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Note

Rapid purification of β-endorphin by high-performance liquid chromatography

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The opioid peptide β-endorphin has been isolated from porcine and camel pituitary glands by traditional chromatographic methods[1,2]. The amount of pituitary tissue needed to yield the β-endorphin has made such procedures inappropriate for routine preparative purification on the laboratory. In a previous report from this laboratory[3], we described the partial purification of a large opioid peptide from porcine melanocyte-stimulating hormone (MSH) concentrate, a fraction obtained in the commercial preparation of adrenocorticotropic hormone (ACTH)[4]. We have now purified this peptide to homogeneity and identified it as β-endorphin. A system of reversed-phase liquid chromatography was developed to facilitate the purification. The yield and percent recovery of opioid activity indicate that MSH concentrate is a good source of natural β-endorphin.

MATERIALS AND METHODS

Bio-Gel P-6 (Bio-Rad Labs., Richmond, Calif., U.S.A.) columns (52 × 2.5 cm I.D.) were used for initial gel filtration fractionations. Amberlite CG-50 columns, 14.5 × 1.2 cm I.D. (Mallinckrodt, St. Louis, Mo., U.S.A.) equilibrated with 0.1 *M* sodium borate buffer (pH 8.0) and then washed with distilled water to neutrality, were used for ion-exchange fractionation. Bio-Gel P-2 (Bio-Rad Labs.) columns (41 × 1.4 cm I.D.) were used for desalting. A Waters Assoc. high-performance liquid chromatograph equipped with two pumps, an ultraviolet detector (254 nm) and a μBondapak C_{18} column (30 cm × 4 mm I.D.) was used for reversed-phase chromatography. All chromatography was done at room temperature.

Synthetic camel β-endorphin was obtained from Peninsula Labs. (San Carlos, Calif., U.S.A.), methanol (HPLC grade) was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.), naloxone was obtained from Endo Labs. (Garden City, N.Y., U.S.A.) and porcine MSH concentrate was the generous gift of Dr. J. Fisher, Armour Co. All other chemicals were reagent grade from various sources. Glass-distilled water was used throughout the purification.

Opioid activity was assayed with the guinea pig ileum–myenteric plexus preparation[5], and was confirmed by reversal and blockade of the inhibition by 100 n*M* naloxone. Activity is reported as the concentration of synthetic β-endorphin or nor-

morphine giving equivalent inhibition. One nmole of β-endorphin is equivalent to 1.6 nmoles of normorphine in this bioassay.

RESULTS

The opioid material was extracted from 2.5 g of dry MSH concentrate with 10 ml of water and centrifiged at 8000 *g* for 10 min. The supernatant layer was reserved and the pellet was re-extracted successively with 2 ml and 1 ml of water. The combined supernatant layers were applied to a Bio-Gel P-6 column equilibrated with 50 m*M* Tris–HCl (pH 7.4) as previously described[3]. Most of the opioid activity eluted as a single peak between 140 and 190 ml. These fractions were pooled, lyophilized, and applied to the same Bio-Gel P-6 column re-equilibrated with 0.25 *M* acetic acid. The elution position of the opioid activity in acid did not differ from that obtained in Tris–HCl, but much of the contaminating material was retarded on the column during acid elution, resulting in a 5-fold purification as well as desalting the opioid peak (Fig. 1). The active fractions were pooled and lyophilized. The dried material was taken up in 25 ml of water, applied to an Amberlite CG-50 column and eluted successively with 25 ml of water, 25 ml of 0.04 *M* sodium borate buffer (pH 8.0) and 50 ml of 0.1 *M* sodium borate buffer (pH 8.0). The active fraction obtained in the final eluate, containing 46% of the initial opioid activity, was desalted on a Bio-Gel P-2 column equilibrated with 0.25 *M* acetic acid. The desalted material was lyophilized and re-dissolved in 150–250 μl of 10 m*M* formic acid and injected onto the μBondapak C_{18} column equilibrated with 10 m*M* formic acid–methanol (50:50). The column was eluted at a flow-rate of 1 ml/min (Fig. 2). The opioid activity emerged in a broad peak between 6.5 and 10 min ($k' = 2.8$). The methanol was blown off under nitrogen, and the remaining material was lyophilized.

