Isolation and characterization of dipeptidyl peptidase IV from human meconium

Functional role of β-casomorphins

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Dipeptidyl aminopeptidase IV (DAP-IV) (EC 3.4.14.1) was purified from meconium particles sedimenting at 105000 × *g*. Its molecular properties and activity on synthetic and natural substrates (casomorphin and procasomorphin) were investigated.

Dipeptidyl peptidase IV *Brush border enzyme* *Meconium* *Casomorphin* *Procasomorphin* *Fast atom bombardment mass spectrometry*

1. INTRODUCTION

Dipeptidyl peptidase IV (DAP-IV), an aminopeptidase which cleaves X-Pro dipeptides, has been purified and characterized from many animal tissues [1–8]. The human enzymes studied were from placenta [8], submaxillary gland [6] and kidney [1]. β-Casomorphins [9,10], the morphine-like acting fragments present in the sequence of all β-caseins so far investigated [10–14], might be natural substrates for this peptidase. Therefore, it seems interesting to investigate the DAP-IV activity of human intestinal brush border on these peptides arising from food in view of their postulated physiological and/or pathological role. Here we report the purification and characterization of DAP-IV from human meconium which is an easily accessible source of human intestinal brush border peptidases. The enzyme activity on buffalo procasomorphin (Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Lys) [15] and casomorphin-9 (Des-Val-1 procasomorphin) was also investigated. The use of fast atom bombardment (FAB) mass spectrometry is suggested as a simple, rapid and sensitive technique to analyze peptide digestion by proteolytic enzymes.

2. MATERIALS AND METHODS

2.1. *Materials*

Reagents and substrates used were as described in [16]. Materials for gel electrophoresis and electrofocusing were obtained from LKB, Bromma, FRG; crystalline neuraminidase of *Clostridium perfringens* (type VI) and thioglycerol were obtained from Sigma, St. Louis, USA. Standard proteins for SDS gel electrophoresis and gel filtration, DEAE-Sephacel, Sephadex G-200 and FPLC apparatus were obtained from Pharmacia, Uppsala, Sweden; in addition to the standard protein kit from Pharmacia, aminopeptidase M from hog kidney (M_r 280000) (Boehringer, Mannheim, FRG) was utilized.

2.2. *Methods*

Purification procedure: meconium samples of at

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term newborns were collected during the first day of life and stored at −20°C for less than 3 months. Meconium samples were thawed and lyophilized and 200 g were used for enzyme purification. The dry powder was homogenized in saline (10%, w/v) in the presence of 0.02% NaN_3; the homogenate was centrifuged at 1500 × *g* for 30 min, then at 105000 × *g* for 60 min; the sediment was suspended and homogenized in 200 ml of 5 mM Tris-HCl buffer, pH 8, containing 2% (v/v) Triton X-100. After centrifugation at 105000 × *g* for 90 min solid ammonium sulphate was added to the supernatant. The 50–80% ammonium sulphate precipitate, dissolved in and dialysed against 5 mM Tris-HCl, pH 8, was added to 40 ml of packed DEAE-Sephacel equilibrated with the same buffer; the mixture was gently stirred for 15 min and filtered. The adsorbed enzyme was recovered from the ion exchanger by washing with 100 ml of 50 mM sodium acetate buffer, pH 3.6, 150 mM NaCl. The filtrate, after dialysis against 5 mM Tris-HCl, pH 8, was chromatographed on a DEAE-Sephacel column (1.6 × 20 cm) previously equilibrated with the same buffer and then developed with 400 ml of 50 mM acetate, pH 5, plus a linear saline and pH gradient (100 ml of 50 mM acetate, pH 5, plus 100 ml of 50 mM acetate, pH 3.6, 150 mM NaCl). Active fractions were pooled, concentrated to 5 ml and chromatographed on a Sephadex G-200 column (2.6 × 100 cm) equilibrated and eluted with 5 mM Tris-HCl, pH 8, 150 mM NaCl. The peak of activity was pooled and submitted to FPLC on a Mono Q column developed with a linear gradient of NaCl (0–0.5 M) in 20 mM Tris-HCl buffer, pH 7 (20 ml total volume). Finally, the enzyme was obtained in a homogeneous form by rechromatography on a Mono Q column developed with 5 ml of 20 mM sodium phosphate buffer, pH 6, plus a linear gradient of NaCl (0–0.25 M) in the same buffer (20 ml).

