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# Immunologic Characterization of Human Seminal Leucine Aminopeptidase (LAP) and Its Medicolegal Use*

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**Summary.** A new method for identification of seminal stains is described, based on the immunologic demonstration of leucine aminopeptidase (LAP), which is extremely abundant in human semen and specific for the prostate as well as semen.

An antiserum against human seminal plasma was obtained by repeated immunization of rabbits with seminal plasma and Freund's adjuvant. Ouchterlony's double immunodiffusion test and Culliford's precipitin electrophoresis were performed to demonstrate specific proteins of seminal plasma. LAP activity was visualized with L-leucyl-β-naphthylamide as substrate and with Fast Garnet GBC as coupler.

The immunologic analysis of LAP produced two precipitin lines with enzyme activity. One was observed in kidney, jejunum, pancreas, prostate, as well as in semen, and was completely absorbed with kidney homogenates. The other was found only in semen and the prostate and was not absorbed with kidney homogenates. When the anti-seminal plasma serum absorbed with the kidney was used, the semen-specific LAP could be demonstrated by precipitin electrophoresis only in seminal stains stored for up to 2 months, whereas it was not demonstrated in stains from other human body fluids. By means of precipitin electrophoresis the detection of the semen-specific LAP was possible at semen dilutions of up to 1:32.

The method described here greatly enhances the value of semen identification and is quite recommendable for the examination of stains in medicolegal practice.

**Key words:** Immunologic analysis of semen, LAP - Examination of stains, identification of semen - Identification of seminal stains, LAP

**Zusammenfassung.** Es wird über eine neue Methode zum Nachweis von Spermaspuren berichtet, die auf immunologischer Darstellung der Leucinaminopeptidase (LAP) beruht, die im menschlichen Sperma besonders reich-

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lich vorhanden und sowohl für das Sperma als auch für die Prostata spezifisch ist.

Ein gegen menschliches Spermaplasma gerichtetes Antiserum wurde durch wiederholtes Immunisieren von Kaninchen mit Hilfe von Freunds Adjuvans gewonnen. Zur Demonstration der spezifischen Proteine des Spermaplasmas wurden der Doppelimmunodiffusionstest nach Ouchterlony und die Überwanderungselektrophorese nach Culliford benutzt. Die Darstellung der LAP erfolgte mit L-Leucin-β-Naphthylamid als Substrat und mit Fast Garnet GBC als Kuppler.

Die immunologische Analyse der LAP ergab zwei Präzipitatlinien mit enzymatischer Aktivität. Die eine wurde im Sperma sowie in Nieren, Dünndarm, Pankreas und Prostata beobachtet und mit Nierenhomogenat vollständig absorbiert. Die andere war nur im Sperma und in der Prostata zu finden und ließ sich mit Nierenhomogenat nicht absorbieren. Bei Verwendung des mit Nieren absorbierten Antiserums konnte die spermaspezifische LAP mittels der Überwanderungselektrophorese nur in Spermaspuren bis zu einer Lagerungszeit von 2 Monaten nachgewiesen werden, aber sie kam in Spuren aus anderen menschlichen Körperflüssigkeiten nicht vor. In der Überwanderungselektrophorese war die Darstellung der spermaspezifischen LAP bis zu einer Spermaverdünnung von 1:32 möglich.

Die hier angegebene Methode erhöht den Beweiswert der Feststellung des Spermas beträchtlich und ist somit für die Spurenuntersuchung in der rechtsmedizinischen Praxis recht empfehlenswert.

**Schlüsselwörter:** Immunologische Spermaanalyse, LAP - Spurenuntersuchung, Spermanachweis - Spermaspurennachweis, LAP

An aminopeptidase hydrolyzing L-leucyl-β-naphthylamide (leucine aminopeptidase, LAP) is widely distributed in human tissues and body fluids. We showed previously that human seminal plasma contained an extraordinary amount of LAP as compared with any other body fluid and proposed an assay of LAP for medicolegal identification of seminal stains [1]. Subsequently, we developed a simple qualitative method detecting the enzyme as a preliminary test [2], which proved more specific, although less sensitive than the acid phosphatase test [3].

