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Determination of Effects and Binding Properties of N-Nitrosomorpholine and N-Nitrosopyrrolydine on Human Acrosin by Thin Layer Chromatography

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Aim: Acrosin is a unique trypsin-like acrosomal proteinase that is required for mammalian fertilization. Nitrosamines are a large group of chemical compounds that have been found to be carcinogenic in all species of animals tested and thus suspected to be a human carcinogen. The purpose of the our study was to investigate the *in vitro* effect of nitrosamine, on human acrosin activity and some of the main amino acids (tryptophan, phenylalanine, histidine, tyrosine, pyroline, cysteine, methionine and arginine),
Material and Methods: *In vitro* effect of nitrosamine, on human acrosin activity and some of the main amino acids, which are responsible for the constitution of acrosin, was investigated by thin layer chromatography.
Results: Our results showed that nitrosamines can have non-competitive inhibitory role on enzyme activity. Results of effect of nitrosamines on some of the main amino acids showed that 24 hours incubated amino acid-nitrosamine mixtures areas were smaller then free amino acid, free NMOR and free NPYr, indicating that reaction velocity was increased as in a time-dependent manner, therefore the amount of product was increased,
Conclusion:It was observed that the reaction velocity increased with time and that the resulting product increased with time. In other words, binding of amino acids to nitrosamine increases in a time depended manner. The present results indicated the non-competitive inhibitory effect of nitrosamines on acrosin.

Key Words: Human acrosin, Thin layer chromatography, Nitrosopyrrolydine, Nitrosomorpholine, Enzyme kinetic, Amino acid.

N-Nitrosomorfolin ve N-Nitrosopirolidin'in İnsan Akrosinine Bağlanma Özelliğinin ve Etkisinin İnce Tabaka Kromatografisi ile belirlenmesi.

Amaç: Akrozin kendine özgü, tripsin benzeri bir akrozomal proteinaz olup, memeli fertilizasyonunda gereklidir. Nitrozaminler oldukça geniş bir kimyasal bileşik grubudur ve test edildiği bütün hayvan grublarında kansere sebebiyet verdiği gösterilmiştir ve insanlar üzerinde de aynı etkiye sahip olabileceği konusunda düşünceler vardır. Çalışmanın amacı, nitrozamin'in *in virto* etkisinin insan akrozin aktivitesi ve bazı amino asitler (triptofan, fenilalanin, histidin, tirozin, prolin, sistin, metionin ve arjinin) üzerine olan etkisini incelemektir.
Gereç ve Yöntem: Akrozinin yapısında yer alan bu amino asitlere nitrosaminin bağlanması ince tabaka kromatografisi ile incelenmiştir.
Bulgular: Çalışmada 24 saat inkübasyon sonucunda, nitrozamin aminoasitlerin üzerine olan etkisinin zamana bağlı olarak arttığı kontrol grubu olan serbest amino asid, serbest NPOR ve serbest NPYR ile karşılaştırılarak gösterilmiştir.
Sonuç: Reaksiyon hızının zamana bağlı olarak arttığını ve sonucunda da oluşan ürünün zamana bağlı olarak arttığı görülmüştür, diğer bir deyişle amino asidlerin nitrozamine bağlanması zamana bağlı olarak artmaktadır. Elde edilen sonuçlar; nitrozaminlerin akrozin üzerine non-kompatatif inhibitör etkisine sahip olduğunu göstermiştir.

Anahtar Kelimeler: İnsan akrozini, İnce tabaka kromatografisi, Nitrozoprolidin, Nitrozomorfolin, Enzim kinetik, Amino asid.

Acrosin is a unique trypsin-like acrosomal proteinase that is required for mammalian fertilization.[1] Nitrosamines are a large group of chemical compounds that have been found to be carcinogenic in all species of animals tested and thus suspected to be a human carcinogen.[2, 3]

Acrosin is a 'trypsin-like' serine proteinase extrinsically associated with membranes of the mammalian sperm acrosome an intracellular organelle.[4, 5, 6]

The acrosin content is most accurately evaluated by immunological techniques, and the acrosin activity by extraction and measuring its interaction with substrate. From a clinical standpoint, it is most likely preferable to measure the acrosin activity rather than the acrosin content of the spermatozoa because it is the acrosin activity that determines the ability of the spermatozoa to function.[7, 8, 9] The ability of acrosin inhibitors to prevent both *in vitro* and *in vivo* fertilization has stimulated interest in the development of agents for specific acrosin inhibition as well as interest in mechanism of acrosin regulation.