At various steps in this procedure, aliquots were removed, lyophilized and assayed to determine the recovery of opioid activity. To minimize the variability of

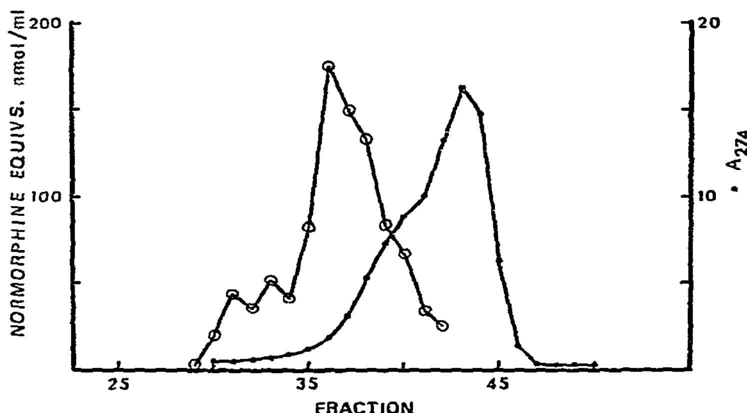

Fig. 1. Gel filtration of the opioid peak on Bio-Gel P-6 in 0.25 *M* acetic acid. The opioid peak, obtained from gel filtration on Bio-Gel P-6 in Tris–HCl, was chromatographed on the same P-6 column after re-equilibration with 0.25 *M* acetic acid; 5-ml fractions were collected. The opioid activity (open circles) of each fraction was assayed on the guinea pig ileum using normorphine as the standard. Ordinate (left): opioid activity expressed as normorphine equivalents, nmole/ml; ordinate (right), absorbance at 274.5 nm.

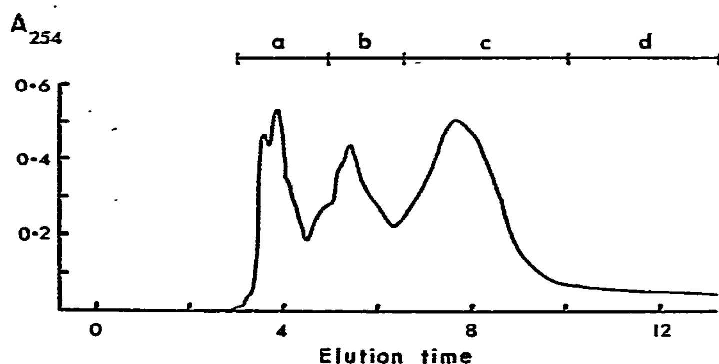

Fig. 2. High-performance liquid chromatography of the opioid peak after cation exchange. The opioid activity from the 0.1 *M* borate buffer elution on CG-50 was subjected to reversed-phase high performance liquid chromatography as described in the text. UV absorbance was measured at 254 nm. Fractions a, b, c and d were collected, dried, taken up in 2 ml of 10 m*M* HCl and assayed on the guinea pig ileum preparation. Fraction a contained no endorphin; b, 29 nmoles; c, 1245 nmoles; and d, 104 nmoles. The material in fractions c and d was pure.

the bioassay, all aliquots were assayed on the same four ileum–plexus strips. The results are given in Table I. It was not possible to assay the original extract of the MSH concentrate because of interfering spasmogenic activity. The final yield of β-endorphin was 4.7 mg from 2.5 g of MSH concentrate, and the final recovery of opioid activity, based on the activity of the initial gel filtration peak, was 25%.

TABLE I

RECOVERY OF OPIOID ACTIVITY IN THE PURIFICATION OF β-ENDORPHIN FROM PORCINE-MSH CONCENTRATE

Lyophilized aliquots of the product of each purification step were taken up in appropriate volumes of 10 m*M* HCl and assayed for opioid activity on four guinea pig ileum–myenteric plexus strips. The values given are the means of the four values thus obtained, expressed as equivalents of camel β-endorphin (nmole). Protein content was estimated by optical absorbance at 274.5 nm. Recovery at each stage has been expressed relative to the activity in the first gel filtration eluate, since the activity could not be accurately determined in the original extract.

