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Evidence that "Vaginal Peptidase" Is a Bacterial Gene Product

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ABSTRACT: A peptidase has been described in vaginal samples, termed "vaginal peptidase." This enzyme has been proposed as a tissue specific marker for vaginal debris. We have explored the presence of this enzyme in vaginal swabs from alleged sexual assault victims and volunteer donors as well as bacterial cultures. These studies reveal that "vaginal peptidase" is composed of a family of peptidase isozymes that originate from several bacterial species. The characterization of "vaginal peptidase" as a tissue specific marker for vaginal debris is premature.

KEYWORDS: criminalistics, peptidases, body fluids, criminal sex offenses

The analysis of sexual assault evidence usually focuses on the identification and genetic typing of semen. Semen evidence in sexual assault cases commonly contains vaginal material. The presence of vaginal material may be obvious from the nature of the specimen. For example, it would be very unusual for vaginal swabs and stains in the crotch of female underpants not to contain vaginal fluid. In addition, analytical evidence for the presence of vaginal secretion material can be obtained from the microscopic observation of nucleated epithelial cells in the absence of significant levels of amylase activity.

The discovery of a tissue specific protein marker for vaginal fluid would be useful in those situations where the primary focus of attention is the identification or quantitation of vaginal debris. Such a marker would be useful in providing rigorous evidence of recent sexual intercourse in penile swabs taken from the male, in the identification of vaginal material on foreign objects alleged to have been inserted into the vagina, and in assisting in the estimation of vaginal fluid contribution to mixed semen stains.

Recently, Divall identified a peptidase that appeared to be unique to vaginal material. This enzyme has been termed "vaginal peptidase" [*1,2*]. "Vaginal peptidase" is characterized by a rapid anodal electrophoretic mobility and substrate specificity for L-valyl-L-leucine and several other dipeptides. Divall did not detect this enzyme in blood, semen, saliva, feces, urine, tears, perspiration, nasal secretion, or penile swabs.

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Previous work by Harris's group at the Galton Laboratory has characterized the expression of seven peptidase loci in human body fluids and tissues; these loci have been termed Pep S, A, B, C, D, E, and F in order of increasing anodal electrophoretic mobility [3–7]. Each enzyme has been determined to be the product of a distinct genetic locus based upon direct genetic evidence or differences in substrate specificity. The only human peptidase loci found in these studies to have activity using L-valyl-L-leucine as substrate are Pep A and Pep S, where Pep S has a slower anodal electrophoretic mobility than Pep A. Further, in an extensive survey of human tissues, Rapley et al. failed to detect an anodal peptidase with the properties described by Divall for vaginal peptidase [7]. Thus, it would appear that "vaginal peptidase" represents a newly discovered human peptidase locus that is confined in its expression to vaginal material.

While Divall's studies suggest that vaginal peptidase may be a new human peptidase locus, there are a number of observations that indicate an alternative explanation for these findings. Proteins and enzymes which have localized tissue expression usually serve some essential biological function in that tissue. Such proteins are always found in that tissue, usually at high levels. Hemoglobin is a classic example of a tissue specific protein. Furthermore, there have been no previous reports of a tissue specific protein that is found in one individual one day but not in that same individual the next day with the possible exception of proteins that are under strict hormonal regulation. It is significant, therefore, that in Divall's study "vaginal peptidase" was absent in 113 (38.6%) of 293 samples from 3 donors where the presence or absence of the peptidase activity was not related to the menstrual cycle [1]. These data lead us to believe that there might be an alternative explanation for the occurrence of this unusual peptidase in vaginal material.

Bacteria are a potential alternative source for vaginal peptidase activity. Bacteria and mammalian cells use some of the same metabolic pathways; and, therefore, frequently contain some of the same enzyme specificities. In addition, it is known that bacterial enzymes can be significantly more acidic and consequently demonstrate a more rapid anodal mobility than their human analogs. For example, bacterial phosphoglucomutase (PGM) can be demonstrated in cultured biological specimens and in some vaginal samples at the anodal end of PGM electrophoresis plates [8, 9]. Bacterial PGM activity is demonstrated best by reducing the electrophoresis time normally used for human PGM typing by one-half. In this report we present evidence that "vaginal peptidase" is a bacterial gene product.

