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Note

A Quantitative Chromatographic Method for the Determination of Leucine Aminopeptidase Activity

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A micro method for the determination of leucine aminopeptidase activity was developed. Leucinamide was used as substrate. The liberated leucine was quantitatively determined using paper chromatography and reflectance-densitometrical scanning of the spots. From the spot area of leucine the enzyme activity was evaluated. This method is useful for the determination of leucine aminopeptidase activity in supernatants obtained after centrifugation of homogenized organs.

A number of methods is known for the determination of leucine aminopeptidase activity. Mostly, they involve the colorimetric measurement of β-naphthylamide which is liberated from L-leucyl-β-naphthylamide under the catalytic action of leucine aminopeptidase[1–7]. L-Leucyl-glycine and L-leucinamide have been also used as substrates[8]. The majority of authors is of the opinion that leucine aminopeptidase splits many substrates. Recently different enzymes were found which split the mentioned substrates to a different degree[9–11].

The colorimetric methods have numerous advantages but they require a considerable amount of substrate and enzyme. There exists an ultramicro colorimetric method[12], but it is more complicated than the chromatographic method. Haschen[13] developed a partly chromatographic method for the quantitative determination of peptidase activity, but the final step involves the colorimetric measurement. Our intention was to develop an entirely chromatographic method, which should be simple, reliable enough and applicable to the ultramicro scale. A similar method was described for the determination of intracellular proteinases[14].

MATERIAL AND METHODS

Leucinamide-hydrochloride was used as substrate. Leucine aminopeptidase shows a maximal activity at *p*H = 8.0 and magnesium ion is needed as activator. For the calibration curve either leucine or leucinamide hydrochloride were dissolved in 0.01 *M* phosphate buffer (*p*H = 8.0) containing 0.01 *M* of magnesium sulphate. Different concentrations of substrate were prepared because always the same volume of substrate solution (5 µl), was applied to the chromatographic paper. For the determination of leucine aminopeptidase activity the solution of leucinamide was used containing 1 mg. of leucinamide in 1 ml. of phosphate buffer. It was found that 1 hr. was the optimal incubation period for the determination of the enzyme activity in supernatants dialyzed against the buffer *p*H = 8.0 containing magnesium ions. Under these conditions approximately 2 µg. of leucine was liberated from 5 µl of reaction mixture. These values are then in the lower part of the calibration curve.

Quantitative chromatographic separation of the reaction mixture was carried out using strips of Schleicher-Schüll No 2043 b Mgl paper, 2 cm broad. The distance from the starting line to the front of the solvent was 18 cm. After development the chromatograms were sprayed with 0.2% ninhydrin solution and heated for 15 min. in moist atmosphere at 80° C. The chromatograms developed with ninhydrin were left for maximum 2 hr. at room temperature and then the spots were scanned with a reflectance densitometer. The area under the curves was measured planimetrically. The calibration curve made with the known amounts, served for the reading of the amount of leucine, which was liberated in the enzyme reaction. The area in cm^2 was plotted against the amount of leucine (in μg.).

RESULTS AND DISCUSSION

The best results were achieved with 80% acetone as the chromatographic solvent. The separation of leucine from leucinamide was very good, so that traces of peptides arising from the enzyme sample and located between the two spots, did not interfere with the scanning. The solvent was moving fast and the time for the development of the chromatogram was 3.5 hr.

The calibration curves for leucine and leucinamide are shown in Fig. 1. A linear dependence of the leucinamide amount from the area (in $cm.^2$) was observed. In the case of leucine the slope of the curve decreases. From the calibration curve reliable results can be obtained up to 7 μg. of leucine and up to

