrated with 50 mM HAc at pH 5.0. The column was eluted with a linear gradient between 1500 ml of 50 mM HAc and 1500 ml of 50 mM HAc containing 1.0 M NaCl. Fractions of 10 ml were collected and analysed for the content of CART peptides by HPLC. The chromatogram from the ion exchange chromatography is shown in Fig. 3. Three pools (A, B and C) were generated on the basis of the HPLC analysis of the individual fractions and CART peptides corresponding to these pools were purified by preparative HPLC on a Vydac 214TP1022 reverse phase C4 preparative HPLC column (2.2×25 cm) as previously described [8]. The total yields of CART peptides from 4.25 l of fermentation supernatant were: peptide A: 33 mg, peptide B: 200 mg and peptide C: 280 mg.

The fermentation supernatant (4.6 l) from yeast strain YES1789 expressing Glu-Glu-Ile-Asp-CART(55–102) was dialysed against 60 l of water at 4°C for 96 h. The pH was adjusted to 4.3 and the solution was pumped onto an SP-Sepharose (Pharmacia) column (5×15 cm) with a flow rate of 300 ml/h. Prior to the application the column was equilibrated with 50 mM HAc buffer pH 4.25. The column was washed with 3 l of 50 mM HAc buffer pH 4.25. EEID-CART(55–102) was eluted from the column by a linear gradient between 1.5 l of 50 mM HAc buffer pH 4.25 and 1.5 l of 50 mM HAc buffer pH 4.25 containing 1 M NaCl. Fractions (10 ml) were collected at a flow rate of 100 ml/h and the absorbance was measured at 280 nm. The EEID-CART(55–102) molecule eluted at 0.5 M of NaCl and fractions containing the peptide were dialysed against 25 l of 50 mM HAc buffer pH 4.5 at 4°C for 96 h. L-cysteine was added to the solution (560 ml) to give a final concentration of 1 mM, and 4.5 ml dipeptidylaminopeptidase-1 (DAP-1, Cathepsin C from chicken liver, EC 3.4.14.1, Unizyme Laboratories) was added. The resulting concentration of DAP-1 was 20 units/ml. The digestion of EEID-CART(55–102) was carried out at 37°C and aliquots were analysed by HPLC each half hour. After incubation for 4.5 h more than 98% of the precursor was converted to CART(55–102). The CART(55–102) peptide was purified essential as fragments A, B and C described above. The total yield of CART(55–102) from 4.6 l of fermentation supernatant was 705 mg.

2.6. Characterisation of CART peptides

The isolated CART peptides all gave rise to a single peak when analysed by HPLC (results not shown).

N-terminal amino acid sequences were determined by automated Edman degradations using an Applied Biosystem Model 494 Protein Sequencer essentially as described by the manufacturer.

Mass spectrometric analysis was performed on a Voyager RP MALDI-TOF instrument (Perceptive Biosystems Inc., Framingham, MA) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction, and the accelerating voltage in the ion source was 25 kV.

Sample preparation was done as follows: 1 μl sample solution was mixed with 10 μl matrix solution (alpha-cyano-4-hydroxy-cinnamic acid dissolved in a 5:4:1 (v/v/v) mixture of acetonitrile:water:3% (v/v) TFA) and 1 μl was deposited on the sample plate and allowed to dry. Calibration was performed using external standards and the accuracy of the mass determinations is within 0.1%.

2.7. Disulphide bond configuration in CART

The mature CART(1–102) molecule contains six cysteine residues in the C-terminal part of the molecule in positions 68, 74, 86, 88, 94 and 101. These six cysteine residues can in principle form three disulphide bonds in 15 different arrangements [9]. The following procedure was

Table 1
N-terminal sequence and mass spectrometry analyses of isolated CART peptides

Peptide	N-terminal sequence	Mass found	Mass calculated
CART(54–102)	RIPIYEKKYG...	5418.0	5415.4
CART(61–102)	KYGQVPMXDA...	4516.5	4515.3
CART(62–102)	YGQVPMXDAG...	4389.9	4387.1
CART(55–102)	IPIYEKKYGQ...	5257.1	5255.5

used for the disulphide mapping: CART(62–102), 1 mg, was dissolved in 300 μl of 50 mM *N*-ethylmorpholine buffer at pH 8.0. Five nmole *Armillaria mellea* protease, an enzyme cleaving on the N-terminal side of lysine residues [10], was dissolved in 50 μl of 50 mM *N*-ethylmorpholine buffer at pH 8.0 and was added to the solution. The digestion was carried out for 17 h. A further 10 nmole of *Armillaria mellea* protease was added and the digestion continued for an additional 5 h. Peptide mapping was carried out by injection of the digest mixture onto a Vydac 214TP54 column (4.6×250 mm) equilibrated at 30°C at a flow rate of 1 ml/min with 0.1% (v/v) TFA. The concentration of acetonitrile in the eluting solvent was raised to 40% (v/v) over 40 min. From the sequence and mass spectrometry analysis the structure of the peptides representing the main peaks of the chromatogram (Fig. 4) were deduced as follows:

Fraction 3 (Fig. 4): Lys-Gly-Ala-Arg-Ile-Gly. Fractions 4 and 5 (Fig. 4): Tyr-Gly-Gln-Val-Pro-Met-**Cys**-Asp-Ala-Gly-Glu-Gln-**Cys**-Ala-Val-Arg, Lys-Leu-**Cys**-Asp-**Cys**-Pro-Arg-Gly-Thr-Ser-**Cys**-Asn-Ser-Phe-Leu-Leu and Lys-**Cys**-Leu. Fractions 6 and 7 (Fig. 4): undigested CART(62–102).

The first of these fragments is a small hexapeptide without any cysteine residues. The fragment isolated from fractions 4 and 5 is a 3-chained molecule still held together by the three disulphide bonds. The reason for the existence of this peptide at two different HPLC positions is the presence of a methionine residue, which appears in an oxidised and a non-oxidised form.

The 3-chained CART(62–77, 84–99, 100–102), 100 μg, was dissolved in 300 μl of 50 mM phosphate buffer at pH 8.0 and digested with 2 μg Endoproteinase Asp-N (Boehringer, Cat. No. 1054 589). The incubation was carried out at 37°C for 24 h. The resulting peptide map is shown in Fig. 5. From the sequence and mass spectrometry analyses the structure of the peptides representing the main peaks of the chromatogram were deduced as follows:

Fractions 3 and 4 (Fig. 5): Tyr-Gly-Gln-Val-Pro-Met-**Cys** and Lys-Leu-**Cys**. Fraction 5 (Fig. 5): Asp-Ala-Gly-Glu-Gln-**Cys**-Ala-Val-Arg, Asp-**Cys**-Pro-Arg-Gly-Thr-Ser-**Cys**-Asn-Ser-Phe-Leu-Leu and Lys-**Cys**-Leu.

The fragments isolated from fractions 3 and 4 represent a 2-chained molecule held together by a single disulphide bond. Thus cysteine residues I and III of the original molecule must be linked. The fragments isolated from fraction 5, which contain cysteine residues II, IV, V and VI of the original molecule in a 3-chained structure linked by two disulphide bonds, was further digested with trypsin. The following procedure was used: 10 μg of CART(69–77, 87–99, 100–102) was dissolved in 275 μl of 0.1 M Tris-HCl buffer at pH 8.5 and digested by 2 μg trypsin (Boehringer, Cat. No. 1047 841) dissolved in 20 μl of 0.01% (v/v) TFA at 37°C for 4.5 h. The peptide map is shown in Fig. 6 and the structure of the peptides representing the main peaks of the chromatogram were deduced as follows: Fraction 1 (Fig. 6): Asp-**Cys**-Pro-Arg and Lys-**Cys**-Leu. Fraction 2 (Fig. 6): Asp-Ala-Gly-Glu-Gln-**Cys** and Gly-Thr-Ser-**Cys**-Asn-Ser-Phe-Leu-Leu. Fraction 3 (Fig. 6): Asp-Ala-Gly-Glu-Gln-**Cys**-Ala-Val-Arg and Gly-Thr-Ser-**Cys**-Asn-Ser-Phe-Leu-Leu.

From the above results it is clear that Cys-II and Cys-V are linked (fractions 2 and 3) and that Cys-IV and Cys-VI are linked (fraction 1).

By combining all of the above results the entire primary and secondary structure of the C-terminal part of the CART molecule can be deduced. Thus the disulphide bond in the C-terminal part of the CART molecule exists in a I–III, II–V and IV–VI configuration (Fig. 7).