Methods and Materials

Two populations of vaginal swabs were used in this study. One set of vaginal swabs was obtained from alleged sexual assault victims. These swabs were dried after collection and stored frozen (−20°C) before analysis. Since these specimens were obtained from inactive cases, most of them were 2 to 4 years old at the time of analysis. In this set there were 362 samples from 283 individuals. A second set of vaginal swabs was obtained from a volunteer donor population. This set consisted of samples collected approximately every other day over the course of a menstrual cycle. These samples were stored frozen and were 1 to 2 months old at the time of analysis. This set consisted of 67 swabs from 6 donors.

Semen samples were obtained from the Reproductive Biology Unit, Department of Human Anatomy, School of Medicine, University of California, Davis, CA and from volunteer donors. These samples were stored frozen before analysis.

Tissue specimens were obtained at autopsy from three women of reproductive age. Samples were collected from the vaginal, cervical, and uterine wall. These specimens were homogenized in two volumes of the solution used to extract vaginal swabs. The tissue homogenates were centrifuged and the supernatant solutions were used for peptidase and PGM electrophoresis.

Yogurt specimens were obtained from a local grocery store.

Bacterial cultures were prepared using thioglycolate broth (Difco); the broth was prepared according to the manufacturer's instructions. Pilot broth cultures were prepared by inoculating 3 mL of broth with a small amount of sample. The initial vaginal swab pilot cultures were inoculated with a small piece of swab material from specimens that had previously produced anodal peptidase activity. Semen pilot cultures were inoculated with one to two drops of semen taken at random from our semen collection. Yogurt pilot cultures were inoculated with 0.1 to 0.2 mL of yogurt. The pilot broth cultures were allowed to incubate for two to three days at 37°C, following which the broth was tested for anodal peptidase activity using the electrophoresis procedure described below. Samples which demonstrated peptidase activity were recultured in 100 mL of thioglycolate broth using 0.5 mL of the pilot culture as an inoculum. After three days the broth was harvested. The broth was centrifuged and the supernatant material was concentrated at 4°C under pressure using a molecular filter with a molecular weight barrier of 10 000 daltons (Amicon). The final volume of the concentrated culture material was 1 to 2 mL. This process usually produced a sample with high enzyme activity. Note that thioglycolate is a general purpose culture medium that will grow most bacteria including those from the vagina. No attempt was made to identify the bacteria obtained from these cultures.

Electrophoretic analysis of peptidase activity was conducted using 1-mm-thick agarose gels employing a modified procedure originally designed for the concurrent typing of peptidase A and carbonic anhydrase [*10*]. The tank buffer was composed of 0.056*M* monosodium phosphate and 0.05*M* Tris base (tris[hyroxymethyl]aminomethane), pH 7.4. The gel buffer contained 10m*M* Tris, 4.3m*M* maleic acid and 5m*M* magnesium chloride, pH 7.5. The gels were prepared using 1% agarose (Sigma, type II, $-M_r = 0.17$). In the conventional use of this system, the samples are applied 8 cm from the cathode end of the gel; peptidase A activity migrates toward the anode and carbonic anhydrase activity migrates toward the cathode. Electrophoresis is conducted at 20 V/cm for 2.5 h on cooling plates. In our experiments, the samples were applied 2 cm from the cathode end of the gel and electrophoresis was conducted for 1.5 h at 20 V/cm. This modification was necessary to prevent anodal peptidase activity from migrating off the anodal end of the gel. Peptidase activity was detected using the MTT staining procedure of Suguira et al. [*11*] modified by substituting Meldola blue (8-dimetheylamino-2,3-benzophenoxasine) for PMS (phenazine methosulfate).[3] Meldola blue (1 mL) was added to the stain mixture from a stock solution containing 0.5 mg/mL in water. In our initial screening studies, L-valyl-L-leucine was used as substrate for peptidase activity. In later studies we also used leucyl-leucyl-leucine and leucyl-glycyl-phenylalanine as substrates. Electrophoresis chemicals were obtained from Sigma.