Nitrosamines are a large group of chemical compounds that have been classified as suspect human carcinogens even though direct causal evidence is lacking. [3, 10, 11] The n-nitrosomorpholine (NMOR) and n-nitrosopyrrolydine (NPYr) are both cyclic nitrosamine, which naturally occur in foods, tobacco and rubber. They are formed from secondary amines and nitrite ions.[2,10] In the human environment, nitrosatable amine precursors to N-nitroso compounds and nitrosating species such as; nitrite and oxides of nitrogen are abundant. It is known that human foods may contain trace amounts of carcinogenic N-nitrosamines and that the largest known human exposures to exogenous N-nitrosamines occur in the work place via nearly all industries.[2] Originally, it was thought that the use of nitrite as a curing agent for flesh foods was the major source of these trace compounds in the diet.[12] As a result, the formation of N-nitroso compounds and human exposure to these compounds show a rather complex pattern.[13]

The purpose of the our study was to investigate the *in vitro* effect of nitrosamine, found to be carcinogenic and particularly present all most everywhere with higher levels, on human acrosin activity and some of the main amino acids (tryptophan, phenylalanine, histidine, tyrosine, pyroline, cysteine, methionine and arginine), which are responsible for the constitution of acrosin. For this, thin layer chromatography (TLC) technique was used which is known as reliable by reducing the contamination risk in minimum levels and also all the spots can be analyzed separately.

MATERIAL AND METHODS

Materials: N-nitrosomorphiline, n-nitrosopyrrolydine, Benzamidine hydrochloride and Ficoll (type 400) were purchased from Sigma Chemical Company (St. Louis, MO). Sephadex G-75 and dextran blue were obtained from Pharmacia. TLC Scanner (Shimadzu CS 9000), Centrifuge (refrigerated Damon/IEC DIVISION), pH meter (Beckman), Peristaltic Pomp (Eyela MP-3) were used. All solutions and buffers were prepared in tridistilled water and all glass materials were washed with a deionised water, left for 24 hours in 20% HNO_3 and rinsed three times with a deionised water prior to each use unless otherwise specified. All other regents were of the highest quality commercially available.

Human semen was obtained from apparently normal and healthy 30 volunteers by self-masturbation aged between 20-30 and known to be father in our region. They were collected in sterile deionised cylindrical tubes (2.5 x 5 cm) and shipped in dry ice and stored at –70 °C until being used.[14] In addition, to eliminate the possibility of intestinal parasites could be effective on antioxidant enzyme activities[7] all subjects were examined for intestinal parasites. For this, wet mount preparations in 0,9 % NaCl, diluted Lugol's iodine and flotation technique in saturated saline solution were used and parasite negative subjects were selected for the study.

Isolation and purification of human acrosin: The sperm were dispersed and diluted in physiological saline containing 50 mM benzamidine. Layered over 10 ml 11% ficoll containing 50 mM benzamidine and 0.9% NaCl and centrifuged at 1000 x g for 30 min at room temperature. The sperm concentration in the resulting pellet was measured with a hemacytometer and 6 x 10^9 cells were found. Afterwards the resulting pellets were resuspended in 1 ml solution containing 10% glycerol and 50 mM benzamidine in 1 mM HCl. The final pH of the sperm suspension was adjusted to pH 3.0 with 1 M HCl. The sperm were extracted at 4 °C for 4 hours and removed by centrifugation at 1000 x g for 30 min. The subsequent supernatant was dialyzed (Spectrophor #2) against two changes of a 2000-vol excess of 1 mM HCL at pH 3.0. The dialyzed preparation was further applied to a Sephadex G-75 superfine column by the method of Siegel MS et al Polakoski et al.[15,16] Briefly; The Sephadex G-75 superfine column was packed and equilibrated with 1 mM HCl, pH 3.0. The height of the gel bed (90 cm) in the column (12 mm wide) and the flow rate (30 drops per min)

remained constant throughout the experiment. Plastic collecting tubes (13 x 100 mm; falcon) were used to a void the protein adsorption to glass tubes. The protein content of the fractions was measured using Lowry et al.'s method.[17]

Determination of binding properties of NMOR and NPYR on human acrosin activity by Thin Layer Chromatography: Pure acrosin and 0.50 μg/ml BSA (same amount as enzyme protein) were mixed with 10 μg/ml NMOR and NPYR respectively to give the ratio of 1/1.

These mixtures, firstly, without incubating and afterwards having been incubated at 25°C were applied to TLC plate with pure acrosin, BSA, NMOR and NPYr respectively. TLC method was also used for the determination of the effect of NMOR and NPYr on some of the main amino acids (tryptophan, phenylalanine, histidine, tyrosine, pyroline, cysteine, methionine, arginine) which are responsible for the constitution of the enzyme.

