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SEPARATION OF ISORENIN ACTIVITY FROM NERVE GROWTH FACTOR (NGF) ACTIVITY IN MOUSE SUBMAXILLARY GLAND EXTRACTS

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Abstract—Submaxillary glands of adult male mice contain large amounts of isorenin and of nerve growth factor (NGF). Since the NGF preparations available so far contain isorenin activity, we tried to separate the two biological activities. By means of Sephadex fractionation and chromatographic methods an NGF preparation free from isorenin activity and an isorenin preparation free from NGF activity were obtained. On the basis of acrylamide-gel electrophoresis a molecular weight of between 35,000 and 40,000 was calculated for isorenin, which is similar to that of kidney renin from various species. The close association of isorenin and NGF and their dependence on the male hormone suggest that the two factors are produced in the same cells, probably located in the tubules.

MOUSE submaxillary gland extracts show a marked renin-like activity, which has been ascribed to an isoenzyme.[1] The isorenin activity of the glands is sex-dependent, being much higher in the male than in the female; it is reduced by castration and is markedly increased in both the female and the castrated male by the administration of androgens.[2] In these respects, isorenin behaves like the nerve growth factor (NGF) of mouse submaxillary glands, which is also androgen-dependent.[3] Purified preparations of NGF (either commercially available or prepared by different laboratories) have been found to contain significant amounts of isorenin activity; these observations suggested the possibility that the two biological activities might occur in the same protein molecule.† On the other hand, renin or isorenin preparations from sources other than mouse salivary glands show no NGF activity whatsoever.‡ It was the aim of the experiments reported here to clarify whether or not isorenin and NGF activities could be completely separated. The results obtained show that the two biological activities are in fact due to two distinct protein molecules.

MATERIALS AND METHODS

Adult male mice (Swiss strain) were the source of submaxillary glands in all experiments. NGF was purified according to the method of Bocchini and Angeletti.[4] Submaxillary glands were dissected, freed from adherent connective and adipose

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† F. Gross: unpublished observation.

‡ P. U. Angeletti: unpublished observation.

tissue and homogenized in ice-cold distilled water (1:5 w/v). The homogenate was centrifuged for 30 min at 10,000 *g*. One vol. of 0·2 M streptomycin sulphate in 0·1 M Tris buffer (pH 7·5) was slowly added to 9 vol. of the supernatant. The mixture was allowed to stand in ice for 30 min and then re-centrifuged. The clear supernatant was then lyophilized. Gel filtration was performed on Sephadex G-100 columns, equilibrated with Tris–HCl buffer (0·05 M, pH 7·5) containing 0·5 mM EDTA.

Ion exchange chromatography was performed on CM-52 and DE-52 cellulose columns equilibrated at various pH's, as indicated in Results section. Fractions from the gel filtration on ion exchange chromatography were checked for protein by u.v. absorption at 280 nm using a Zeiss model II spectrophotometer. The protein content in selected pools was estimated by the method of Lowry,[5] bovine albumin being used as a standard.

Polyacrylamide gel electrophoresis was performed as indicated by Davis.[6] The gel systems used were at pH 4·3 and pH 8·6. Gel electrophoresis on dodecyl–sulphate–polyacrylamide was performed according to Weber and Osborn.[7]

NGF activity was assayed in tissue culture by the method of Levi-Montalcini.[8] One biological unit was referred to as the amount of NGF required to give a 3+ response as judged by the halo of nerve-fibre outgrowth from explanted ganglia. Protease activity was measured by the Kunitz method, casein being used as a substrate.[9] Esterase activity was measured by a photometric method in which benzoyl–arginine ethyl ester (BAEE) was used as substrate.[10]