Vaginal swab specimens were prepared for electrophoresis by extraction of 1/4 swab in 10 to 20 uL of 50m*M* dithioerythritol and 10m*M* magnesium chloride containing 5% glycerol. Fluid was collected from cotton swabs by placing the wetted swab in a small plastic centrifuge tube with a hole in the bottom, placing this tube on top of a second tube without a hole in a "piggyback" arrangement, and subjecting this arrangement to centrifugation. The swab remains in the upper tube while fluid from the swab is collected in the lower tube. The supernatant fluid then can be removed for further analysis. Culture samples were centrifuged to remove insoluble material. Samples were applied to electrophoresis gels on one to two cotton threads.

Results and Discussion

Anodal Peptidase Activity in Vaginal Swabs

In the survey of vaginal swabs from case specimens, anodal peptidase activity was detected in 104 (28.7%) out of 362 swabs. The anodal peptidase activity consisted of a family of pepti-

[3]Gary Shutler, Royal Canadian Mounted Police Laboratory, Ottawa, personal communication, 1986.

dase isozymes that are significantly anodal to Pep A. This family of peptidase isozymes may be grouped into 2 subsets where 1 set consists of a group of very anodal isozymes and the second set consists of 1 or 2 isozymes midway between the first set and peptidase A. The occurrence of anodal peptidase activity did not depend on the age of the sample at the time of analysis nor did it depend on the presence of semen or blood. Some of the electrophoretic variation in the anodal peptidases is illustrated in Fig. 1*a* through 1*d*.

No anodal peptidase activity was detected in a survey of 67 vaginal swabs collected from 6 volunteer donors. Absence of anodal peptidase activity in these samples did not appear to be the result of sample collection technique or sample age at the time of analysis. It is noteworthy that 1 of the donors provided 12 blood and semen free swabs; and all 12 contained PGM activity and 4 of these contained peptidase A activity.

Anodal peptidase activity was absent from tissue homogenates of the vaginal, cervical, and uterine wall. All of these tissues contained high levels of both peptidase A and PGM activity. The presence of high levels of peptidase A and PGM activity in these samples demonstrates that the absence of anodal peptidase activity is not the result of the sample preparation procedure.

It is difficult to account for these findings in terms of a vaginal specific human peptidase locus. The hypothesis that a unique peptidase locus is expressed in vaginal material predicts that the product of that locus should be present in all individuals and that its phenotypic expression should be understandable in terms of one or more alleles at that locus. The anodal peptidase observed in these vaginal specimens does not meet either of these criteria. Anodal peptidase activity is not consistently detected in all individuals nor is it consistently

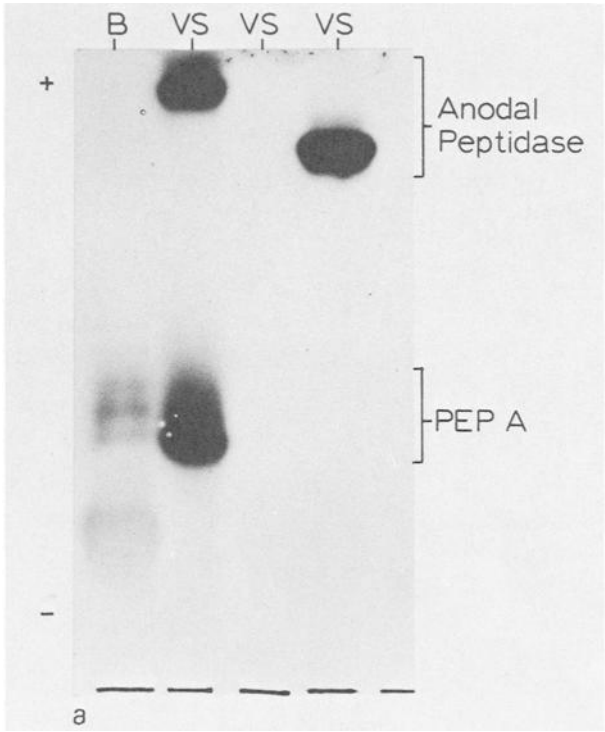

FIG. 1*a–d*—*The occurrence of various anodal peptidase isozymes in vaginal swabs collected from alleged sexual assault victims.* B *is a Pep A 2-1 blood reference.* VS *are vaginal swab specimens from different individuals.*

