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Enzyme Assays for the Identification of Gastric Fluid

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ABSTRACT: Simple, reliable procedures for the assay of pepsin and rennin-like enzyme activities are described as a means of identifying gastric fluid-containing samples in forensic science laboratories. These samples are usually vomitus, or stomach contents originating from wounds that perforate the stomach. They may be encountered at scenes or on articles submitted for examination, in fresh form or as dried stains. The pepsin activity assay is based on proteolytic activity with bovine albumin as substrate and the rennin-like activity assay is based on the coagulation of milk protein.

KEYWORDS: forensic science, body fluids, enzymes, gastric fluid, stain, body fluid identification, stomach contents, pepsin, rennin, enzyme assay

Gastric fluid and gastric fluid stains are uncommon types of physiological fluid evidence at crime scenes or on items submitted for examination. Recently, however, the identification of gastric fluid residues became important in several major homicide investigations in order to disprove a suspect's alibi, support witness testimony, and reconstruct events. Gastric fluid is most frequently encountered in vomitus, but may also be a component of stomach content residues produced by stomach wounds. The literature contains very few references to the identification of gastric fluid, and no papers devoted to the subject were found [*1-3*]. Microscopical examination of suspected gastric fluid material is currently the method employed to aid in identification. Various cells, fat globules, and food materials identified microscopically provide an indication that the sample may consist of stomach contents; however, a positive identification of gastric fluid cannot be made in this way.

Several enzyme assay procedures have been used for gastric fluid analysis in clinical laboratories at various times [*4-6*]. These procedures required large quantities of gastric fluid samples, and were quite cumbersome. They would not, therefore, meet the requirements of a routine procedure for casework samples in the forensic science laboratory. We have modified assay procedures for gastric fluid pepsin and rennin-like enzyme activities, and report here the successful application of these assay procedures to the routine identification of gastric fluid and gastric fluid stains.

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Materials and Methods

Sample Preparation

Vomitus samples containing gastric fluid were collected from normal adults. Stomach contents samples were collected from cadavers at autopsy. Each sample was divided into two equal volumes. Half the sample was divided into 1-mL portions, and deposited onto either clean cotton cloth or glass plates to form dried stains. These stains were kept at room temperature for various lengths of time. The other half of each sample was centrifuged at 2000 rpm in a clinical centrifuge to remove solids. The clear supernatant was then divided into 1-mL aliquots, which were stored frozen at −20°C as positive controls. For assay, a portion of dried vomitus or stomach contents sample was weighed, then resuspended in 1-mL 0.2*N* hydrochloric acid at 4°C for 18 h. It was then centrifuged at 2000 rpm for 5 min, and the clear supernatant used for enzyme assays.

Rennin-Like Activity Assay

A sample stain extract of 0.5 mL was mixed with 0.5-mL whole milk, and the mixture incubated at 37°C for 10 min. A positive control contained 0.5-mL known liquid gastric fluid material in place of stain extract; a negative control contained 0.5-mL 0.2*N* hydrochloric acid in place of the extract. Following incubation, the samples were checked for coagulation, which was "scored" according to the degree of curding in the mixture: 4+, a single complete curd; 3+, several large curds; 2+, a number of medium size curds; 1+, a larger number of small curds; *w*, a few small, flake-like curds; and −, no curds (negative).

Pepsin Activity Assay

Protein substrate solution (PSS) consisted of 5.6-g bovine serum albumin/100-mL distilled water, adjusted to pH 2 with hydrochloric acid. For assay, four tubes were prepared: (1) 0.5-mL gastric fluid or stain extract + 2.5-mL PSS; (2) 0.5-mL 0.2*N* hydrochloric acid + 2.5-mL PSS; (3) 0.5-mL 0.2*N* hydrochloric acid + 2.5-mL 0.35*N* trichloroacetic acid; and (4) 0.5-mL gastric fluid or stain extract + 2.5-mL 0.35*N* trichloroacetic acid. The tubes were incubated at 37°C for 15 min, after which the contents of (1) are added to (3) and mixed thoroughly, and the contents of (2) are added to (4) and mixed thoroughly. The two tubes, (1) + (3) representing the assay tube, and (2) + (4) representing the control tube, were then incubated for an additional 5 min at 37°C, then centrifuged at 2000 rpm for 5 min to remove any precipitate. Of the clear supernatant 0.5 mL from each tube was then transferred to new tubes containing 5-mL 0.25*N* sodium hydroxide and 0.25-mL Folin-Ciocalteu reagent [*7,8*]. After 15 min, the absorbancy value of the tubes was determined spectrophotometrically at 680 nm.

A standard phenol control for the Folin-Ciocalteu reagent was prepared by mixing 0.5-mL phenol standard (5-mg phenol/100-mL distilled water), 5-mL 0.25*N* sodium hydroxide and 0.25-mL Folin-Ciocalteu reagent and determining the absorbancy at 680 nm after 15 min at room temperature.