Enzymatic activities on synthetic substrates were assayed as previously described [16]. Proteins were determined according to Lowry et al. [17,18]. Polyacrylamide gel electrophoresis (PAGE), SDS-PAGE and analytical gel isoelectric focusing (pH range 2.5–6) were performed as described in [19], [20] and [21], respectively. The enzyme activity was revealed on the gels according to Skovbjerg et al. [22]. M_r determinations were performed on a Sephadex G-200 column (1.6 × 100 cm) equilibrated and developed with 5 mM Tris-HCl buffer, pH 8, 150 mM NaCl at flow rate of 8 ml/h. Purified DAP-IV was incubated with neuraminidase according to [23] or to [24]. Casomorphin-9 was obtained, by Edman degradation, from procasomorphin which was purified as in [15]. FAB mass spectra were recorded on a double-focusing VG ZAB HF mass spectrometer equipped with a FAB source of the same company and an M-Scan gun. Xenon as primary ionizing beam was utilized.

3. RESULTS AND DISCUSSION

The purification procedure summarized in section 2 gave a final yield of about 15%. The enzyme, homogeneous on PAGE (fig.1) and SDS-PAGE (fig.2), had a specific activity of 15 U/mg and an optimal pH of 7.8, using Gly-Pro-2-naphthylamide as substrate. When analysed by isoelectric focusing, the purified protein showed 3 bands of enzymatic activity with isoelectric points 5.4, 5.2 and 4.9 (not shown). The M_r studies were per-

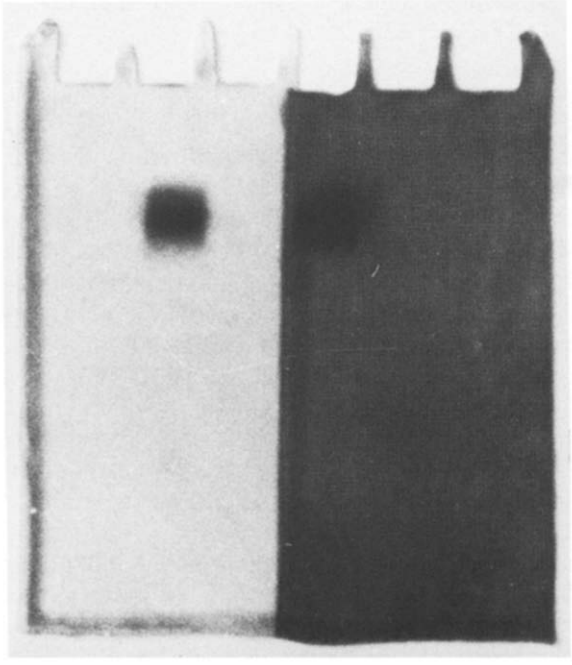

Fig.1. PAGE: Purified enzyme (10 μg) after protein staining (left) and activity staining (right). Cathode is at the top.

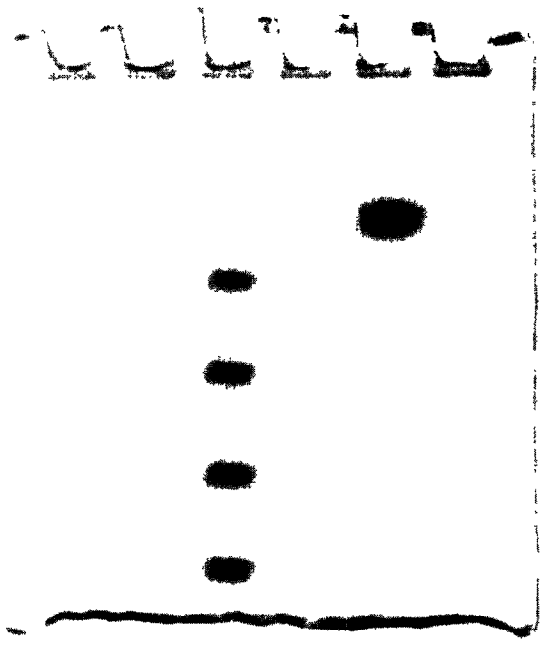

Fig.2. SDS-PAGE: On the left 10 μg of each standard protein; from the top: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase. On the right: 25 μg denatured purified enzyme. Cathode is at the top.