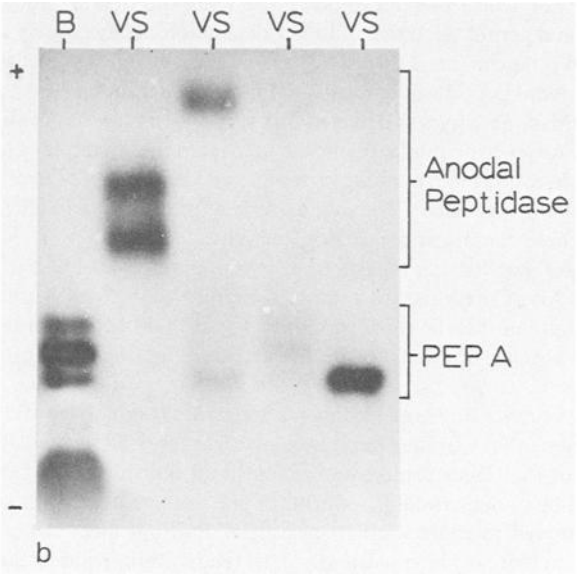

FIG. 1—*Continued.*

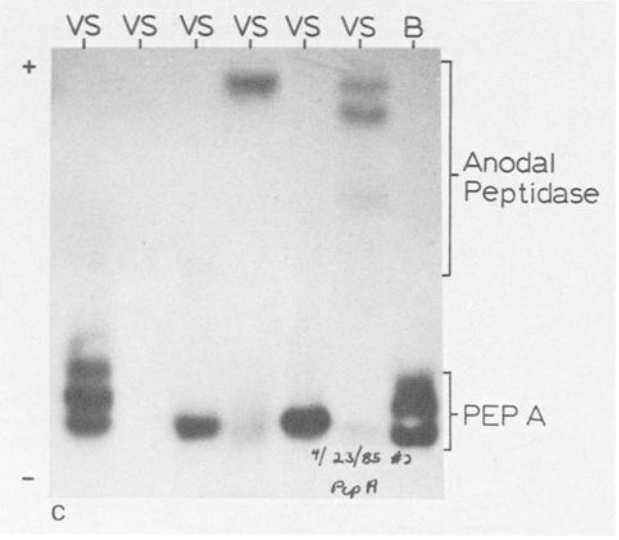

FIG. 1—*Continued.*

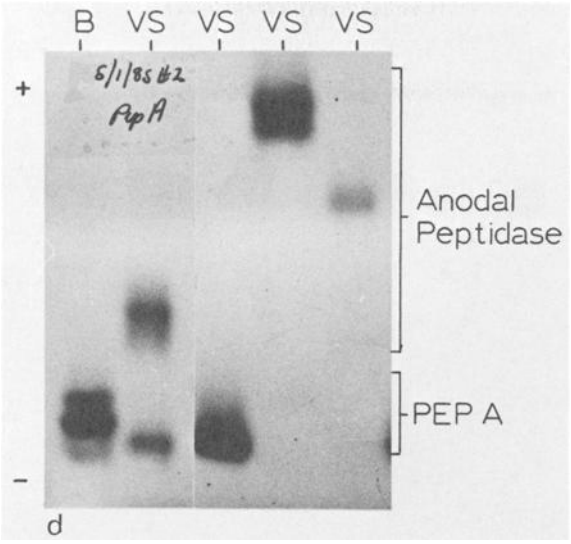

FIG. 1—*Continued.*

detected in samples taken from the same individual at different times. The anodal peptidase activity found by Divall does not appear at one stage of the menstrual cycle and disappear at another stage. Furthermore, the pattern of electrophoretic variation that is observed with anodal peptidase in our studies cannot be readily explained in terms of multiple alleles at a single genetic locus; nor can we demonstrate anodal peptidase activity in female reproductive tract tissues. We conclude, therefore, that the explanation for anodal peptidase activity must lie elsewhere.