During recent years, various antigen-antibody reactions in gel diffusion have been used to demonstrate tissue-specific enzymes. Using such techniques, Hermann [4] demonstrated that human seminal plasma contained two aminopeptidases, both of which exhibited an immunologically identical reaction with the renal enzyme. Mattila [5] also found two aminopeptidases in the prostate: one was immunologically identical with the renal aminopeptidase, whereas the other appeared to be the enzyme characteristic of the prostate tissue.

More recently, an immunologic technique of precipitin reaction in agar gel electrophoresis (precipitin electrophoresis, counterimmunoelectrophoresis) has been developed, and the technique has been shown to be considerably more sensitive than the conventional immunodiffusion method [6–8]. By means of this technique, we have presented evidence that the origin of human seminal LAP is the prostate, by which the enzyme is secreted into the seminal plasma [9]. This

paper describes the immunologic characterization of human seminal LAP and its medicolegal use.

## Materials and Methods

### *Assays*

LAP activity was estimated by the method of Takenaka [10], using L-leucyl-β-naphthylamide. HCl as substrate and *p*-dimethylaminobenzaldehyde as color-developing reagent. The activity (units) was expressed in terms of the amount (mg) of β-naphthylamine liberated by 100 ml of sample in 1 h.

Protein was measured by the biuret method using crystalline bovine serum albumin as standard [11].

### *Tissue Homogenates*

Cerebrum, cerebellum, thyroid, heart, lungs, liver, spleen, pancreas, kidneys, stomach, jejunum, colon, and prostate were obtained from three male cadavers who were medicolegally autopsied within 12 h after death. Homogenization was performed in 9 vol. of 0.1 *M* sodium phosphate buffer (pH 7.0) at 4° C with an Ultra-Turrax homogenizer (Janke & Kunkel KG, Staufen im Breisgau, FRG). Supernatants were separated by centrifuging the homogenates at 12,000 × *g* for 10 min, condensed at 1:10 of the original volume with Amicon macrosolute concentrators (Amicon Co., Lexington, USA) and subjected to immunologic analysis.

### *Semen Samples*

Ejaculates were obtained by masturbation from 20 male volunteers, and the seminal plasma was separated from the sperm cells by centrifugation at 12,000 × *g* for 10 min. The plasma was pooled and stored at −20°C until use.

### *Preparation and Absorption of Antiserum*

Five rabbits received i.m. injections of an emulsion of 1 ml of pooled seminal plasma and 1 ml of Freund's complete adjuvant. Injections were given 5 times at weekly intervals. Blood was collected 2 weeks after the last injection, and the anti-human seminal plasma serum was separated.

One volume of the condensed supernatant of kidney homogenates having about 5,000 U of LAP activity was added to 3 vol. of the antiserum. The mixture was allowed to stand overnight at 4°C, and the resulting precipitate was discarded by centrifuging at 12,000 × *g* for 10 min. The antiserum thus absorbed was stored at −20° C until use.

### *Precipitin Reaction Techniques*

Ouchterlony's double immunodiffusion test [12] was carried out on 7 × 7 cm gel plates in 1 mm thickness, using 1% agarose in 0.05 *M* Veronal buffer (pH 8.6). Six wells were punched around a central well. The wells were 3 mm in diameter, and the center-to-center distance between the central and peripheral wells was 5 mm. Antigens were placed in the peripheral well; the antiserum in the central well. Diffusion was done in a moist chamber at room temperature overnight.

Precipitin electrophoresis was performed essentially according to the method of Culliford [6]. Sixteen wells, 3 mm in diameter, were punched in the gel plates in pairs 6 mm apart. Antigens were placed in the cathodic well; the antiserum in the anodic well. Electrophoresis was run at 4°C at a constant current of 10 mA/7 cm for 20 min.