2.8. Biological activity of recombinant CART peptides

The appetite suppression activity of the purified CART peptides was analysed using the following methods: Female NMRI mice were fasted for 24 h after exposure to the test diet, a nutritionally complete formula milk. Thirty minutes before testing, 10 μl of a solution containing CART peptide or vehicle (phosphate buffered saline) was injected ICV in the lateral ventricle. Each mouse was placed in a 15 cm^2 test box with a grid floor and a glass drinking tube containing the formula milk. Food consumption was measured for 10 min by electronically recording the total amount of contact with the milk during the test session. The degree of appetite suppression produced by a dose of peptide was evaluated by comparing the duration of consumption by control mice with that of treated mice. Each group's feeding response is expressed as percent relative to the control response (100%).

3. Results

3.1. Expression and purification

The expression level for recombinant CART(1–102) and Glu-Glu-Ile-Asp-CART(55–102) in the present yeast system were approximately 200 mg/l and 250 mg/l, respectively. This is a factor 2–3 times higher than we have previously found for other small disulphide linked peptides [8,11]. When the full length CART(1–102) sequence was inserted in the expression plasmid several CART peptides could be isolated from the fermentation broth (Fig. 2). The dominating fragments were: CART(54–102), CART(61–102) and CART(62–102) which were separated and purified individually from the fermentation broth (Fig. 3). The fermentation broth also contained other fragments e.g. CART(49–102), but these were minor components and were not further purified and characterised in the present study. The above peptides all represent fragments generated by processing either between or after dibasic sequences in the CART(1–102) by the action of yeast proteases.

Since the naturally occurring CART(55–102) could not be isolated from the above construction we modified the expression plasmid to encode Glu-Glu-Ile-Asp-CART(55–102) instead of the full length CART(1–102). The extensive processing at dibasic sequences observed for the full length construction was not seen for this precursor, and only the Glu-Glu-Ile-Asp-CART(55–102) peptide could be isolated from the fermentation broth. The subsequent in vitro digestion of the Glu-Glu-Ile-Asp-CART(55–102) peptide to CART(55–102) using dipeptidylaminopeptidase-1 was carried out with a yield close to 100%. The process was favoured by the presence of a proline residue at position 2 of the CART(55–102) peptide, blocking the DAP-1 enzyme from further processing.

The overall purification yields from both fermentations were approximately 60%, and the loss was mainly due to the complex medium in which the yeast cells were grown.

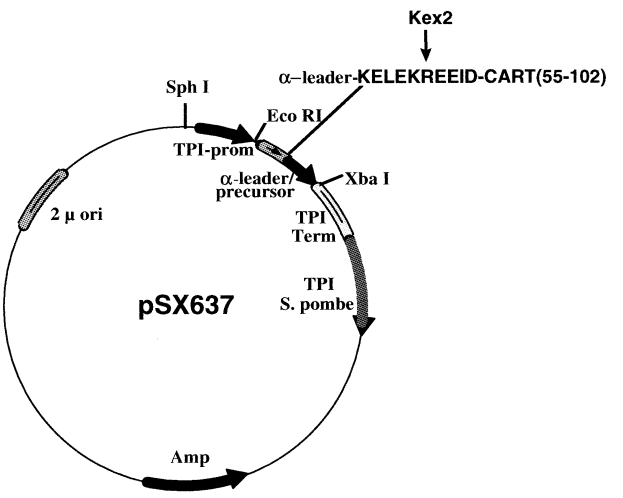

Fig. 1. *S. cerevisiae* plasmid for the expression and secretion of Glu-Glu-Ile-Asp-CART(55–102). TPI-prom. and TPI-term. are *S. cerevisiae* triosephosphate isomerase transcription promotor and terminator sequences, respectively. TPI S. pombe is the *Schizosaccharmyces pombe* triosephosphate isomerase gene. Only restriction sites relevant for the plasmid construction have been indicated.

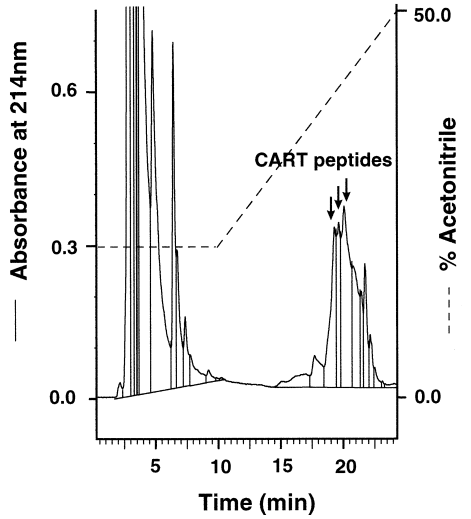

Fig. 2. Reversed phase HPLC on a Vydac 214TP54 C4 column of supernatants from yeast strain ME1487 expressing CART(1–102).