Anodal Peptidase Activity in Vaginal Swab Cultures

Direct evidence that bacteria produce anodal peptidase activity is obtained from cultured swab specimens. Cultures of swabs that previously demonstrated anodal peptidase activity also reveal anodal peptidase activity. The activity in the cultures cannot be due to simple admixture of the original swab since this specimen is significantly diluted by the culture broth. The pattern of electrophoretic variation of anodal peptidase isozymes from the swab cultures is similar to that observed from swab specimens tested directly. The occurrence of different peptidase isozymes in different cultures indicates that different bacterial species may dominate any particular culture. Examples of anodal peptidase isozymes from cultured vaginal swabs are illustrated in Fig. 2.

Anodal Peptidase Activity in Cultures of Semen and Yogurt

Despite the demonstration that anodal peptidase is not a human gene product, the question remains whether the various bacterial species capable of producing these isozymes are found only in the vagina. Our studies show that cultures of semen specimens and yogurt samples are capable of producing various anodal peptidase isozymes as illustrated in Figs. 3 and 4. Some of these peptidase isozymes are similar in electrophoretic mobility to the peptidase isozymes obtained from vaginal swab cultures (Fig. 5). These results indicate that bacteria exist outside the vagina that produce peptidase isozymes with similar electrophoretic mobility to peptidase isozymes from vaginal bacteria.

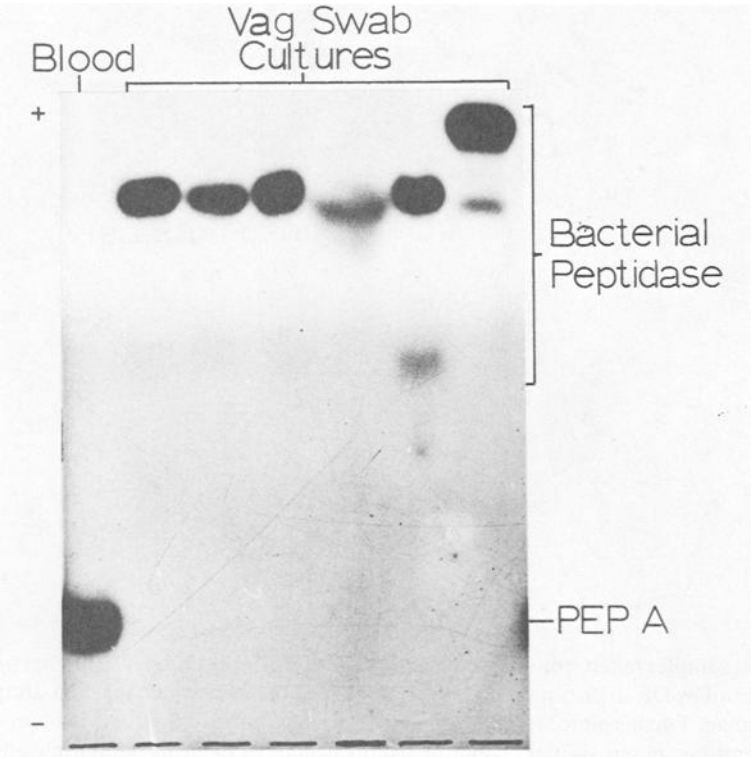

FIG. 2—*Anodal peptidases in cultures of vaginal swabs. The blood reference specimen is Pep A type 1-1.*

Anodal Peptidase Activity Revealed by Different Substrates

Culture samples were examined for the presence of peptidase activity using the tripeptide substrates, L-leucyl-leucyl-leucine and L-leucyl-glycyl-phenylalanine, and these were compared to the peptidase activity observed with L-valyl-leucine (Fig. 6*a* and *b*). In some samples the tripeptides reveal a series of isozymes at the anodal region of the gel. Some of these isozymes are clearly different from some of the isozymes revealed by L-valyl-leucine; and some of the isozymes revealed by L-valyl-leucine are not detected or are detected poorly with the tripeptides. The isozymes revealed by L-leucyl-leucyl-leucine are similar to the isozymes revealed by L-leucyl-glycyl-phenylalanine. The presence of anodal tripeptidase activity in some cultures but not in others provides further evidence that more than one bacterial species is capable of producing peptidase activity.

