ACTIVITY OF ARYLAMIDASES IN SOME BOVINE ORGANS AND THEIR CHROMATOGRAPHIC SEPARATION ON SEPHADEX G-200

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Received February 2nd, 1972

The arylamidase activity was examined in homogenates and supernatants of various bovine organs, using L-leucyl-, L-lysyl-, glycyl-, α-L-glutamyl-, L-phenylalanyl- and S-methyl-L-cysteinyl-*p*-nitroanilides as substrates. The highest enzyme activity was observed in all organs, with the exception of liver, toward L-leucyl-*p*-nitroanilide, the pronouncedly highest activities having been found in homogenates of placenta and of kidney. Low activities were found toward α-L-glutamyl- and S-methyl-L-cysteinyl-*p*-nitroanilides in all organs studied. The lowest activities in general were found in the serum. Using gel filtration on Sephadex G-200 the homogeneity of the enzymes was investigated. Enzymes present in the soluble supernatant fraction of the homogenates of the various organs were separated mostly into two distinct enzyme fractions with unequal activities toward the substrates used.

Aminopeptidase (arylamidase) enzymes are being studied extensively with the aid of amino acid derivatives of β-naphthylamine[1] or *p*-nitroaniline[2], originally designed and used for the assay of leucine aminopeptidase. Some authors have reached the conclusion that enzymes catalyzing the splitting of the N-terminal amino acid from the arylamine group are different from aminopeptidases hydrolyzing amides or small peptides of different amino acids[3–12]. The reasons for distinguishing arylamidases from classical leucine aminopeptidase[5] are different molecular weight[13,14], various chromatographic and electrophoretic behaviour[9,13,15,16] and different effects of divalent cations on enzyme activity[17–20]. The occurrence of arylamidases in sera and tissues of different animals is rather varied. Considerable differences in activities of these enzymes were observed in the sera of various species[21] as well as in different tissues of a single species[19,22]. High arylamidase activities were found in the kidney[23], liver[24], brain[10] but also in other tissues[25].

In the present communication we describe the levels of arylamidases in bovine organs and serum, their sensitivity to different amino acid derivatives of *p*-nitroaniline and their chromatographic properties on Sephadex G-200.

EXPERIMENTAL

Material. Fresh bovine organs and serum were obtained from the slaughter-house from 5 healthy animals, with the exception of placenta which was obtained within 5 h of birth from the field. The following organs were used: liver, kidney, heart, muscle, spleen, brain, pancreas and placenta. Before processing, the material was stored at 0–4°C. To examine the enzyme activities, the following substrates were used: L-leucyl-, L-lysyl-, glycyl-, L-phenylalanyl-, α-L-glutamyl

and S-methyl-L-cysteinyl-*p*-nitroanilides. With the exception of L-lysyl-*p*-nitroanilide, the substrates are poorly soluble in water and, therefore, they were prepared as 25 mM solutions in 10 mM-HCl. To estimate the enzyme activity of the various fractions after gel filtration and agar electrophoresis, 5 mM substrates were used. The Sephadex G-200 was from Pharmacia, Uppsala, other chemicals were of analytical purity.

TABLE I

Arylamidase Activities in Individual Bovine Organs and Serum

Enzyme activity was estimated toward L-leucyl-, L-lysyl and glycyl *p*-nitroanilides and is expressed in μmol *p*-nitroaniline released during 1 h incubation at 37°C per mg protein nitrogen.

Organ	L-Leucyl-*p*-nitroanilide		L-Lysyl *p*-nitroanilide		Glycyl-*p*-nitroanilide	
	Homogenate	Supernatant	Homogenate	Supernatant	Homogenate	Supernatant
Muscle	61·3	168·6	58·7	137·1	15·8	72·2
Heart	109·1	168·6	109·1	168·6	37·9	145·8
Liver	63·6	103·3	88·7	132·3	30·5	72·2
Spleen	264·0	188·4	121·2	213·6	66·0	73·7
Kidney	900·0	390·6	331·5	262·2	222·3	103·5
Pancreas	—	151·2	—	74·4	—	38·6
Brain	226·8	158·4	206·3	111·3	66·3	40·5
Serum	—	17·8	—	13·2	—	13·9
Placenta	2 244·0	2 172·0	118·2	290·7	53·7	171·6

TABLE II

Arylamidase Activities in Individual Bovine Organs and Serum

Enzyme activity was estimated toward α-L-glutamyl, L-phenylalanyl, and S-methyl-L-cysteinyl *p*-nitroanilide and is expressed as in Table I.