Isorenin activity was assayed in nephrectomized rats anaesthetized with the sodium salt of 5-ethyl-5-(1′-methyl-propyl)-2-thiobarbituric acid (Inactin®, Promonta) and given pentolinium tartrate (5 mg/rat intraperitoneally) to block autonomic reflexes. Relative specific activities (RSA) were calculated by comparison of pressor responses to the unknown with the response to a known amount of angiotensin II-amide (Hypertensin®, CIBA), according to the following formula:

$$\frac{\text{Response to the unknown (mm Hg)}}{\text{Response to 5 ng angiotensin (mm Hg)}} \times \frac{\text{Dilution of the unknown}}{\text{Milligrams of the unknown}} = \text{RSA}.$$

RESULTS

A typical Sephadex G-100 fractionation pattern of the salivary gland homogenate after streptomycin precipitation is shown in Fig. 1. Most of the NGF activity was recovered in the fractions indicated by the dashed area. The vertical bars indicate the distribution of proteolytic activity. The fractions in the dashed area were collected, pooled, and further purified as follows: after dialysis for 24 hr against acetate buffer (0·05 M, pH 5) the material was centrifuged and then applied to a CM-52 cellulose column equilibrated with the same acetate buffer. Elution was performed with a salt gradient from 0 to 1 M NaCl. The chromatographic separation is shown in Fig. 2. Most of the NGF activity was found in the last protein peak, which accounts for about 3 per cent of the proteins loaded. Acrylamide-gel electrophoresis showed that this protein peak was homogeneous, and only at high protein concentration could traces of protein contaminants be detected.

This NGF preparation, which may be considered pure for all practical uses, has a very high specific activity as measured by tissue culture.

When different NGF preparations were tested for pressor activity in nephrectomized

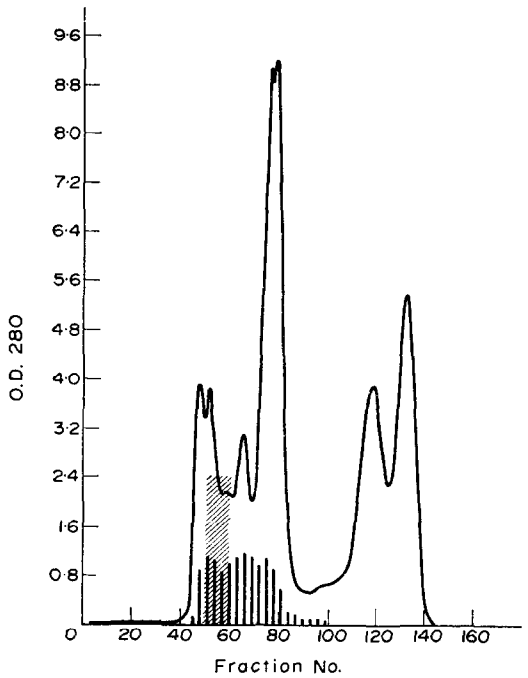

FIG. 1. Fractionation of mouse salivary gland extracts on Sephadex G-100. The dashed area indicates the fractions containing NGF activity. Distribution of protease activity is indicated by vertical bars.

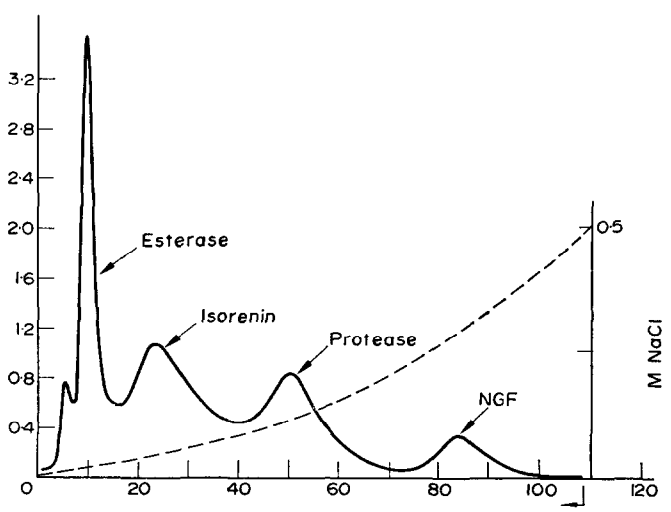

FIG. 2. CM-cellulose separation of the active NGF pool obtained from Sephadex G-100. The localization of four different biological activities is indicated.

