ISOLATION AND IDENTIFICATION OF THE PLATELET AGGREGATION INHIBITOR PRESENT IN THE ONION, *ALLIUM CEPA*

H. WEISENBERGER, H. GRUBE, E. KOENIG and H. PELZER
Department of Biochemistry, Dr. K. Thomae GmbH, 795 Biberach/Riss, Germany

Received 13 July 1972

1. Introduction

Enhanced fibrinolytic activity in the blood after ingestion of onions was reported by Gupta [1] and Menon [2–4]. As we are interested in platelet aggregation, a few *in vitro* experiments were performed to evaluate if platelet aggregation could also be influenced by onion constituents. The marked inhibition of platelet aggregation which we demonstrated stimulated us to isolate and to identify the inhibitor present in onions.

2. Materials and methods

2.1. *Onion extracts*

Peeled onions were homogenized for 2 min in a blendor and particulate material removed by filtration through cheese-cloth. After 2–4 hr at 4°, a precipitate formed which was removed by centrifugation without loss of aggregation-inhibiting activity in the supernatant.

2.2. *Platelet rich plasma (PRP)*

PRP was prepared by differential centrifugation of blood drawn from healthy volunteers by venipuncture and anticoagulated by 3.13% trisodium citrate dihydrate (9:1 v/v). Siliconised (Bayer H) glassware was used throughout PRP preparation.

2.3. *Platelet aggregation*

This was measured by a turbidimetric technique according to Born [5]. After incubating 20 μl of the preparation to be tested for 2 min at 37° in 0.5 ml PRP, aggregation was induced by adding ADP. Depending on the PRP, 10–40 μl of ADP solution had to be added to yield irreversible aggregation at a final ADP concentration of 1–4 μM. ADP and adenosine were purchased from Boehringer Mannheim, Biogel P-2 (200–400 mesh) from BioRad, and ultrafiltration membranes from Sartorius Membranfilter Gesellschaft Goettingen.

3. Results and discussion

Preliminary ultrafiltration and dialysis experiments employing membranes of different pore sizes suggested a molecular weight of well below 10 000 for the platelet aggregation inhibitor. Therefore high-molecular-weight proteins were removed by ethanol precipitation. An equal volume of 96% ethanol was added to the supernatant of the first centrifugation step, and the precipitate which formed within 1 hr at 4° removed by centrifugation. The clear yellowish supernate was diluted 1:4 with water and lyophilized. Reconstitution of the lyophilisate with water to its original volume did not cause loss of activity.

Several attempts to achieve a satisfactory separation of the reconstituted lyophilisate by filtration over different ion exchange resins resulted in partial (Dowex 50 WX 8, Na^+ form) or complete (Dowex 1 X 8, Cl^- form) loss of activity. Peptide rich fractions as seen by the ninhydrin reaction contained practically no activity. Therefore we tried to separate the platelet aggregation inhibitor and ninhydrin-positive fractions.

Finally, Biogel P-2 column chromatography (60 cm × 1.6 cm ∅) proved to be most promising. 5 ml reconstituted lyophilisate was applied to the column and eluted with water at 4°. The optical

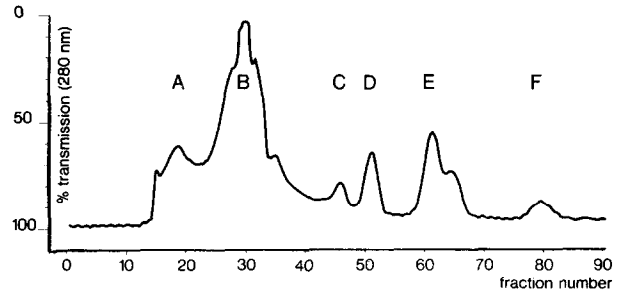

Fig. 1. Elution pattern of lyophilized onion homogenate supernatant from Biogel B-2 with water. The main peaks are designated A to F.

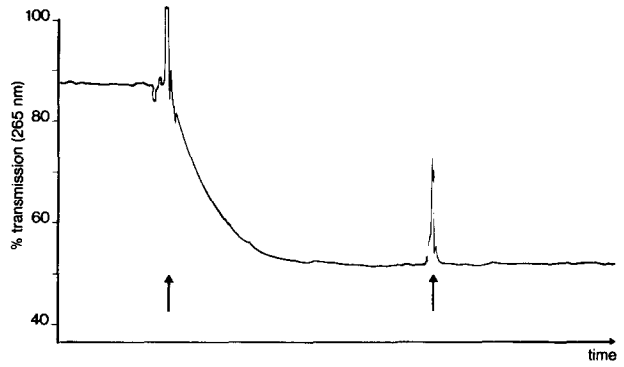

Fig. 2. Enzymatic deamination with adenosine deaminase (ADA) of the isolated compound. At the times marked with an arrow, ADA was added.

density at 280 nm was recorded with a Uvicord II (LKB Producter) and 15 ml fractions collected. The fractions corresponding to the 6 main peaks seen on the record (fig. 1) were individually combined, lyophilized and reconstituted to 5 ml with water. Activity was tested with 20 μl of each of the 6 fractions as described under Methods. Only the fraction "E" inhibited platelet aggregation, and addition of other fractions did not improve the results.