Preparation of the samples: All above mentioned amino acid solutions were prepared to give 10 μg/ml concentration in distilled water. These amino acid solutions were mixed with either 10 μg/ml of NMOR or NPYr separately and incubated for 24 hours at room temperature. In separate tubes, NMOR and NPYr mixtures of these amino acid solutions were prepared, without incubating, each amino acid was applied to TLC plate same as with the incubated ones. For the application, aluminum base (10 x 10 cm) Silica-gel 60_F254 plate was used. 1 μl of sample was applied to plate with Hamilton syringe and left for a while to dry. Tank was incubated with alcohol and H_2O (70+30) mixture about 30 min to provide solvent-vapor balance before plate was placed into the tank. Samples were run 8 cm in 45 min. Plate was left to dry in air after chromatography. R_f values and their areas were calculated with linear scanning technique by a Shimadzu CS 9000 TLC scanner.

RESULTS

Determination of NMOR-Acrosin and NPYr-Acrosin Bindings by TLC: R_f values and their calculated areas were determined with linear scanning technique by Shimadzu CS 9000 TLC scanner. Chromatographic result of the enzyme-NMOR and enzyme-NPYr binding studies by TLC is shown in figure 1.

Calculated R_f values, maximum wavelengths and areas of these interactions were given in table 1. As it is seen from the table that under both unincubated and incubated (with enzyme) conditions, R_f values and maximum wave lengths of both nitrosamines were found to be the same, however, calculated areas were found to be relatively different between each other.

Figure 1: Determination of NMOR-enzyme and NPYr-enzyme interaction by TLC 1.[Ψ] Pure enzyme, 2. Free NPYr, 3. Enzyme-NPYr (unincubated), 4. Enzyme-NPYr (incubated), 5. Free NMOR, 6. Enzyme-NMOR (unincubated), 7. Enzyme-NMOR (incubated), 8.[Ψ]BSA, 9. BSA-NPYr (unincubated), 10. BSA-NPYr (incubated), 11. BSA-NMOR (unincubated), 12. BSA-NMOR (incubated). [Ψ]: Spots can not be seen under the UV light on thin layer chromatogram.

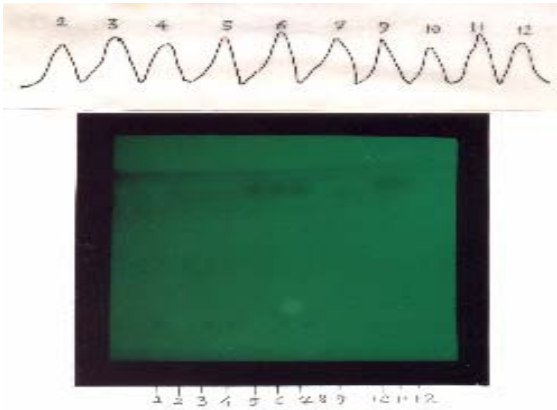


Table 1. R_f values of enzyme-NPYr and enzyme-NMOR interactions measured by TLC.

	R_f	*Area*	*Max (nm)*
Free NPYr	**86.0**	**16292**	**240**
Enzyme-NPYr (unincubated)	86.0	12834	240
Enzyme-NPYr (incubated)	86.0	10667	240
Free NMOR	88.0	16910	245
Enzyme-NMOR (unincubated)	88.0	15396	245
Enzyme-NMOR (incubated)	87.0	13685	245

In comparing with the free nitrosamine areas (control), we found a clear difference between incubated and unincubated enzyme-nitrosamine mixtures; therefore, we concluded that there is a clear interaction i.e., a binding between enzyme and each nitrosamine. The left areas were belonged to excess nitrosamines, which were present after binding.

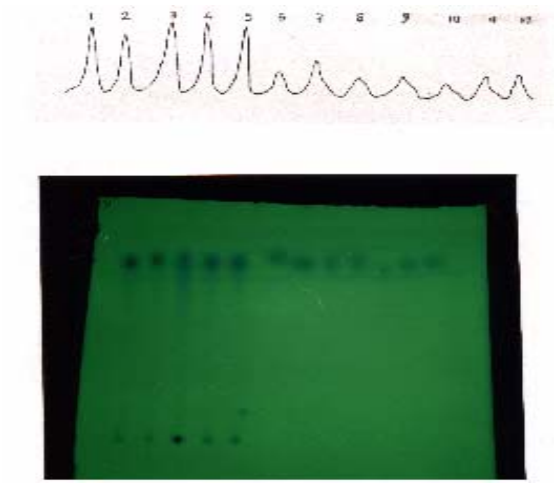
Binding of NMOR-BSA and NPYr-BSA was shown also by TLC and values were presented in table 2. As it is seen from the table, although there was a small change in R_f values and maximum wave lengths, incubated BSA-NMOR, BSA-NPYr areas were found smaller then free NMOR, free NPYr, unincubated BSA-NMOR and BSA NPYr areas. In conclusion, affect of BSA-NMOR and BSA-NPYr

interactions were less then enzyme-NMOR and enzyme-NPYr interactions.