*LAP Staining*

LAP activity was visualized by the histochemical technique of Burstone and Folk [13]: the gels were immersed and washed in physiologic saline for 2 days, then incubated at 37°C for 30 min in 20 ml of 0.2 *M* Tris-HCl buffer (pH 7.1) containing 5 mg of L-leucyl-β-naphthylamide·HCl and 20 mg of Fast Garnet GBC salt. The activity was visualized as red-pink precipitates.

*Stains*

Various human body fluids including semen were dropped or smeared on filter paper (Toyoroshi No. 2, Tokyo, Japan), allowed to dry at room temperature, and examined by the present precipitin techniques. Seminal stains of various ages were also tested. The stain to be tested was cut in a size of 3 × 3 mm, moistened with small amounts of gel buffer and placed in the antigen well.

## Results and Discussion

### *Distribution of LAP in the Human Body*

Table 1 shows the results for the distribution of LAP activity in various human tissues. The specific activity was the highest in the kidney, followed by prostate and jejunum. We used, therefore, the kidney for absorption of antiserum.

### *Immunologic Analysis of Human Seminal LAP*

The results of Ouchterlony's immunodiffusion analysis of LAP in human tissues and semen developed with the unabsorbed anti-human seminal plasma serum are shown in Fig. 1a, which demonstrates two different types of precipitin reaction obtainable. One was observed in kidney, jejunum, pancreas, prostate, and semen, each showing a reaction of identity and forming a ring around the central well. The other was observed only in the prostate and semen and localized adjacent to the peripheral well, each also showing a reaction of identity. Cerebrum, cerebellum, thyroid, heart, lung, liver, spleen, stomach, and colon gave no visible precipitin reactions. When the antiserum absorbed with kidney homogenates was used, the fused precipitin ring disappeared and only the precipitin line in the prostate and semen adjacent to the peripheral well stained (Fig. 1b).

Figure 2a shows precipitin electrophoresis of LAP in human tissues and semen obtained with the non-absorbed anti-human seminal plasma serum. The prostate and semen produced two precipitin lines with LAP activity near the anodic well: one was adjacent to the anodic well and the other a little toward the cathode. These two precipitin lines were located so closely together that they sometimes looked like a single line. The former type was observed also in kidney, jejunum, and pancreas. No precipitin reactions were found in the other tissues. When the antiserum was absorbed with kidney homogenates, the anodic precipitin line failed to emerge, whereas the cathodic precipitin line in the prostate and semen did not disappear (Fig. 2b).

The above results clearly indicate the existence of two immunologically distinct species of LAP in seminal plasma: one of them is specific for the prostate as well as seminal plasma, while the other is not specific. This finding is mainly com-