The UV spectra of the individual 15 ml fractions within the peak "E" displayed a sudden change of the wavelength of maximal absorption, λ_{max}, from 280 to 260 nm, indicating the elution of a substance other than a peptide. Platelet aggregation inhibiting activity was found only in the fractions with λ_{max} = 260 nm. Complete separation was finally achieved by thin-layer chromatography (Merck precoated plates,

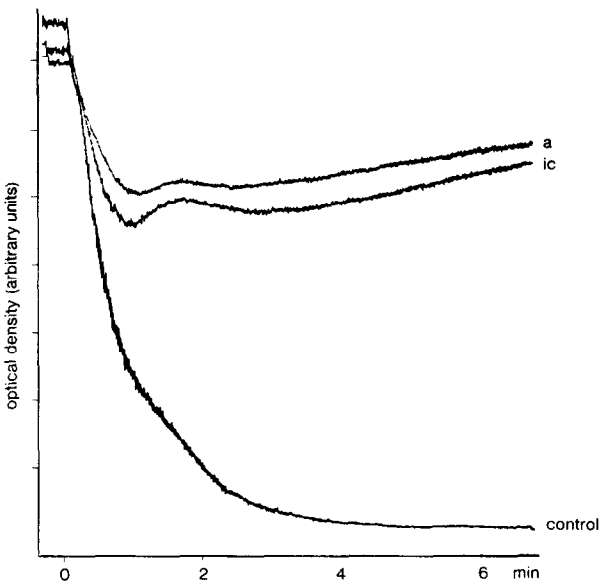

Fig. 3. Superimposed tracings of platelet aggregation induced by 2 μM ADP without further addition (control), and with 10 μM adenosine (a) and 10 μM isolated compound (ic), resp.

silica gel HF 254; solvent, dioxane/water 9:1 v/v), which yielded two distinct bands: one ninhydrin-positive with λ_{max} = 280 nm, and one ninhydrin-negative with λ_{max} = 260 nm. The latter was scraped off, eluted with methanol, and the concentrated solution chromatographed two-dimensionally on cellulose thin-layer plates "Merck", the solvents being methanol/conc. HCl/H_2O 70:20:10 (v/v/v) and n-butanol/methanol/H_2O/conc. NH_3 60:20:20:1 (v/v/v/v). Adenosine served as reference and identical R_f values were obtained. Next, adenosine deaminase was added to a buffered solution of the isolated compound, and the decrease in optical density recorded (fig. 2); further addition of enzyme did not alter the absorbance. Fig. 3 shows the inhibition of platelet aggregation by adenosine and the isolated substance. Finally, after chromatography on a prewashed silica gel thin-layer plate, using redistilled solvents, the isolated compound and adenosine as reference were chromatographed, the spots scraped off and freed from solvent at 1 mm Hg for 5 hr. After elution from silica gel, the subsequent mass spectra showed that the two substances were identical (fig. 4). Thus, the isolated compound was shown to be adenosine by UV spectrum, thin-layer chromatography, enzymatic de-