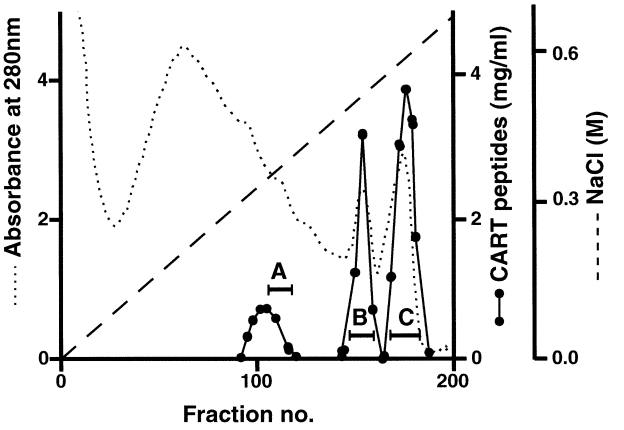

Fig. 3. Ion exchange chromatography on a Fast Flow SP-Sepharose column of partially purified CART peptides. The amount of CART peptide was determined by analytical HPLC. The bars indicate the fractions pooled for further purification. The dashed line shows the concentration of NaCl in the eluting solvent.

3.2. Structural characterisation of CART peptides

The analytical HPLC chromatograms obtained on the purified CART peptides showed that all four peptides eluted with nearly the same retention time. Based on N-terminal sequence analysis of the individual peptides the purity was determined to more than 98%. The four peptides were subjected to amino acid sequence and mass spectrometry analyses (Table 1). By combining these data the structure of the isolated CART fragments were deduced to be: CART(54–102), CART(61–102), CART(62–102) and the naturally occurring CART(55–102). In all cases the molecular weight determined by mass spectrometry analyses was within 3 mass units from the theoretically calculated mass. These results also show that no posttranslational modifications (e.g. *O*-glycosylation) exist in the recombinant peptides.

In order to simplify the elucidation of the disulphide configuration in the CART molecule the shortest of the isolated CART peptides, CART(62–102), was used for these studies. The strategy was to digest CART(62–102) by several specific proteases, using non-reducing conditions, until peptide fragments containing a single disulphide bond were obtained. Using this strategy three peptides: CART(62–68, 84–86), CART(87–90, 100–102) and CART(69–74, 91–99), all containing a single disulphide bond, were isolated and their structure determined by mass spectrometry and sequence analyses. By combining these data the cysteines, when numbered from the N-terminal end, are linked in the configuration: I–III, II–V and IV–VI. This configuration was determined for the CART(62–102) peptide, but it seems highly probable that the same disulphide bond configuration exists in the other CART peptides as well as in naturally occurring CART, although this has not been directly shown in the present study.

Fig. 7 shows the I–III, II–V and IV–VI disulphide bond configuration in CART(55–102).

3.3. Biological activity of recombinant CART peptides

Fig. 8 shows the inhibitory effects on feeding of the four isolated CART peptides.

The most active of these peptides was the naturally occurring CART(55–102). The minimum effective dose of this peptide was between 0.1 μg and 0.2 μg, which inhibited food intake by approximately 50%. However also the longer form, CART(54–102) and the two shorter forms, CART(61–102) and CART(62–102) were biologically active although a somewhat higher dose was necessary to produce a 50% inhibition of food intake.

4. Discussion

In the present study we describe the use of a yeast expression system for the production of mg to g amounts of CART peptides. The expression levels in this system are relatively high (200–250 mg/l) and the purification was straight forward, resulting in an overall yield of approximately 60%. Furthermore the purified peptides are not posttranslationally modified. However, a drawback using this system was the extensive

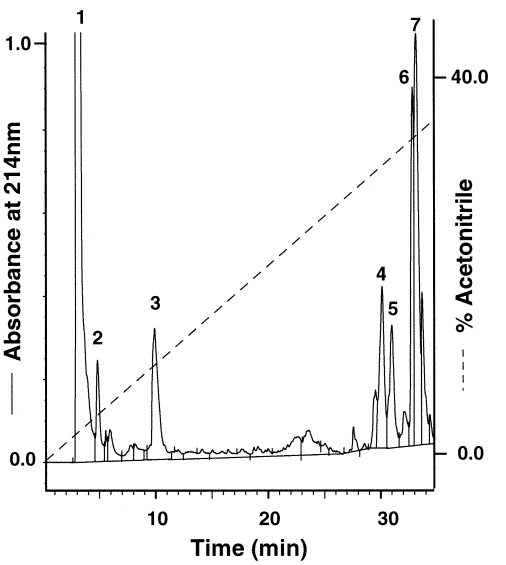

Fig. 4. Peptide map of AMP digested CART(62–102). Fractions corresponding to the individual absorbance peaks were collected as indicated. Peptide material in fractions 4 and 5 corresponding to the 3-chained CART(62–77, 84–99, 100–102) was further digested with Asp-N protease (Fig. 5).