The absorbancy at 680 nm of the standard phenol control was arbitrarily defined as representing 50 U of pepsin activity. Using purified known pepsin (Sigma Chemical Co., St. Louis, MO), it was shown that the assay was linear over a range of about 20 to 125 U per assay tube. All the measurements made on gastric fluid-containing samples fell within the linear portion of the assay, and the arbitrarily defined 50-U standard phenol control exhibited an absorbancy at 680 nm of about 0.4.

Results

In addition to gastric fluid-containing samples, rennin-like and pepsin activities were assayed in liquid samples and stain extracts of a number of other physiological fluids, including

serum, semen, urine, hemolysate, and saliva. The results, shown in Table 1, show that only gastric fluid-containing samples exhibited significant activities in either case.

Table 2 shows representative rennin-like and pepsin activities in ten different gastric fluid stain extract samples (representing ten different individuals). Pepsin activity varied in different samples, from 6.2- to 21-U/mL extract. In assays of all gastric fluid-containing stain samples not more than two weeks old, no extract had a pepsin activity of less than 6 U/mL. Individual differences in rennin-like activity among the ten samples were less pronounced, all samples giving a 3+ or 4+ coagulation result. To determine the persistence of enzymatic activities in stains, gastric fluid-containing sample stains were aged at room temperature for several months, a portion of the stains being taken for rennin-like and pepsin assays at four-week intervals. Figure 1 shows the results for rennin-like activity in four representative samples over a three-month period. Activity decreased with sample age in all the samples tested, but not at a uniform rate. Over the course of twelve weeks, the activity of one sample decreased from 4+ to −, that of another form 3+ to 1+, and that of two others from 4+ to 2+. Figure 2 shows the effect of sample age on pepsin activity. Results are shown for the same four samples in which rennin-like activity was followed (Fig. 1).

In ten stain samples from different individuals assayed periodically for pepsin activity over three months, the mean activity of all samples (± standard deviation) in units/millilitres ex-

TABLE 1—*Comparison of rennin-like and pepsin activities in various physiological fluids.*

Sample	Rennin Activity[a]	Pepsin Activity[b]
Urine (liquid)	—	0.0
Urine (stain)	—	0.3
Semen (liquid)	—	0.0
Semen (stain)	—	0.1
Saliva (liquid)	—	0.0
Saliva (stain)	—	0.5
Serum (liquid)	*w*	0.0
Serum (stain)	*w*	0.3
Hemolysate (liquid)	*w*	0.0
Hemolysate (stain)	*w*	0.1
Gastric fluid (liquid)	4+	23.6
Gastric fluid (stain)	4+	13.2

[a]Degree of coagulation (see Materials and Methods section).
[b]Units per millilitre (liquid) or per millilitre stain extract.

TABLE 2—*Comparison of rennin-like and pepsin activities among different gastric fluid-containing stain extracts.*

Sample	Rennin Activity[a]	Pepsin Activity[b]
1	3+	13.2
2	4+	15.1
3	4+	17.8
4	4+	12.5
5	4+	6.2
6	4+	13.1
7	3+	16.6
8	4+	21.0
9	3+	16.3
10	4+	20.1

[a]Degree of coagulation (see Materials and Methods section).
[b]Units per millilitre stain extract.