**Table 1.** Distribution of LAP in the human body

| Organ | Sample (1 ml) | Case 1 | | | Case 2 | | | Case 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Activity (Units (U)) | Protein (mg) | Specific activity (U/mg) | Activity (Units) | Protein (mg) | Specific activity (U/mg) | Activity (Units) | Protein (mg) | Specific activity (U/mg) |
| Cerebrum | Homogenate | 117.0 | 12.3 | 9.5 | 118.3 | 12.3 | 9.6 | 115.7 | 13.2 | 8.8 |
| | Supernatant | 48.8 | 4.9 | 10.0 | 85.8 | 4.1 | 20.9 | 93.6 | 5.1 | 18.4 |
| Cerebellum | Homogenate | 81.9 | 11.3 | 7.2 | 105.3 | 12.3 | 8.6 | 113.1 | 10.8 | 10.5 |
| | Supernatant | 50.1 | 3.7 | 13.5 | 78.0 | 4.3 | 18.1 | 80.6 | 4.2 | 19.2 |
| Thyroid | Homogenate | 70.2 | 21.6 | 3.3 | 26.0 | 22.5 | 1.9 | 35.1 | 22.5 | 1.7 |
| | Supernatant | 41.6 | 13.1 | 3.2 | 18.2 | 13.3 | 1.4 | 26.7 | 18.4 | 1.5 |
| Heart | Homogenate | 62.4 | 18.1 | 3.4 | 67.6 | 19.1 | 3.5 | 63.7 | 19.1 | 3.3 |
| | Supernatant | 48.1 | 7.6 | 6.3 | 45.5 | 5.9 | 7.7 | 52.7 | 7.1 | 7.4 |
| Lung | Homogenate | 110.5 | 21.1 | 5.2 | 31.0 | 24.5 | 1.3 | 59.8 | 21.6 | 1.4 |
| | Supernatant | 65.0 | 14.9 | 4.4 | 23.4 | 13.5 | 1.7 | 49.4 | 15.8 | 3.1 |
| Liver | Homogenate | 156.0 | 20.6 | 7.6 | 98.9 | 20.1 | 4.9 | 208.3 | 21.6 | 9.6 |
| | Supernatant | 66.3 | 14.1 | 4.7 | 49.4 | 11.0 | 4.5 | 87.1 | 13.3 | 6.5 |
| Spleen | Homogenate | 111.8 | 39.2 | 2.9 | 87.1 | 25.0 | 3.5 | 137.8 | 16.7 | 8.3 |
| | Supernatant | 74.8 | 26.5 | 2.8 | 65.0 | 16.3 | 4.0 | 110.5 | 11.6 | 9.5 |
| Pancreas | Homogenate | 195.8 | 20.1 | 9.7 | 260.0 | 15.7 | 16.7 | 305.5 | 14.2 | 21.5 |
| | Supernatant | 105.3 | 12.2 | 8.6 | 182.0 | 6.3 | 28.9 | 130.0 | 6.9 | 18.8 |
| Kidney | Homogenate | 1267.5 | 23.5 | 53.9 | 936.0 | 19.1 | 49.0 | 949.0 | 16.7 | 56.8 |
| | Supernatant | 253.5 | 8.2 | 30.9 | 214.5 | 11.0 | 19.5 | 253.5 | 6.0 | 42.3 |
| Stomach | Homogenate | 79.3 | 26.0 | 3.1 | 57.2 | 14.7 | 3.9 | 114.4 | 18.1 | 6.3 |
| | Supernatant | 50.1 | 11.6 | 4.3 | 28.6 | 4.5 | 6.4 | 32.5 | 6.7 | 4.9 |
| Jejunum | Homogenate | 507.0 | 17.2 | 29.5 | 260.0 | 12.3 | 21.1 | 539.5 | 17.2 | 31.4 |
| | Supernatant | 204.8 | 12.4 | 16.5 | 143.0 | 4.7 | 30.4 | 182.0 | 8.0 | 22.8 |
| Colon | Homogenate | 52.0 | 18.6 | 2.8 | 53.3 | 17.6 | 3.0 | 83.2 | 13.7 | 6.1 |
| | Supernatant | 46.5 | 9.8 | 4.7 | 31.2 | 4.9 | 6.4 | 51.4 | 7.3 | 7.0 |
| Prostate | Homogenate | 148.9 | 13.0 | 11.5 | 146.9 | 12.5 | 11.8 | 312.0 | 12.7 | 24.6 |
| | Supernatant | 135.9 | 7.0 | 19.4 | 106.6 | 5.1 | 20.9 | 258.7 | 5.4 | 47.9 |