formed under both native and dissociating conditions. Meconial DAP-IV emerged from an analytical Sephadex G-200 column at an elution volume corresponding to M_r 265000. SDS-PAGE gave a single protein band of M_r 130000. Therefore the enzyme seems to consist of two polypeptide chains identical in size. Meconial DAP-IV, unlike the placental enzyme [8], contains little or no sialic acid as the neuraminidase treatment did not modify the electrophoretic mobility of the purified enzyme (not shown). The K_m values for Gly-Pro-2-naphthylamide and Ala-Ala-2-naphthylamide were 6.6×10^{-4} and 8.2×10^{-4} M, respectively. The proline-containing substrate (V_{max} = 19 U/mg) was hydrolysed about 10-fold faster than the other substrate (V_{max} = 2.2 U/mg). These results show that meconial DAP-IV is very similar to the enzymes purified from other sources [1–8]. The products of hydrolysis of procasomorphin and β-casomorphin-9 by DAP-IV were identified according to their M_r values by FAB mass spectrometry. The positive ion FAB mass spectra of procasomorphin (*m/z* 1114) at 0, 1 and 2 h of incubation with DAP-IV are reported in fig.3. As can be observed after 1 h incubation a peak at *m/z* 852, corresponding to the loss of the Val-Tyr dipeptide, appeared. At 2 h incubation the peak corresponding to procasomorphin completely disappeared while the peak at *m/z* 852 was still present demonstrating that further digestion did not occur. In the same experimental conditions, the quasi-molecular ion of β-casomorphin-9 completely disappeared after about 5 min incubation (not shown). This result shows that β-casomorphins, as expected, are very good substrates for the intestinal DAP-IV enzyme. So far the physiological role for β-casomorphins has been postulated on the basis of their resistance to proteolytic enzymes [9]. Present and previous studies with intestinal brush border [15] and plasma enzymes [25] seem to indicate that these peptides could hardly reach concentrations of physiological significance. In this light, the resistance to DAP-IV activity of procasomorphin which, unlike casomorphins, is released from β-casein by gastric and pancreatic enzymes [15], has to be underlined. Despite the recognized sensitivity of these peptides to intestinal peptidase, it appears that they can escape the brush border barrier and reach the blood stream; as a matter of fact, Kreil et al. [25] claimed the presence, in the plasma of newborn calves, of a β-casomorphin immunoreactive material larger than β-casomorphins. As β-casomorphins seem to be quickly degraded in the plasma by a DAP-IV activity [25], it could be suggested that these peptides can survive in the blood and reach their putative brain receptors in the peptidase-resistant precursor form, i.e., procasomorphin.

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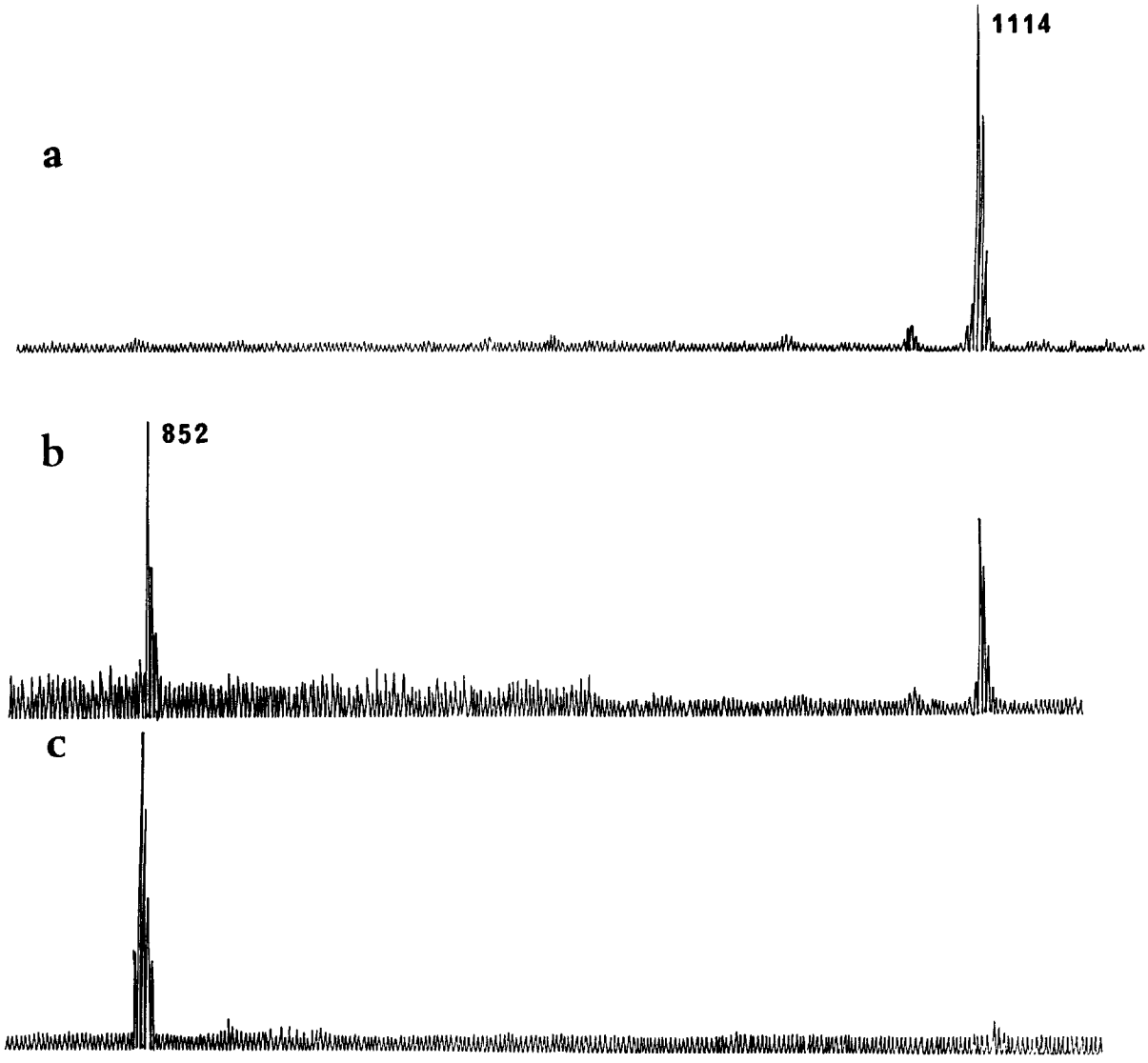