rats, considerable amounts of a renin-like activity were detected. The high variability of the renin-like activity in different samples suggested the possibility of a contaminant. In order to test this hypothesis, NGF was further purified by three successive passages through a CM-52 column, under the conditions described above. After each passage, only the central part of the emerging protein peak was collected and re-chromatographed. By this procedure the renin-like activity was progressively decreased and, after the third cycle, was undetectable (Table 1).

TABLE 1. RELATIVE ACTIVITY OF NGF PREPARATION AFTER ONE, TWO AND THREE CYCLES ON A CM-CELLULOSE COLUMN

NGF	Relative specific activity
1st CM-chromatography	12
2nd CM-chromatography	5
3rd CM-chromatography	0

Attempts were then made to isolate the isorenin activity from the other fractions collected after chromatography on the first CM-cellulose column. The chromatographic pattern and the localization of various biological activities (esterase, protease and isorenin) are shown in Fig. 3.

The fractions displaying pressor activity were pooled, dialyzed against acetate buffer (pH 5), and re-chromatographed on a CM-52 column. A single, broad peak was obtained, in which the pressor activity was mainly localized in the first part (Table 2). Only the fractions with the highest specific activity were collected and pooled. Subsequently, this active pool was dialyzed against distilled water, concentrated by vacuum dialysis, and applied to a Sephadex G-100 column equilibrated with Tris–HCl buffer (0·01 M, pH 7·2). Three main peaks were obtained; renin-like activity was localized in the major peak (Fig. 4). Electrophoresis of this component

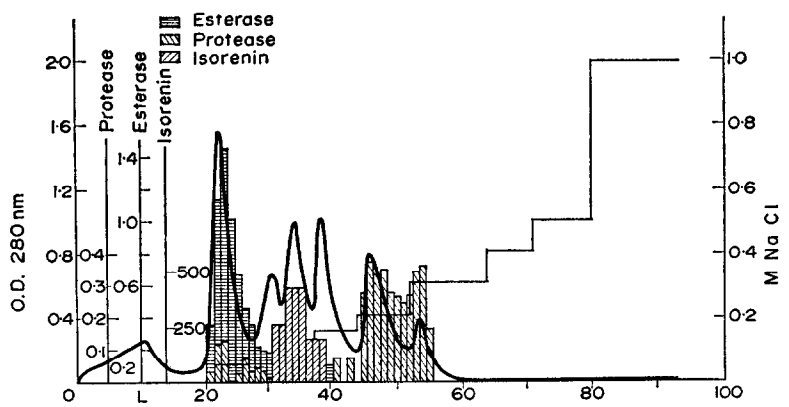

FIG. 3. Re-chromatography of the fractions collected after the first CM-column, those containing NGF activity having been removed. Elution by step-wise gradient. Isorenin activity is mainly localized in the third protein peak.

TABLE 2. RELATIVE SPECIFIC ACTIVITY OF ISORENIN IN THE POOLS COLLECTED AFTER CM-CHROMATOGRAPHY*

Samples	Relative specific activity
CM-1	456
CM-2	879
CM-3	340
CM-4 (NGF)	5

* See Fig. 2.