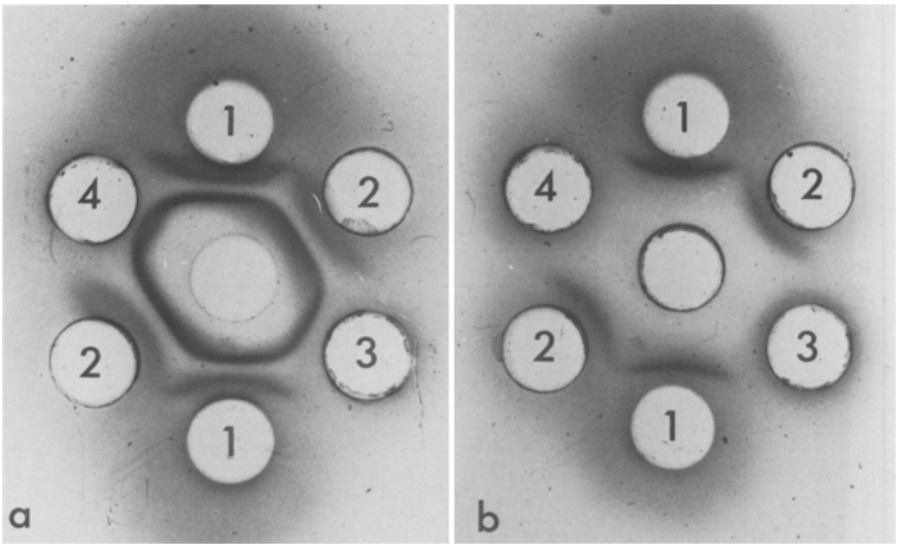


**Fig. 1a, b.** Double immunodiffusion test of LAP in semen *(1)*, prostate *(2)*, kidney *(3)*, and jejunum *(4)* using non-absorbed anti-human seminal plasma serum (**a**) and anti-human seminal plasma serum absorbed with kidney homogenates (**b**)

patible with the view of Mattila [5]. In a previous immunologic analysis of LAP in the male reproductive system developed with the unabsorbed antiserum, however, we were able to detect a single precipitin arc specific for the prostate and seminal plasma [9]. This was probably caused by having diluted them to possess the same enzyme activity as the other male reproductive tissues. In the present experiments, the latter type of LAP occurring not only in the prostate and semen, but also in kidney, jejunum, and pancreas could not be demonstrated in the other tissues, which is supposed to be ascribable to their low concentration. We feel that all human tissues and body fluids share this specificity.

*Identification of Seminal Stains*

The techniques described above were directly transposed to the examination of 20 seminal stains and five stains from various body fluids using the anti-human seminal plasma serum absorbed with kidney homogenates. The results of immunodiffusion and of precipitin electrophoresis are shown in Figs. 3 and 4, respectively. The precipitin line of LAP specific for the prostate and semen was demonstrated only in the seminal stains. No precipitin reactions were observed in the stains from blood, saliva, nasal discharge, tear, milk, perspiration, urine, vaginal secretions, and feces. Consequently, the semen-specific LAP is a consistent marker for the discrimination of seminal stains from other sources. The methods are superior to the spot LAP test [2, 14], because by the latter method weak positive reactions were seen in some fecal stains.

To compare the sensitivity between the two immunologic techniques, five ejaculates were serially diluted with physiologic saline, dropped on filter paper,