Fig.3. FAB mass spectra of procasomorphin at 0 (a), 1 (b) and 2 (c) h incubation with meconial DAP-IV. The digestion mixture contained 0.3 μmol of peptide and 5 mU peptidase in 0.1 ml ammonium bicarbonate (0.1%). Aliquots of 1–2 μl were withdrawn and directly loaded into the spectrometer.

REFERENCES

[1] Hama, T., Okada, M., Kojima, K., Kato, T., Matsuyama, M. and Nagatsu, T. (1982) Mol. Cell. Biochem. 43, 35–42.
[2] Wolf, B., Fischer, G. and Barth, A. (1978) Acta Biol. Med. Germ. 37, 409–420.
[3] Yoshimoto, T., Fischl, M., Orlowski, R.C. and Walter, R. (1978) J. Biol. Chem. 253, 3708–3716.
[4] Fukasawa, K.M., Fukasawa, K. and Harada, M. (1978) Biochim. Biophys. Acta 535, 161–166.
[5] McNair, R.D.C. and Kenny, A.J. (1979) Biochem. J. 179, 379–395.
[6] Kojima, K., Hama, T., Kato, T. and Nagatsu, T. (1980) J. Chromatogr. 189, 233–240.
[7] Fukasawa, K.M., Fukasawa, K., Hiraoka, D.Y. and Harada, M. (1981) Biochim. Biophys. Acta 657, 179–189.

[8] Puschel, G., Mentlein, R. and Heymann, E. (1982) Eur. J. Biochem. 126, 359–365.
[9] Henschen, A., Lottspeich, F., Brantl, W. and Teschemacher, H. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1217–1224.
[10] Brantl, V. (1984) Eur. J. Pharmacol. 106, 213–214.
[11] Ribadeau-Dumas, B., Brignon, G., Grosclaude, F. and Mercier, J.C. (1972) Eur. J. Biochem. 25, 514–515.
[12] Richardson, B.C. and Mercier, J.C. (1979) Eur. J. Biochem. 99, 285–297.
[13] Petrilli, P., Addeo, F. and Chianese, L. (1983) Ital. J. Biochem. 32, 336–344.
[14] Greenberg, R., Groves, M.L. and Dower, H.J. (1984) J. Biol. Chem. 259, 5132–5143.
[15] Petrilli, P., Picone, D., Caporale, C., Addeo, F., Auricchio, S. and Marino, G. (1984) FEBS Lett. 169, 53–56.
[16] Auricchio, S., Greco, L., De Vizia, B. and Buonocore, V. (1978) Gastroenterology 75, 1073–1079.
[17] Lowry, O.H., Rosebrough, N.J., Randall, R.J. and Farr, A.L. (1951) J. Biol. Chem. 193, 265–275.
[18] Wang, C.S. and Smith, R.L. (1975) Anal. Biochem. 63, 414–417.
[19] Maurer, H.M. (1971) in: Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis (De Gruyter, ed.) pp.43–44, New York.
[20] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[21] Karlsson, C., Davies, H., Ohman, J. and Anderson, U.B. (1973) in: LKB Application note 75.
[22] Skovbjerg, H., Noren, O. and Sjostrom, H. (1978) Scand. J. Clin. Lab. Invest. 38, 723–729.
[23] Miki, K., Suzuki, H., Iimo, S., Hoda, T., Hirano, K. and Sugiura, M. (1977) Clin. Chim. Acta 79, 21–30.
[24] Mulivor, R.A., Hannig, V.L. and Harris, H.C. (1978) Proc. Natl. Acad. Sci. USA 8, 3909–3912.
[25] Kreil, G., Umbach, M., Brantl, V. and Teschemacher, H. (1983) Life Sci. 33, 137–140.