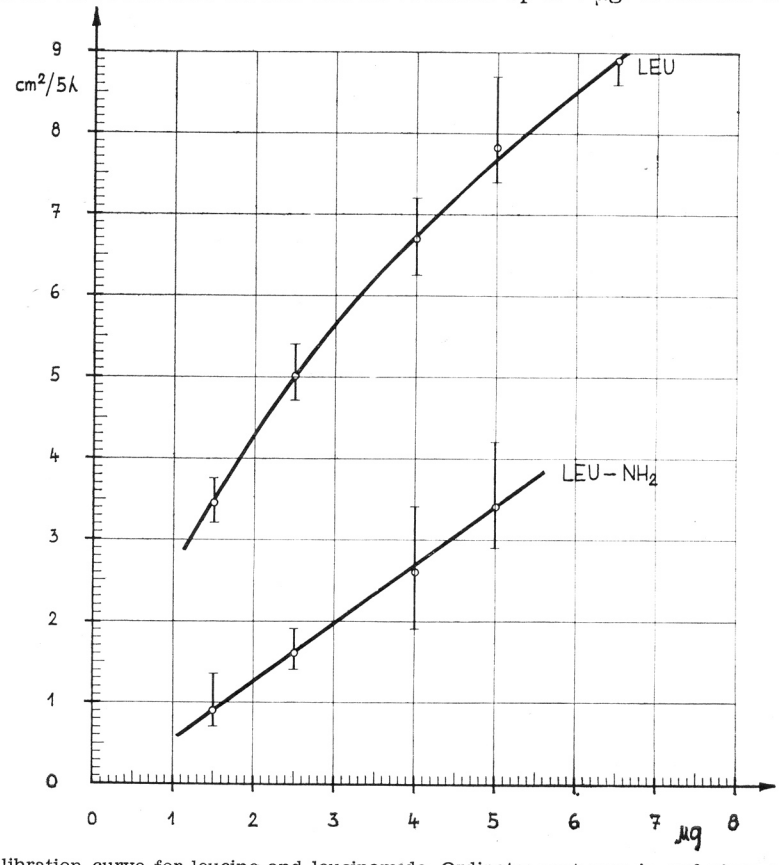

Fig. 1. Calibration curve for leucine and leucinamide. Ordinate: spot area in cm^2 given by 5 μl of sample (reaction mixture). Abscise: micrograms of leucine and leucinamide.

5 μg. of leucinamide, respectively. Each point in the diagram represents the mean value of 20 experiments. Maximal deviations were 6% at lower concentrations and 8% at higher concentrations. From our measurements we can conclude that the reproducibility of this method is comparable with other published methods.

The units in our calibration curve are given in μg. of liberated leucine in one hour and they can easily be converted into other units described in the literature. In our laboratory the quantity of leucine (in μM) liberated in one hour at 37° C from the amount of enzyme, containing 1 mg. of protein nitrogen, is used as the unit.

Using this method we were able to determine leucine aminopeptidase activity in some organs of Wistar albino rats. In liver the leucine aminopeptidase activity from 0.95 to 2.23 enzyme units was found, in lungs from 0.40 to 0.57, in spleen from 0.10 to 0.59, in kidney from 0.68 to 0.95, in mucosa of the small intestine from 0.78 to 1.28 and in mucosa of the large intestine from 0.32 to 0.48.

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REFERENCES

1. J. A. Goldbarg and A. M. Rutenburg, *Cancer* **11** (1958) 283.
2. M. N. Green, Kwan-Chung Tsu, R. Bressler, and A. M. Seligman, *Arch. Biochem. Biophys.* **57** (1955) 458.
3. J. A. Goldbarg, E. P. Pineda, and A. M. Rutenburg, *Am. J. Clin. Pathol.* **32** (1959) 571.
4. O. Räsler and H. Schön, *Clin. Chim. Acta* **6** (1961) 583.
5. J. E. Folk and M. S. Burstone, *Proc. Soc. Exp. Biol. Med.* **89** (1955) 473.
6. O. Braun-Falko and K. Salfeld, *Arch. Klin. Exp. Dermatol.* **204** (1957) 407.
7. H. E. Arst, R. T. Manning, and M. Delp, *Am. J. Med. Sci.* **238** (1959) 598.
8. E. L. Smith and D. H. Spackman, *J. Biol. Chem.* **212** (1955) 255.
9. E. L. Smith and D. H. Spackman, *J. Biol. Chem.* **212** (1955) 271.
10. E. K. Paterson, S. H. Hsiao, and A. Keppel, *J. Biol. Chem.* **238** (1963) 3611.
11. G. A. Fleischer, M. Pankow, and C. Warmka, *Clin. Chim. Acta* **9** (1964) 259.
12. G. A. Fleischer, M. Pankow, and C. Warmka, *Clin. Chim. Acta* **9** (1964) 254.
13. R. J. Haschen, *Clin. Chim. Acta* **1** (1956) 242.
14. D. Lebez and M. Kopitar, *Clin. Chim. Acta* **16** (1967) 267.

IZVOD

Kvantitativna kromatografska metoda za određivanje leucin aminopeptidaze

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Razvili smo mikrometodu za određivanje leucin-aminopeptidaze. Kao supstrat upotrijebili smo leucinamid. Leucin, koji nastaje cijepanjem kvantitativno smo odredili pomoću papirne kromatografije i refleksijsko-denzitometrijskog određivanja njegove količine. Iz površine mrlje nastaloga leucina kvantitativno smo odredili aktivnost encima. Metodu možemo upotrijebiti za dokazivanje leucin-aminopeptidaze u supernatantima, dobivenim centrifugiranjem homogeniziranih organa.

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