Organ	α-L-Glutamyl-*p*-nitroanilide		L-Phenylalanyl-*p*-nitroanilide		S-Methyl-L-cysteinyl-*p*-nitroanilide	
	Homogenate	Supernatant	Homogenate	Supernatant	Homogenate	Supernatant
Muscle	6·2	21·7	26·7	88·2	19·9	25·7
Heart	11·5	37·1	43·0	163·8	25·8	44·6
Liver	10·6	28·1	27·4	60·0	22·4	34·7
Spleen	13·7	36·7	80·8	98·6	111·9	47·7
Kidney	282·8	76·4	236·4	95·6	220·8	47·4
Pancreas	—	16·2	—	48·0	—	29·5
Brain	7·4	19·1	99·6	89·8	50·3	27·4
Serum	—	5·8	—	12·4	—	6·6
Placenta	72·6	198·3	283·5	355·5	10·4	18·8

Preparation of tissue homogenates and supernatants. All the organs except muscle, brain and pancreas were sliced into thin sections and rinsed with precooled 0·1M-Tris-HCl buffer of pH 7·1. Equal weight parts of every organ from 5 animals were pooled and homogenized with a knife disintegrator in 4 volumes of 0·1M-Tris-HCl buffer of pH 7·1. To prepare supernatant fractions of every organs, ultracentrifugation at 106 000 *g* for 60 min at 2°C was done in a preparative VAC 60 Janetzki centrifuge. Samples intended for fractionation by gel filtration were stored at −20°C.

Estimation of enzyme activity. The arylamidase activity of homogenates and supernatants of the individual organs was estimated by spectrophotometric measurement of the liberated *p*-nitroaniline at 400 nm after a 60 min incubation at 37°C. The enzyme reaction took place in 2·5 ml of 0·1M-Tris-HCl buffer of pH 7·1, 0·2 ml 25 mM substrate and 0·2 ml enzyme. The

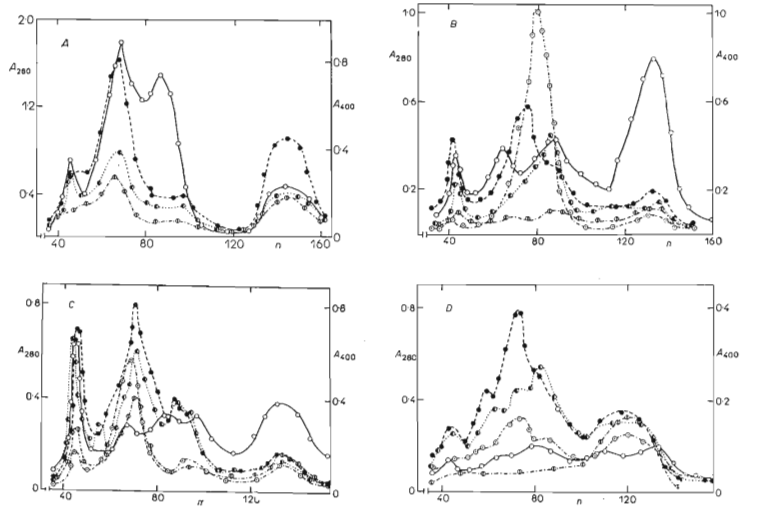

FIG. 1

Gel Filtration of Enzymes of Serum (*A*) and of Supernatant Fractions of Liver (*B*), Kidney (*C*) and Heart (*D*) Homogenates