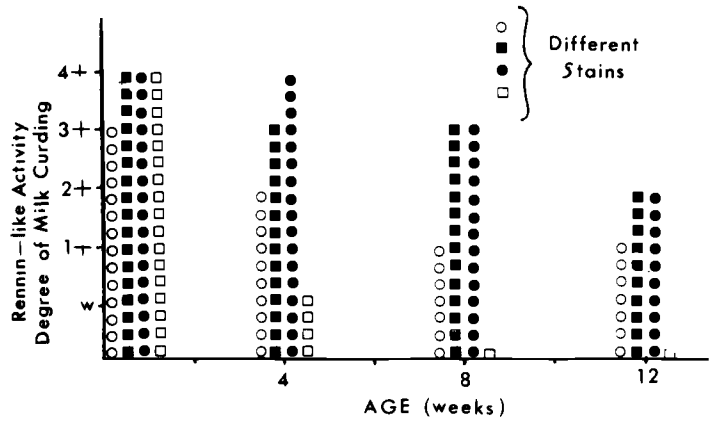

FIG. 1—*Effect of age on rennin-like activity in representative stains.*

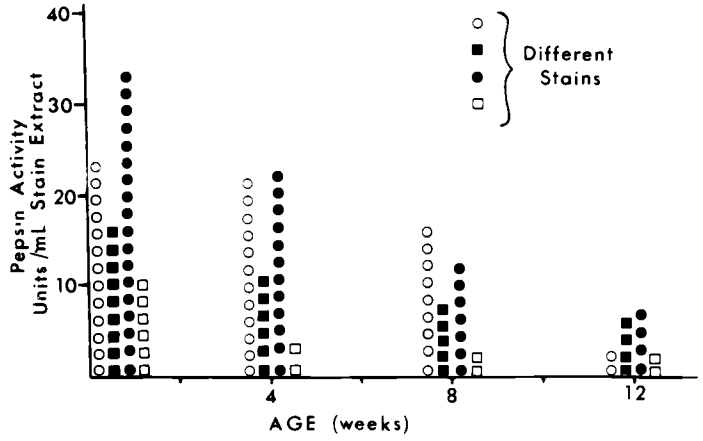

FIG. 2—*Effect of age on pepsin activity in representative stains.*

tract was: 23.79 ± 6.66 (< one week); 16.50 ± 5.70 (four weeks); 10.43 ± 4.84 (eight weeks); and 5.98 ± 2.69 (twelve weeks).

A pattern of activity loss similar to the rennin-like results is seen with pepsin. There is an overall loss of activity in all the samples studied, but no consistent rate of decrease is apparent. Over twelve weeks time, one sample activity decreased from 23.6 to 2.8 U/mL, another from 16.5 to 6.4, a third from 10.7 to 2.2, and the last one from 33.9 to 7.8. The mechanisms underlying the losses of rennin-like and pepsin activity in these samples are not known, and remain to be studied.

While both activities exhibited decreases over the three-month study period, the data indicate that the losses are independent. In the sample that lost all rennin-like activity over twelve weeks, for example, pepsin activity declined about 21%, while the sample that showed the greatest decline in pepsin activity (about 39%) exhibited an intermediate loss in rennin-like activity.

Discussion

The gastric acid acts upon food proteins in vivo to produce denatured protein. Pepsin acts upon such denatured proteins as well as upon natural proteins in an acidic environment to produce low molecular weight peptides and protein derivatives. Pepsin is one of a number of proteolytic enzymes, unusual because of its very low pH optimum. One of the oldest known enzymes, it is found in the gastric secretions of many vertebrate species [*9, 10*].

Pepsin assays are ordinarily based on pepsin's proteolytic activity. The procedure presented here represents a modification of an older procedure that used a standard hemoglobin solution as substrate [*11*]. It represents a simple, reliable method for estimating pepsin activity in gastric fluid-containing samples. Rennin is an enzyme that can be isolated from the fourth stomach of the calf, and its ability to coagulate or curd milk has been known for many decades. Rennin is also sometimes called chymosin, the latter term having been suggested as preferable to avoid confusion with renin, an unrelated enzyme [*12*]. Current thinking is that human gastric fluid does not contain rennin [*13*], although there was considerable discussion about the matter years ago. Studies indicate that the milk coagulating activity of human gastric fluid may be attributable to pepsin [*14*], which can curd milk as effectively as rennin itself. Some experimental support for this view results from our observations in the present studies that purified porcine pepsin gave 4+ milk coagulation in the rennin-like activity assay over a range of 10 to 50 U. Lower pepsin concentrations yielded lower degrees of curding. Higher pepsin concentrations, in the range of 100 to 200 U per assay, gave lower degrees of curding as well, an observation that deserves further study. Additionally, the pepsin and rennin-like activities of human gastric fluid showed parallel inhibition with the pepsin inhibitor pepstatin A, and quantities of inhibitor sufficient to inhibit proteolytic activity completely also gave complete inhibition of milk curding activity. Thus, the "rennin-like activity" measured in the present studies is probably another manifestation of pepsin, and accounts for our use of the terminology, "rennin-like" in referring to it, reserving "pepsin activity" to refer to the proteolytic reaction.

Our data show that, among the common physiological fluids tested along with extracts of their stains, significant pepsin and rennin-like activities are associated exclusively with gastric fluid-containing samples. Further, significant pepsin activity may be demonstrated in extracts of some gastric fluid-containing stains kept for up to twelve weeks at room temperature. Rennin-like activity was likewise present in extracts of stains up to twelve weeks old in many of the samples, and in all samples up to two weeks old. Although the absolute activities vary in different samples, they were significantly higher than those of the negative controls and of other physiological fluids in all fresh samples, in all the stains aged up to two weeks at room temperature, and in many of the stains aged three months at room temperature.

These assay procedures provide a simple, reliable method for the identification of gastric fluid-containing samples in forensic science laboratories in those case situations where it is necessary. It is worthy of note that gastric fluid-containing samples from a variety of vertebrates would be expected to show significant pepsin and rennin-like activities. Therefore, in any case situation or material where the origin of the sample was not obviously human, an immunological species test to demonstrate human origin would be required.

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