Implications for the Identification of Vaginal Material

These studies raise a number of questions concerning the reliability of anodal peptidase activity as a tissue specific marker for vaginal secretions. Anodal peptidase activity is not found in samples from all individuals nor is it consistently found in the same individual at different times. Thus, the absence of anodal peptidase activity has no information value and reference specimens from an alleged vaginal donor are not likely to clarify the issue. The source of anodal peptidase activity is bacterial rather than human and the various bacteria responsible for this activity can be found outside the vagina. While biological evidence is not

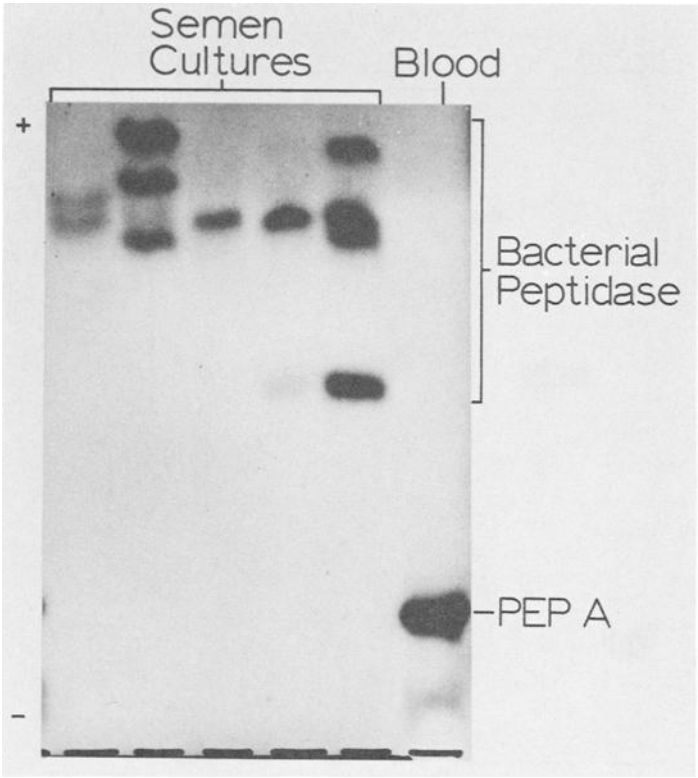

FIG. 3—*Anodal peptidase activity in cultures of semen samples. The blood reference specimen is Pep A type 1-1.*