Three ml of serum or supernatant fractions were chromatographed on a column of Sephadex G-200 (3·1 × 60 cm) which was washed with 0·1M-Tris-HCl buffer of pH 7·1. The protein concentration was estimated at 280 nm (○) the enzyme activity determined by the release of *p*-nitroaniline at 400 nm. Activity towards L-leucyl *p*-nitroanilide (●), toward L-phenylalanyl-*p*-nitroanilide (◐), toward glycyl *p*-nitroanilide (⊙) and toward L-lysyl-*p*-nitroanilide (⊙).

reaction was stopped by adding 0·5 ml 1M-HCl. In control samples, enzymes were inactivated before substrate addition by boiling on a water bath for 15 min. Before measuring absorbance, particles were removed by centrifugation. The nitrogen content of the individual samples was estimated by microkjeldahlization. The enzyme activity of homogenates and supernatants is expressed in μmol liberated *p*-nitroaniline per 1 h of incubation per mg protein nitrogen.

Gel filtration on Sephadex G-200 of the supernatant enzymes from the individual organs was done on a column of Sephadex G-200 (60 × 3·1 cm) which was washed with 0·1M Tris-HCl buffer, pH 7·1 at a rate of 12·5—13·0 ml/h. Three-ml samples were separated. Agar electrophoresis of the supernatant fractions of homogenates and of serum was done in the micromodification of Večerek and coworkers[26]. Electrophoresis was done in a veronal–citrate buffer of pH 7·7 and an ionic strength of 0·05 μ at 5 V/cm and 2 mA/cm. After separation, the agar plate was sectioned transversely into 2 mm strips and the enzymes were eluted with 0·5 ml 0·1M-Tris-HCl buffer of pH 7·1 and incubated with the substrate at 37°C.

RESULTS AND DISCUSSION

Enzymes of aminopeptidase character that split the peptide bond of chromogenic synthetic compounds and called arylamidases[3] are distributed in many organs of different animal species. The activities of these enzymes estimated toward different amino acid derivatives of *p*-nitroaniline in bovine organs are shown in Table I and II. It may be seen that arylamidases are present in all organs, in most cases the enzyme activity in the supernatant fraction being higher than that bound to insoluble cell

TABLE III

Localization of Arylamidase Activities in Soluble Proteins of Bovine Organs and Serum after Agar Electrophoresis

Separation was done at 2—4°C for 2·5 h. Electrophoretic mobility of enzyme fraction I migrating at the tail of albumins was taken as 1, mobilities of other fractions were normalized to this value.

Organ	Anode				Cathode
	I 1·00	II 0·60	III 0·18	IV 0·00	V 0·26
Liver		+	+	+	
Kidney		+	+	+	+
Heart	+	+	+	+	
Muscle		+	+	+	
Spleen		+	+	+	+
Brain		+		+	
Pancreas		+	+	+	
Placenta	+	+		+	+
Serum	+	+	+		

particles. An exception is formed by the kidney where most of the activity under the given experimental conditions is not present in the supernatant fractions but remains bound in the subcellular particles. In the case of placenta, in contrast with the findings of Rehfeld and coworkers[25] (using alanyl β-naphthylamide as substrate), we found a higher activity of enzymes splitting L-leucyl-*p*-nitroanilide as compared with other organs.

In another section of experiments we examined the homogeneity of arylamidase enzymes in the supernatant fraction of homogenates of the individual organs and in serum. For this reason, we fractionated the enzymes on Sephadex G-200. The elution pattern of proteins was measured at 280 nm and enzyme activities in the various fractions were determined with L-leucyl, L-lysyl-, L-phenylalanyl- and glycyl-*p*-nitroanilide. The results of fractionation of serum and of soluble supernatant proteins of the various organs and the distribution of enzyme activities in the various