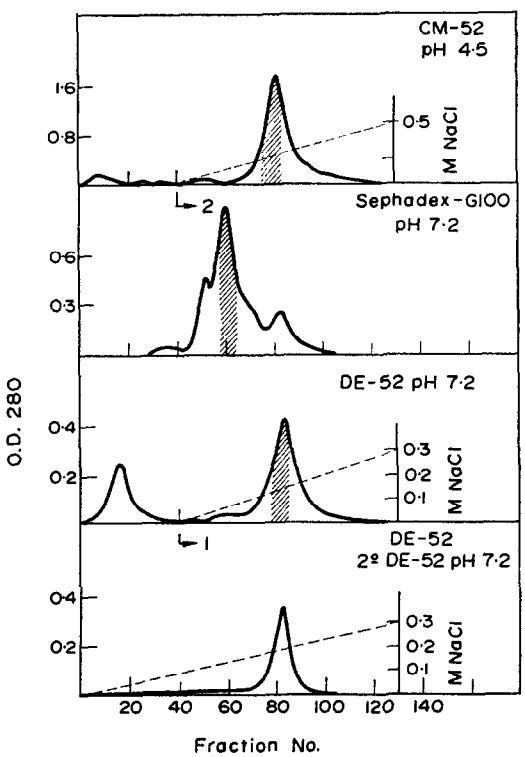

FIG. 4. Re-chromatography of the protein peak containing isorenin activity on a CM-cellulose column (above). The dashed area indicates the fractions displaying the highest activity. These were collected and pooled for further fractionation on a Sephadex G-100 and then on a DE-cellulose column (see text).

revealed the presence of at least three protein bands. Further purification of the active pool, collected after the Sephadex G-100 step, was achieved by chromatography on a DE-52 column at pH 7·2. Two major components were separated under these conditions, the first emerging with the pregradient elution, the second eluted with a salt gradient from 0 to 0·5 M NaCl. All the pressor activity was in this second protein component (Table 3). The most active fractions were pooled, re-chromatographed

TABLE 3. RELATIVE ACTIVITY OF ISORENIN IN THE VARIOUS FRACTIONS COLLECTED DURING THE PURIFICATION PROCEDURE*

Samples	Relative specific activity
Crude submaxillary extract	116
CM-fraction	879
Sephadex G-100 fraction	1794
DE-52 fraction	1953
2nd DE-52 fraction	1927

* See Fig. 4.

under the same conditions, and finally concentrated by vacuum dialysis. On acrylamide-gel electrophoresis at pH 4·5 this active fraction showed one single band; at pH 8·6, however, a major band and a second, migrating closely to it, were observed.

When the purified sample was analyzed on acrylamide-gel electrophoresis in sodium–dodecyl–sulphate, a major protein band was observed: its molecular weight was calculated to be between 35,000 and 40,000. Similar molecular weight estimations were obtained when isorenin was analyzed on Sephadex G-100. This molecular weight is close to that found for renal renin in various other species.[11]

DISCUSSION

The results reported here indicate that the pressor activity displayed by partially purified NGF preparations is not due to the NGF molecule *per se*. This activity can be completely separated from NGF and is due to a protein that may be classified as one of the isorenins present in the mouse submaxillary gland. Attempts to purify this isorenin have led to the isolation of a protein fraction with a very high pressor activity. This high specific activity of isorenin explains why even a minor contamination of NGF with this protein could be detected.

Since, by the fractionation methods used, it was possible to isolate two fractions with completely different biological activities, it is improbable that the isorenin fraction corresponded to one of the subunits of the NGF protein found by other investigators.[12] Furthermore, the fractionation methods used in our experiments differed in various ways from those resulting in the dissociation of the large protein into smaller subunits.

The close association of isorenin with NGF and their similar dependence on androgenic hormones suggest that the two proteins are produced in the same cells of the gland, probably located in the tubular portion. Like NGF, isorenin may be produced by the cells lining the tubuli and secreted in the large secretory granules.[13] The function and significance of these biologically active proteins in the submaxillary gland and of their release remains to be explained.

Further studies are now in progress to completely purify this isorenin and to define its physico–chemical characteristics. Immunochemical analysis with antibodies to the purified enzyme will also be carried out in order to establish similarities and/or differences to the renin-like enzymes in other tissues and in other species.

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