Step	*Treatment*	*β-Endorphin equivalents (nmoles recovered)*	*Absorbance at 274.5 nm*	*Specific activity (nmole/absorbance unit)*	*Recovery (%)*
1	Gel filtration: Bio-Gel P-6, 50 m*M* Tris–HCl, pH 7.4	5450	474	11.5	100
2	Gel filtration: Bio-Gel P-6, 0.25 *M* acetic acid	6700	130	53.1	123
3	Cation exchange: Amberlite CG-50				
	(a) H_2O eluate	1175	—	—	(22)
	(b) 0.04 *M* Na borate eluate	249	—	—	(4)
	(c) 0.1 *M* Na borate eluate	2500	14	179	46
4	Reversed-phase liquid chromatography: μBondapak C_{18} column 10 m*M* formic acid–methanol (1:1, v/v)	1349	1.8	749	25

The N-terminal residue of the peptide after reversed-phase chromatography was determined by dansylation and hydrolysis in 6 *N* HCl. Only bis-Dns-tyrosine and ε-Dns-lysine were observed. The amino acid composition of the peptide given in Table II was determined by AAA Lab. (Mercer Island, Wash., U.S.A.) and is consistent with that expected for porcine β-endorphin[6].

TABLE II

AMINO ACID COMPOSITION OF FRACTION c FROM REVERSED-PHASE LIQUID CHROMATOGRAPHY

Lyophilized aliquots of fraction c from reversed-phase liquid chromatography were sent to AAA Lab. for amino acid analysis. Determinations were done on duplicate samples hydrolysed in 6 *N* HCl for 24 h at 110°. The number of residues expected in porcine β-endorphin was obtained from ref. 6. N.D. = not determined.

Amino acid	*nmoles found**	*Residues determined***	*Residues expected in porcine β-endorphin*
Ala	38.6	1.9	2
Arg	0	0	0
Asx	46.0	2.3	2
Cys	N.D.	N.D.	0
Glx	58.6	2.9	3
Gly	61.6	3.0	3
His	20.0	1.0	1
Ile	16.2	0.8	1
Leu	42.6	2.1	2
Lys	110	5.4	5
Met	13.2	0.6	1
Phe	41.6	2.0	2
Pro	22.6	1.1	1
Ser	39.8	2.0	2
Thr	64.6	3.2	3
Trp	N.D.	N.D.	0
Tyr	16.6	0.8	1
Val	41.4	2.0	2

* The mean of duplicate determinations.

** Based on the assumption of one His residue per molecule.

DISCUSSION

The procedure given here for the purification of β-endorphin is rapid and reproducible and is now used in our laboratory for routine production of β-endorphin in milligram quantities. The recovery of β-endorphin by this procedure has been increased greatly over our earlier methods[3,7] by storage of the material in the dry state under a vacuum between steps in the purification procedure. The losses after lyophilization that have been reported[3,8] appear to be due to adsorption to glass and can be avoided by redissolving the dried material in dilute acids or strong salt solutions. In addition, basic pH conditions are detrimental to the recovery of opioid activity. A substantial loss of activity occurred in our procedure during cation-exchange chromatography. Some peptides with opioid activity eluted from the cation-exchange column before β-endorphin. Previously, we have observed that chromato-

graphy of the extract from MSH concentrate at pH 9 on Bio-Rex 70 (a cation exchanger similar to CG-50) resulted in the appearance of several fractions with opioid activity[3]. Subsequent gel filtration showed that these fractions represented several size classes of endorphins. However, gel filtration of the same extract directly on Bio-Gel P-6 gave only a single major peak, with apparent molecular weight of about 3000 daltons. Therefore, we conclude that some degradation of β-endorphin occurs in basic conditions on cation-exchange resins. However, the cation-exchange step was found to be necessary for the successful purification of β-endorphin and thus the conditions of cation exchange were chosen to minimize this loss.

The recovery of β-endorphin from reversed phase liquid chromatography is a function of the amount injected. Small amounts of β-endorphin (less than 10 nmoles) are not recovered under the conditions of chromatography reported here. The most probable explanation for this loss is non-specific absorption, possibly to unreacted sites on the silica gel. By using solvent systems containing dilute trifluoroacetic acid or ethanol, good recovery of small amounts of β-endorphin can be obtained[9], but the selectivity of these systems is inadequate for complete resolution of the β-endorphin from other components.

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