Table 2. R_f values of BSA-NPYr and BSA-NMOR interactions measured by TLC.

	R_f	Area	Max (nm)
Free NPYr	**86.0**	**16292**	**240**
BSA-NPYr (unincubated)		16125	240
	84.0		
BSA-NPYr (incubated)		14900	236
	85.0		
Free NMOR	88.0	16910	245
BSA-NMOR (unincubated)	88.0	16312	245
BSA-NMOR (incubated)	88.0	14300	245

Figure 2. Determination of NMOR and NPYr with tryptophan and phenylalanine interaction by TLC (Picture was taken under the UV light).
1.**Tryptophan-NMOR, 2.*Tryptophan-NMOR, 3.Tryptophan (free), 4.**Tryptophan-NPYr, 5.*Tryptophan-NPYr, 6. NMOR (free), 7. NPYr (free), 8.**Phenylalanine-NMOR, 9.*Phenyalanine-NMOR, 10. Phenylalanine (free), 11.**Phenylalanine-NPYr, 12.*Phenyalanine-NPYr.
*: Unincubated amino acid-nitrosamine
**: Incubated amino acid-nitrosamine



Determination of NMOR-amino acid and NPYr-amino acid Bindings by TLC: R_f values and their calculated areas were given in table 3. When we compare R_f values of the 24 hours incubated and unincubated NMOR-amino acid and NPYr-amino acid mixtures with the standard amino acid additions, R_f values were found slightly higher in incubated amino acid-nitrosamine mixture (a representative TLC plate was presented in figure 2). In addition, each spot area was calculated. When NMOR/NPYr incubated and unincubated areas of each amino acid were compared with the free amino acid, free NMOR and free NPYr, incubated amino acid-nitrosamine mixtures areas were found smaller then others indicating that reaction velocity was increased in a time-dependent manner, therefore the amount of product was increased, i.e., binding of amino acid to nitrosamine was increased accordingly (table 3).

Table 3. R_f values of NPYr and NMOR interaction with tryptophan, phenylalanine, histidine, tyrosine, pyroline, cysteine, methionine, arginine measured by TLC.

	Rf	Area
Tryptophan**-NMOR	82.0	11930
Tryptophan*-NMOR	83.0	12793
Tryptophan (free)	82.0	20555
Tryptophan**-NPYr	83.0	15846
Tryptophan*-NPYr	83.0	16872
Phenylalanine**-NMOR	84.0	22479
Phenylalanine*-NMOR	84.0	34826
Phenylalanine (free)	81.0	23542
Phenylalanine**-NPYr	84.0	33808
Phenylalanine*-NPYr	86.0	37553
Histidine**-NMOR	88.0	4094
Histidine*-NMOR	88.0	8387
Histidine (free)	-	-
Histidine**-NPYr	86.0	10857
Histidine*-NPYr	86.0	17870
Tyrosine**-NMOR	85.0	71542
Tyrosine*-NMOR	85.0	74507
Tyrosine (free)	-	-
Tyrosine**-NPYr	84.0	56945
Tyrosine*-NPYr	84.0	59972
Pyroline**-NMOR	83.0	11966
Pyroline*-NMOR	83.0	14990
Pyroline (free)	-	-
Pyroline**-NPYr	81.0	6675
Pyroline*-NPYr	81.0	9479
Cysteine**-NMOR	84.0	1062
Cysteine*-NMOR	84.0	19524
Cysteine (free)	-	-
Cysteine**NPYr	81.0	7420
Cysteine*NPYr	82.0	41260
Methionine**-NMOR	84.0	14674
Methionine*-NMOR	84.0	21080
Methionine (free)	-	-
Methionine**-NPYr	83.0	7039
Methionine*-NPYr	82.0	11738
Arginin**-NMOR	85.0	11577
Arginin*-NMOR	85.0	22238
Arginin (free)	-	-
Arginin**NPYr	83.0	8131
Arginin*NPYr	84.0	8395
NMOR (free)	87.0	38954
NPYr(free)	84.0	65385

*: Unincubated amino acid-nitrosamine mixture.
**: Incubated amino acid nitrosamine mixture.
-: Spots are present during the application on TLC plate, but after chromatography they could not be detected under the UV light.