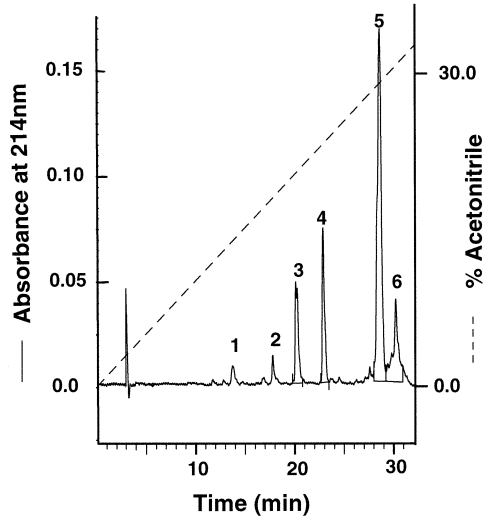

Fig. 5. Peptide map of Asp-N protease digested CART(62–77, 84–99, 100–102). The peptide eluting in fraction 3 corresponded to the 2-chained CART(62–68, 84–86). The peptide eluting in fraction 5 corresponded to the 3-chained CART(69–77, 87–99, 100–102), and was further digested with trypsin (Fig. 6).

processing at dibasic sequences observed when expression of the full length CART(1–102) was attempted. In the present yeast expression system it was necessary to construct a plasmid encoding the Glu-Glu-Ile-Asp-CART(55–102) precursor, to purify this precursor, and to digest it to CART(55–102) to obtain the naturally occurring form of CART. The use of this precursor did not result in any processing e.g. at the dibasic sequence Lys-60-Lys-61 where extensive processing was seen for the full length precursor. One possible explanation for this could be that the Lys-60-Lys-61 sequence in the Glu-Glu-Ile-Asp-CART(55–102) precursor is less exposed to proteolytic cleavage than the corresponding sequence in the full length precursor.

The digestion of the Glu-Glu-Ile-Asp-CART(55–102) precursor with dipeptidylaminopeptidase-1 was very efficient and

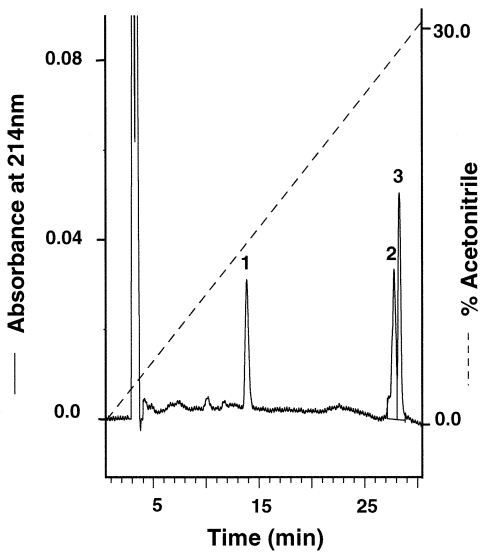

Fig. 6. Tryptic map of CART(69–77, 87–99, 100–102). Fraction 1 corresponded to the 2-chained CART(87–90, 100–102) and fraction 2 to the 2-chained CART(69–74, 91–99).