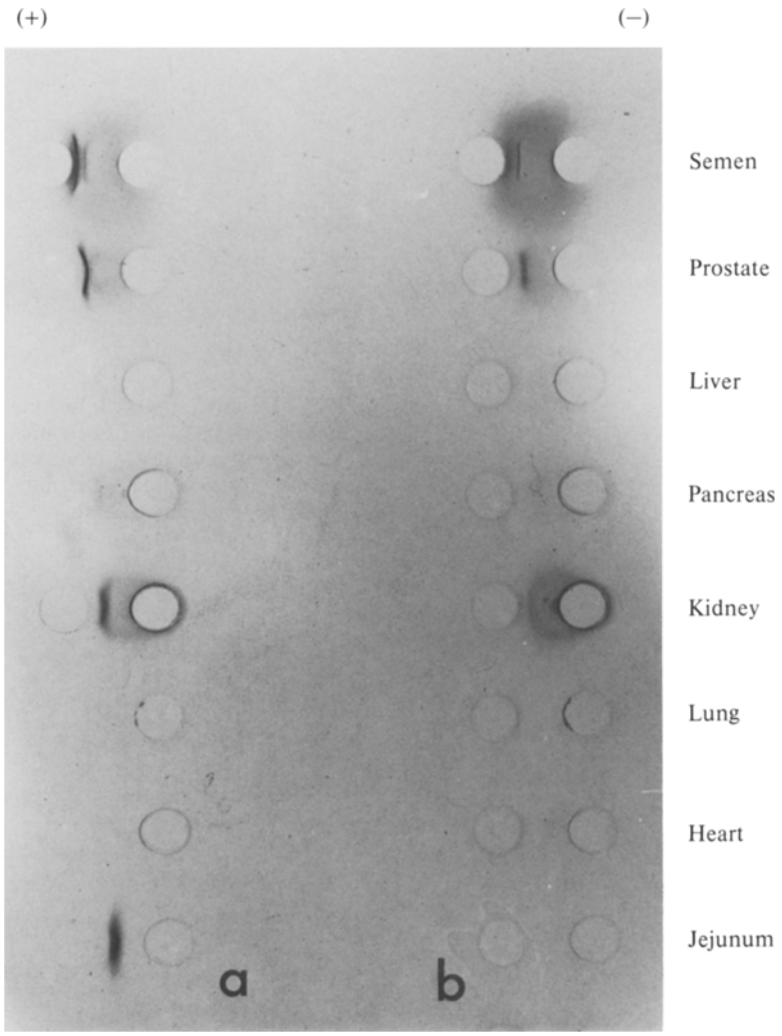


**Fig. 2a, b.** Precipitin electrophoresis of LAP in semen and tissues using non-absorbed anti-human seminal plasma serum (**a**) and anti-human seminal plasma serum absorbed with kidney homogenates (**b**)

and examined. By precipitin electrophoresis the semen-specific LAP was demonstrated at dilutions of up to 1 : 32 (Fig. 5), whereas by immunodiffusion it was no longer detected at a dilution of 1 : 4. The results indicate that precipitin electrophoresis is about 10 times as sensitive as double immunodiffusion. This is of practical value in the examination of vaginal swabs in rape cases, since ejaculates are to some extent diluted with secretions within the vagina.

The results examined by precipitin electrophoresis on 20 seminal stains of various ages are given in Table 2. The semen-specific precipitin line of LAP was demonstrated in all the 20 seminal stains stored for up to 2 months. After a period of 3 months, 14 of the 20 seminal stains exhibited a weak precipitin reaction.

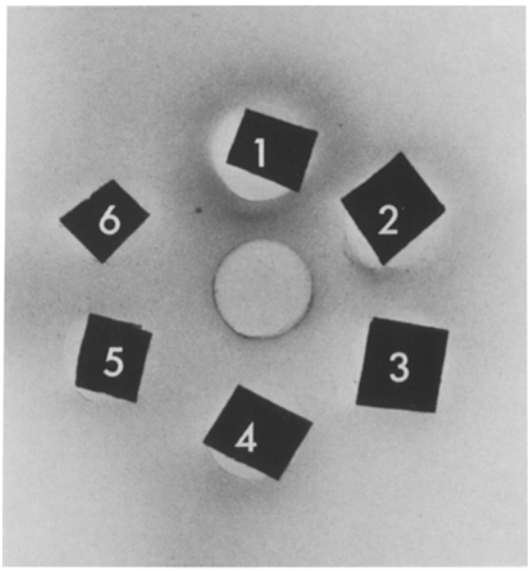


**Fig. 3.** Double immunodiffusion test of LAP in stains from semen *(1)*, feces *(2)*, urine *(3)*, blood *(4)*, saliva *(5)*, and vaginal secretion *(6)* using anti-human seminal plasma serum absorbed with kidney homogenates