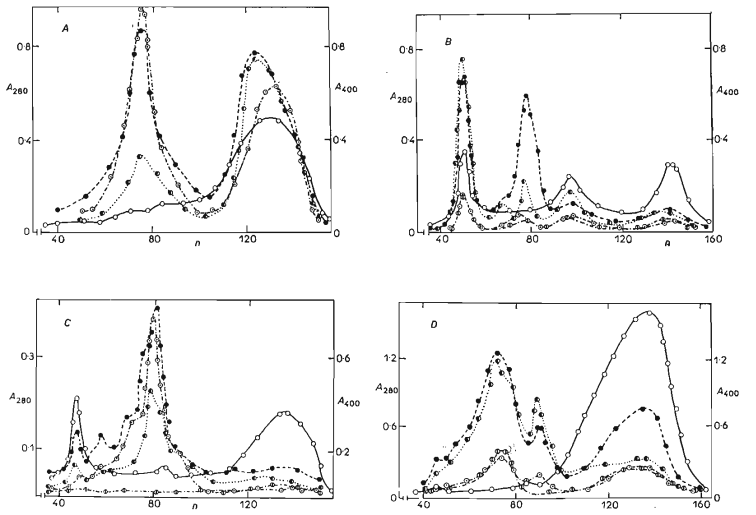

FIG. 2

Gel Filtration of Enzymes of the Supernatant Fractions of Muscle (*A*) Spleen (*B*), Brain (*C*) and Pancreas (*D*) Homogenates

Separation and significance of symbols are as in Fig. 1.

fractions are shown in Fig. 1 and 2. With serum proteins it was observed that, in addition to three protein peaks[27], a fourth fraction is eluted from the column, probably of nonprotein character[28] which was even more pronounced in some organ supernatants. The fastest fraction, so-called macroglobulins (19 S globulins) was pronouncedly in some organs increased as compared with serum (kidney, spleen, brain). On the other hand, the fraction of γ-globulins (7 S globulins) and albumins which form the predominant part of proteins in serum are much less pronounced in these organs.

The results of activity determinations in the fractions after gel filtration (Fig. 1 and 2) show that proteins with enzyme activity toward different chromogenic substrates are not identical but differ in their properties. Enzymes of most organ supernatants were separated after gel filtration into two mutually separated peaks, one of which (with higher molecular weight) was eluted with the front (19 S macroglobulins), the other (with lower molecular weight) emerged between the fractions of γ-globulins and albumins. Fractionation of serum proteins and of soluble supernatant proteins of brain, muscle, pancreas, heart and liver showed that the greatest part of activity is bound in these cases to the lower molecular weight proteins.

Attempts at separation of enzymes hydrolyzing chromogenic substrates in normal human and pregnancy serum by chromatography on Sephadex G-200 resulted in separation of two mutually overlapping fractions in the pregnancy serum, in contrast with the normal serum where the activity was localized only in a single peak[29]. Hopsu and coworkers[30], using the same fractionation procedure, found that the supernatant enzymes of rat liver splitting the basic substrate L-arginyl-β-naphthylamide were separated into two peaks. Similarly to our case, Mäkinen and Hopsu[31] found several enzymically active fractions after gel filtration of enzymes of several rat organs.

Many electrophoretic studies of arylamidase enzymes were done with the purpose of determining these enzymes in the diagnostics of several diseases[32]. Starch electrophoresis of enzymes present in healthy tissues and in serum[33] of rats and in human organs[34] resulted in the separation of at least two unequally mobile, enzymically active fractions. Similarly in our case, the organ and serum enzymes after electrophoretic separation on agar displayed several electrophoretically different enzymes (Table III). It was found that enzymes present in soluble protein fractions of all the organs studied, with the exception of brain, display in agar electrophoresis three electrophoretic variants at the anodic side. A fourth enzyme fraction with highest electrophoretic mobility was found in the serum and in the supernatant of the heart and placenta. Another enzymically active zone was found at the cathodic side with preparations of kidney, spleen and placenta. Results similar to ours were obtained by Rehfeld and coworkers[25] who found after starch electrophoresis of supernatants of some human organs a total of five localities with enzyme activity and different occurrence in the various organs.

On the basis of these resuts it may be concluded that serum and organ arylamidases represent an inhomogeneous polymorphic enzyme system. The separation technique

of gel filtration makes it possible to differentiate between at least two mutually differing enzymes with different activity for the substrates used. The heterogeneity of the enzymes is even more pronounced after agar electrophoresis.

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Translated by A. Kotyk.