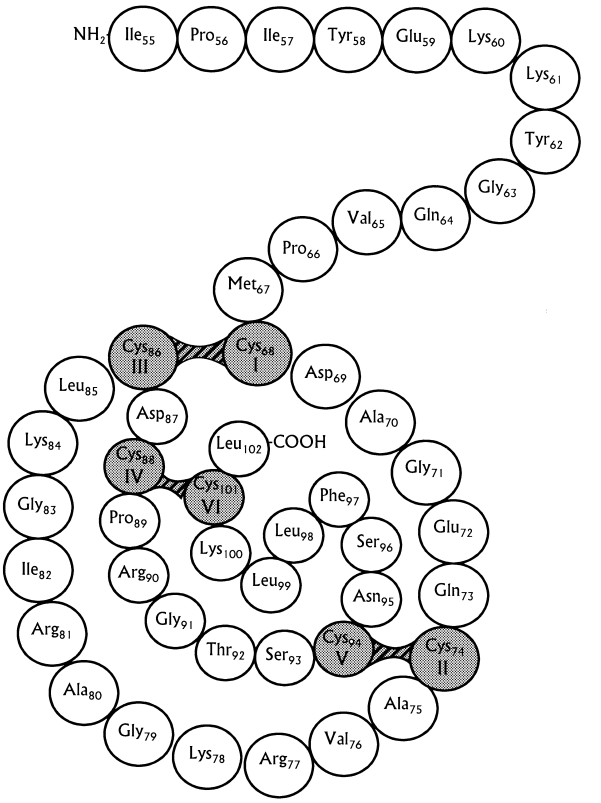

Fig. 7. Proposed structure of the naturally occurring CART(55–102) showing the disulphide bond configurations I–III, II–V, IV–VI.

resulted in a close to 100% yield. This high yield was probably due to the presence of a proline residue in position 2 of the CART(55–102) molecule, which totally blocks the dipeptidylaminopeptidase-1 from attacking this molecule. A similar specificity of dipeptidylaminopeptidase-1 has previously been reported for other peptide substrates as well [12].

Apart from the study by Spiess et al. in 1981 [4], in which they identified the CART(55–102) peptide in ovine hypothalamic extracts, no studies have so far been carried out to determine the form of naturally occurring CART. Using a polyclonal antiserum directed against CART(28–56) Smith and co-workers [13] have localised CART immunoreactivity in the nucleus accumbens of monkey brains. This area of the brain constitute the major part of the ventral striatum, which is thought to be involved in a variety of functions, including regulation of motor and motivational behaviours as well as drug reinforcement [14,15].

Due to the presence of several mono- and dibasic sequences in CART(1–102) (or the corresponding human CART(1–89)) molecule, it seems likely that posttranslational processing occurs in vivo, thus generating several biologically active peptides. In the present study we have focused on the naturally occurring CART(55–102) molecule, but apparently also the shorter peptides CART(60–102) and CART(61–102) as well as the longer CART(54–102) are biologically active (Fig. 8), and the potency of these peptides is almost as high as for CART(55–102). These results seem to indicate that the C-terminal part of the CART molecule containing the three disulphide bridges is important for biological activity.

As a first step in elucidation of the 3-dimensional structure

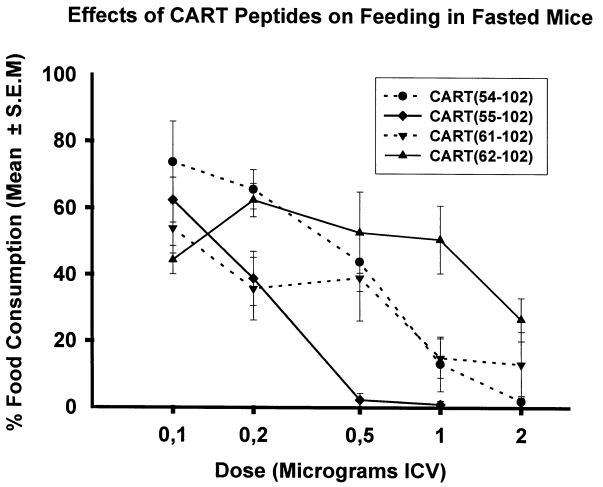

Fig. 8. Biological activity of the isolated CART peptides. The figure shows the dose dependent inhibitory effects on feeding of the four isolated CART peptides. The figure shows the food consumption ±S.E.M. in percent of control (100%). Each data point represents a group of six animals.

of the C-terminal part of CART we have determined the disulphide bond configuration. Six cysteine residues can in principle form disulphide bonds in $5\times3\times1=15$ possible ways, and all of these 15 combinations actually exist in naturally occurring peptides and proteins [9]. The I–III, II–V, IV–VI arrangements found in CART (Fig. 7) have so far only been found in rabbit Ig kappa chains [9]. However no other similarities (e.g. sequence homology) exist between this protein and CART. Thus, the C-terminal part of the CART molecule containing the three disulphide bonds seems to form a new domain not found in any other peptides or proteins.

In summary, the yeast expression system described in the present study may be a valuable tool for generation of mg to g amounts of CART peptides for further biological and physiological studies of this new peptide family.

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