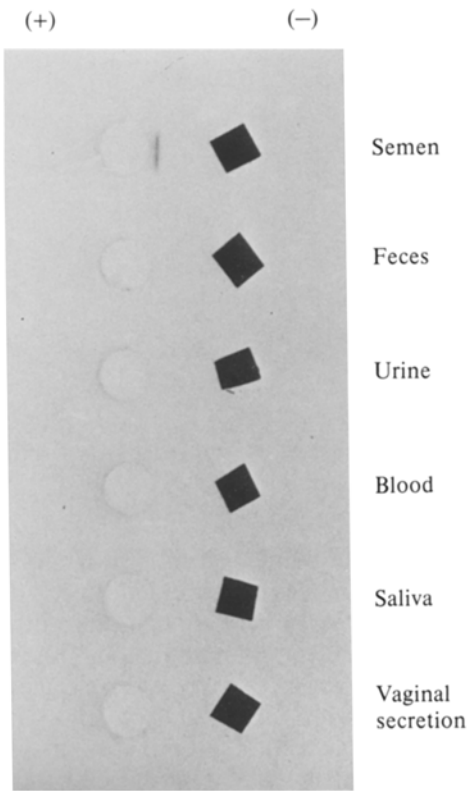


**Fig. 4.** Precipitin electrophoresis of LAP in stains from various body fluids using anti-human seminal plasma serum absorbed with kidney homogenates

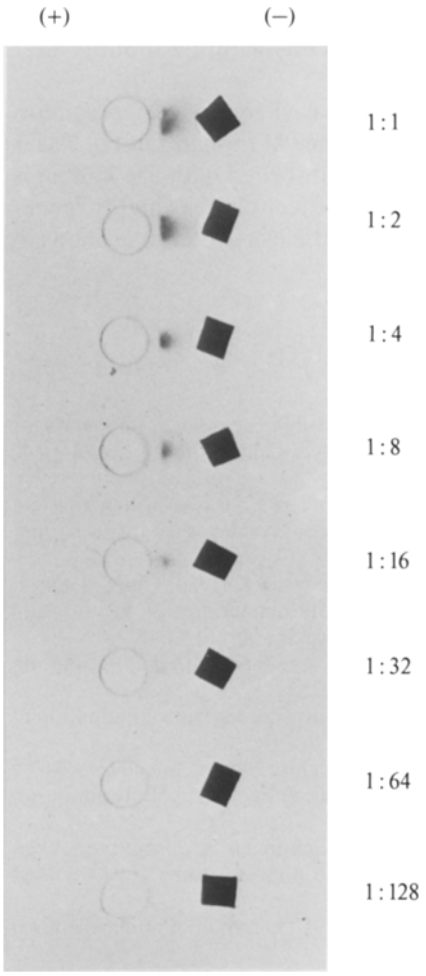


**Fig. 5.** Precipitin electrophoresis of LAP in stains from diluted semen using anti-human seminal plasma serum absorbed with kidney homogenates

**Table 2.** Precipitin electrophoresis of LAP in seminal stains of various ages using anti-human seminal plasma serum absorbed with kidney homogenates

| Age of stains | *n* | Precipitin reaction | |
|---|---|---|---|
| | | Positive | Negative |
| 1 week | 20 | 20 | 0 |
| 2 weeks | 20 | 20 | 0 |
| 3 weeks | 20 | 20 | 0 |
| 1 month | 20 | 20 | 0 |
| 2 months | 20 | 20 | 0 |
| 3 months | 20 | 14 | 6 |
| 4 months | 20 | 0 | 20 |

Detection was no longer possible in 4-month-old stains. The data show that this method can be used for identifying aged seminal stains within 2 months after stain formation.

In conclusion, the detection of the semen-specific LAP by means of precipitin electrophoresis provides a strong proof for the presence of human semen. As far as the anti-human seminal plasma serum properly absorbed with the kidney is used, there is no interference from other sources. The technique is highly specific and sensitive enough and is therefore suitable for the examination of stains in forensic investigations.

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