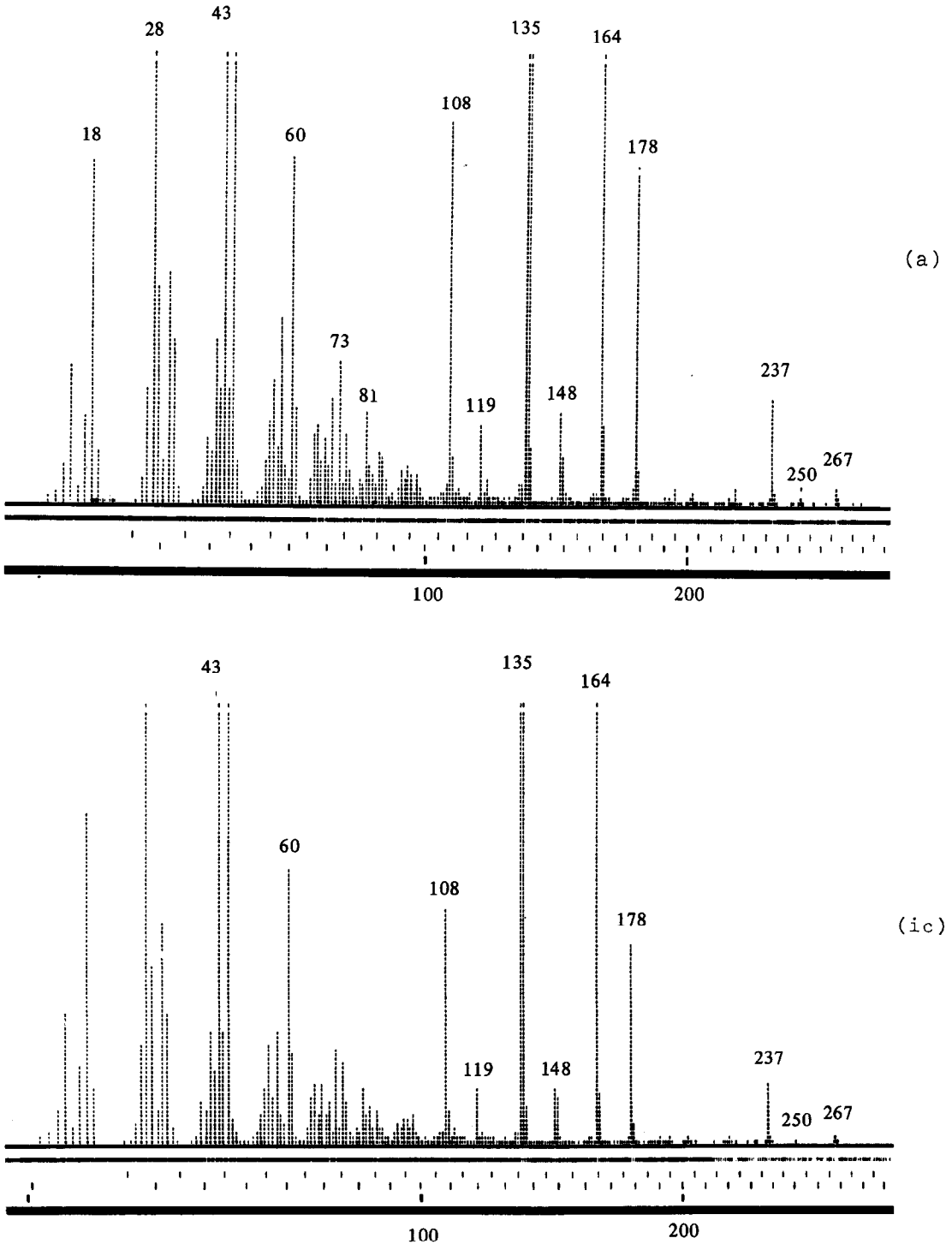

Fig. 4. Mass spectra of (a), authentic adenosine and (ic), isolated compound.

amination, platelet aggregation inhibition and mass spectroscopy.

Having confirmed that inhibition of platelet aggregation by onion homogenates is due to the presence of adenosine, we estimated its concentration according to Bergmeyer [6]. Interestingly, the adenosine content of 1-year-old onions was found to be much higher than in fresh ones (6 μg and 2 μg/ml homogenate, respectively).

Adenosine is a very potent inhibitor of platelet aggregation [7]. Concentrations as low as 1 μM or 0.3 μg/ml produce a marked inhibition, the degree of which depends on the PRP used. Correspondingly, 50 μl of onion homogenate when added to 0.5 ml PRP, gives an adenosine concentration of approx. 0.2 to 0.6 μg/ml, an amount sufficient to inhibit platelet aggregation. *In vivo* effects after ingestion of onions on platelet aggregation, however, can be ruled out, as far as adenosine is concerned.

Acknowledgement

We thank Dr. Prox for the mass spectra and their interpretation.

References

[1] N.N. Gupta, R.M.L. Mehrstra and A.R. Sircar, Ind. J. Med. Res. 54 (1966) 48.
[2] I.S. Menon, R.Y. Kendal, H.A. Dewar and D.J. Newell, Brit. Med. J. 3 (1968) 351.
[3] I.S. Menon, Brit. Med. J. 1 (1969) 845.
[4] I.S. Menon, Brit. Med. J. 2 (1970) 421.
[5] G.V.R. Born and M.J. Cross, J. Physiol. 168 (1963) 178.
[6] H.U. Bergmeyer, ed., Methoden der enzymatischen Analyse (Verlag Chemie, Weinheim, 1962) p. 491.
[7] J.F. Mustard and M.A. Packham, Pharmacol. Rev. 22 (1970) 97.