DISCUSSION

Nitrosamines are a large group of chemical compounds that have been found to be carcinogenic in all species of animals tested and thus suspected to be human carcinogen. [2, 3]

Nearly all industries, which are concerned with the production and/or use of amines, have a related nitrosamine problem. This is particularly evident in the rubber and tyre manufacturing industry and in metal cutting and grinding shops. This contamination may result from the use of contaminated starting materials, in particular amines, or from the formation of N-nitroso compounds during manufacturing processes. A similar problem also exists with agricultural chemicals.

As our knowledge of the occurrence and formation of N-nitroso compounds in the environment increases, preventive measures can be introduced, particularly in manufacturing industries, to reduce the levels of human exposure to nitrosamines in the work place and to protect the consumer from nitrosamine exposure from household commodities.[12,13] Subsequent research has clearly shown that other processing and packaging procedures can also introduce trace amounts of these carcinogens into foods. These procedures include drying foods in direct flame heated air, migration from food contact surfaces and direct addition as contaminants.[12] The greatest capacity to metabolize these nitrosamines to alkylating agents is found in the liver, but other organs, including the esophagus, lung and kidney, are also capable of activation.[11]

Acrosin is a multifunctional enzyme combining several functional properties within a single molecule; the catalytic triad of the proteinase, hydrophobic domains responsible for the special membrane-associating character of the enzyme and the carbohydrate binding sites by which the molecule can bind to the zona pellucida. Acrosin occurs in the sperm acrosome as an inactive precursor, proacrosin with a molecular mass of 53-55 kDa.[4] Several case control studies in patients with unexplained infertility have reported due to defects in capacitation and sperm motion characteristics, binding of the spermatozoa to the zone pellucida, acrosome reaction and including acrosin activity of the spermatozoa. Compounds, which inhibit acrosin, prevent either *in vitro* or *in vivo* fertilization.[18, 19, 20]

The purpose of the our study was to investigate the *in vitro* effect of nitrosamine, found to be carcinogenic and particularly present all most everywhere with higher levels, on human acrosin activity and some of the main amino acids which are responsible for the constitution of acrosin. Thin layer chromatography results were demonstrated that when R_f values, maximum wave lengths and areas of unincubated enzyme-NMOR and enzyme-NPYr mixtures compared with the free NMOR and free NPYr, and also incubated enzyme-NMOR and enzyme-NPYr mixtures compared with the free NMOR and free NPYr mixtures, it has been observed that although R_f values and wave lengths did not change significantly, there were clear differences in the areas of which they cover in themselves. These observations, showed us that there is a possible interaction between enzyme and each nitrosamine, i.e., a binding. Areas were belonged to excess nitrosamines which were left after enzyme-nitrosamine binding. Moreover, the decrease in the enzyme incubated nitrosamine areas in compared with the free nitrosamine areas indicates that nitrosamines bound to enzyme in great access.

When the affect of BSA-nitrosamine interaction was investigated with the same concentration of enzyme in a similar way, it was observed that incubated BSA-nitrosamine areas were found smaller then free NMOR, free NPYr, unincubated BSA-NMOR and BSA-NPYr. Gerhard et al showed that in males, smoking cigarettes did not change sperm number and motility but decreased the acrosin activity very effectively. This also shows us that nitrosamines, which are present in cigarette, can be effective to reduce to acrosin activity. [21]

In this study, effect of nitrosamines on some of the main amino acids (tryptophan, phenylalanine, histidine, tyrosine, pyroline, cysteine, methionine and arginine), which are responsible for constitution of the enzyme, were also investigated. Our results showed that R_f values of both NMOR and NPYr incubated amino acids were increased clearly. In respect to the this increase, we could say that both NMOR and NPYr bind to amino acids and increase the molecular weight and also non-polarity of the amino acid, they bind.

These results show us that nitrosamines can have non-competitive inhibitory role on enzyme activity. It is also known that inhibitory mechanism of the most of the chemical carcinogens is electrophilic. They bind to nucleophilic atoms on the proteins and change the conformation of the enzyme molecule. It has also been indicated that some of the responsible molecules from this process are; methionine and cysteine in sulphur, ring nitrogen in histidine, and third carbon atom in tyrosine.[22]

All these results show us that there is a clear interaction between enzyme and nitrosamine.

Advantages of TLC technique were its fast performance and reliability. It also reduces the contamination risk in minimum levels and all the spots can be analyzed separately.

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