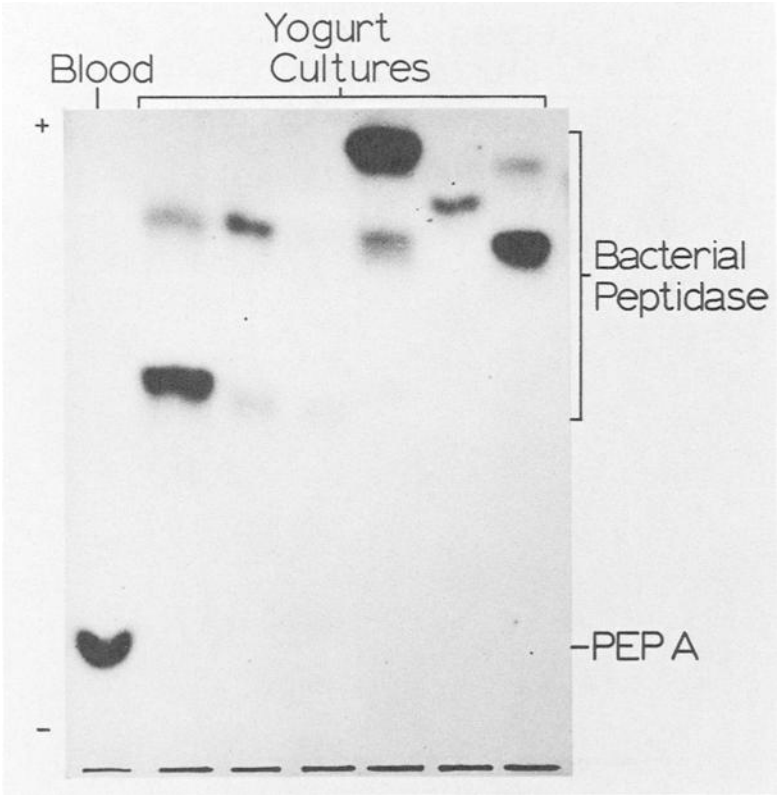

FIG. 4—*Anodal peptidase activity in cultures of yogurt specimens. The blood reference specimen is Pep A type 1-1.*

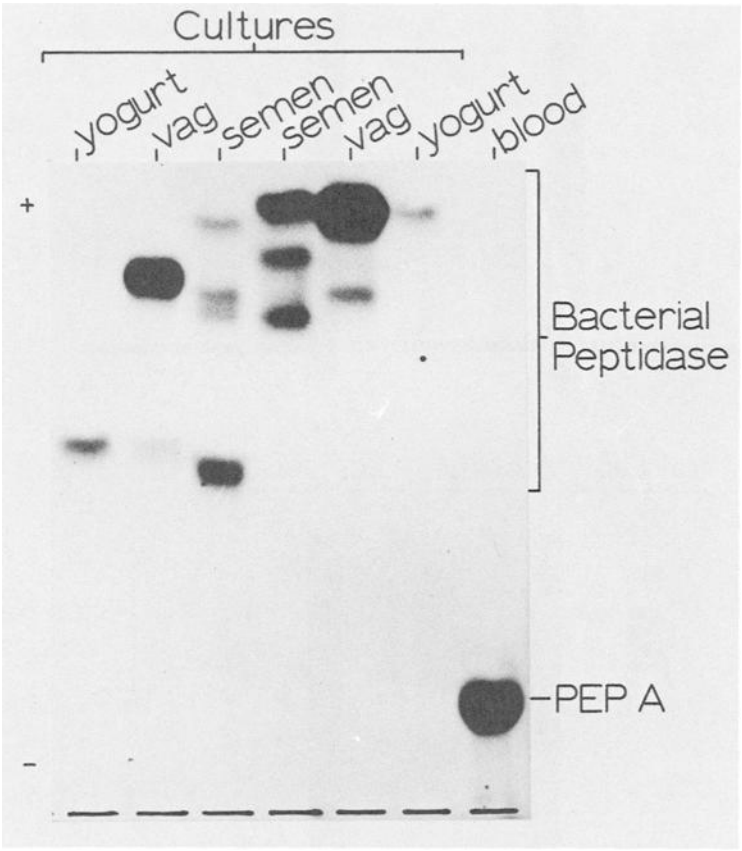

FIG. 5—*Comparison of anodal peptidase activity in cultures of vaginal swabs, semen, and yogurt. The blood reference specimen is Pep A type 1-1.*

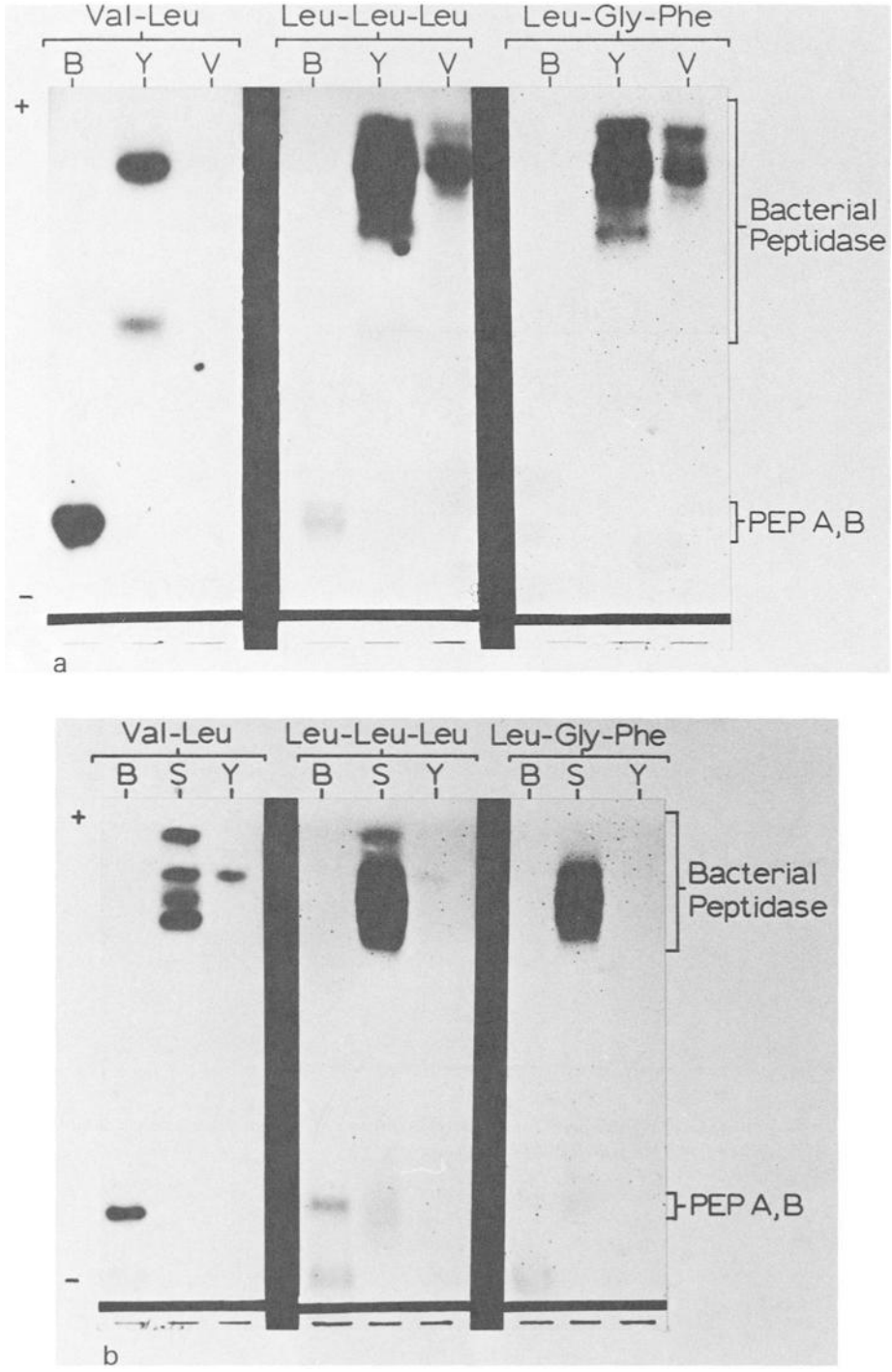

FIG. 6*a* and *b*—*Anodal peptidase activity revealed by different peptidase substrates. The same specimens were applied in triplicate to the gel and then each was developed with a different substrate.* B = *blood,* S = *cultured semen,* Y = *cultured yogurt,* V = *cultured vaginal swab. L-leucyl-leucyl-leucine is a substrate for human Pep B; L-leucyl-glycyl-phenylalanine is a poor substrate for all of the known human peptidase loci.*

normally found in a cultured state, it is worth bearing in mind that virtually any biological specimen can support bacterial growth given an adequate amount of moisture. This growth can be rapid at high temperatures and can also occur at refrigerator temperatures albeit at a slower rate. Therefore, it is important to dry biological evidence as rapidly as possible and to store it frozen before analysis. That anodal peptidase activity signals bacterial growth is clear; whether there is a specific peptidase that signals the presence of a vaginal specific microorganism is less clear. Some progress has been made on the use of isozymes for bacterial species identification [*12,13*]. Until more is learned about bacterial peptidases, it is unwise to rely upon a bacterial gene product to identify a human tissue.

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