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Removal of contaminating hemoglobin from peroxidase in traumatic skin lesions

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Summary. A method is described for the removal of contaminating hemoglobin from the peroxidase enzyme in traumatic skin lesions. The procedure is based on hemoglobin precipitation in a combination of ammonium sulfate half-saturation, and chloroform shaking of the cetyltrimethylammonium-bromide extract. The procedure as such somewhat increases the activity of the peroxidase extract if the extract contains no hemoglobin. On the other hand, the peroxidase activity of the extract decreases as the amount of precipitating hemoglobin increases. On average, about 90% of the peroxidase activity persists after hemoglobin precipitation if the hemoglobin concentration in the extract does not exceed 25 mg/100 ml. In experimental incision wounds, the peroxidase activities obtained with this procedure were the same as when enzyme determinations were done without the removal of hemoglobin or slightly higher. In addition, the amount of peroxidase activity in the wounds was estimated, based on the granulocytes of the contaminating blood.

Key word: Peroxidase activity, removal of hemoglobin

Zusammenfassung. Im Artikel wird ein Verfahren zum Reinigen des Peroxidaseenzyms in Hautverletzungen von kontaminierendem Hämoglobin beschrieben. Die Prozedur gründet sich auf das Ausfallen des Hämoglobins aus dem Cetyltrimethylammoniumbromidextrakt in Verbindung mit kombinierter Ammoniumsulfat-Halbsättigung und Chloroformschütteln. Die Prozedur erhöht einigermaßen die Aktivität in solchem Peroxidaseextrakt, der kein Hämoglobin enthält. Andererseits setzen zunehmende Mengen von ausfallendem Hämoglobin die Peroxidaseaktivität des Extrakts herab. Im Durchschnitt 90% der Peroxidaseaktivität bleiben nach Ausfällung des Hämoglobins erhalten, insofern der Hämoglobingehalt des Extrakts 25 mg/100 ml nicht überschreitet. Bei experimentellen Schnittwunden wurden mit diesem Verfahren Peroxidaseaktivitäten auf gleichem Niveau oder etwas höher als in entsprechenden Enzymbestimmungen erhalten, in denen das Hämoglobin nicht beseitigt wurde. Ferner wurde die von den Granulozyten

im kontaminierenden Blut hervorgerufene Peroxidaseaktivität in Wunden geschätzt.

Schlüsselwort: Peroxidaseaktivität, Beseitigung des kontaminierenden Hämoglobins

Introduction

It has recently been shown that there is a rapid and remarkable increase in peroxidase activity in traumatic skin lesions during the first post-traumatic day, beginning in experimental lesions at 30 min after injury. This peroxidase increase, based presumably on the inflammatory reaction, was demonstrable in both experimental and autopsy specimens taken several days postmortem [1, 2]. Measurements of peroxidase activity might be useful for forensic medical purposes in estimation of the vitality and of the time of infliction of lesions. Before this assumption can be confirmed much more work is needed; in particular a large autopsy material must be collected and methods of eliminating the analytical errors that contaminating blood in the specimens can cause must be developed. These methodological errors are based (1) on the pseudoperoxidase activity of hemoglobin, (2) on the inhibition of myeloperoxidase activity by hemoglobin in the assay reaction, and (3) on the myeloperoxidase activity located in the leukocytes of the contaminating blood [3, 4]. Particularly when the activity levels in the specimens are very low, the contaminating blood may cause significant errors in the analytical results. Most of the inhibition by hemoglobin can be avoided by diluting the extracts made from the specimens, and the pseudoperoxidase activity as such is rather low [3]. If, however, the peroxidase could be purified of the contaminating hemoglobin, both the pseudoperoxidase effect and the inhibition could be avoided.

The present paper describes a simple, rapid method for removing the contaminating hemoglobin from the peroxidase in extracts of traumatic skin lesions, and attempts further, a rough estimate of the amount of myeloperoxidase derived from the granulocytes in the contaminating blood.

Materials and methods

To test the hemoglobin removal procedure mentioned in the Introduction, experimental incision wounds in rats were used. Incision wounds 5 cm long and perforating the skin were made on the right side of the dorsal skin. After different periods of vital time, zones about 1 mm thick were removed from the edges of wounds as specimens. Similar incision wounds of the skin were also made 1 min post mortem; in this case the specimens were taken 1 h later. In all cases, control specimens of normal skin were taken from opposite side of the dorsal skin of the rats. All the specimens were stored at −70°C for from some hours to several days or, if analyzed directly, first frozen. Part of each specimen was used for the peroxidase measurements and part for the hemin determinations.

Myeloperoxidase extraction and the removal of hemoglobin were accomplished as follows: the stored specimens were thawed to room temperature, minced with scissors in a 0.5% solution of cetyltrimethylammoniumbromide (Merck), and homogenized thoroughly at room

temperature using 3 ml 0.5% cetyltrimethylammoniumbromide solution per 100 mg tissue wet weight.

The homogenate was deep-frozen and thawed again, and then sonicated for 5 min in a sonificator with a total output of 120 W (Finnsonic W-181-2T). The freezing and thawing and the sonication were repeated. The homogenate was then centrifuged for 10 min at the maximum speed of the Sorvall table centrifuge with an angle rotor (about 4900 *g*). The supernatant obtained was diluted, if needed, with 0.5% cetyltrimethylammoniumbromide so that the calculated hemoglobin concentration in the supernatant was 25 mg/100 ml or less (see below). To 2.0 ml of the supernatant obtained, 754 mg of dry ammonium sulfate (Merck) was admixed, and the mixture was set aside until the salt dissolved. Then 2.5 ml chloroform (Merck) was added, after which the tube was tightly capped and shaken vigorously for 1 min. The mixture was then centrifuged for 10 min at 4000 rpm. The clear, colorless supernatant (above the precipitated hemoglobin and chloroform; Fig. 1) was removed, care being taken to avoid removing chloroform along with the supernatant. The supernatant obtained was used for the peroxidase assay.

The peroxidase assay was performed as described in our earlier paper [2], except that the 1.8 ml 0.01 *M* phosphate buffer, pH 6.5, with which the 0.3 ml supernatant was mixed in the assay reaction, in addition to 0.0005% hydrogen peroxide (Merck) and 0.223 mg/ml *o*-dianisidine hydrochloride (Sigma), also contained 0.05% cetyltrimethylammoniumbromide. In zero determinations the same amounts of supernatant and reagents were used, but without hydrogen peroxide.

The hemoglobin content in the extracts of the specimens was estimated by using the hemin analysis [5], except that in addition to the hemin standard (Sigma) a hemoglobin standard (Sigma) was also used. The frozen specimens were thawed to room temperature, minced with scissors in a 0.3% solution of saponin (Merck), and homogenized thoroughly at room temperature using 3 ml 0.3% saponin per 100 mg tissue wet weight. The homogenate was deep-frozen and thawed again, and sonicated for 5 min. The freezing and thawing cycle and the sonication were repeated. The homogenate was then centrifuged for 10 min at the maximum speed of the centrifuge described above. The supernatant obtained was used for hemin analysis.

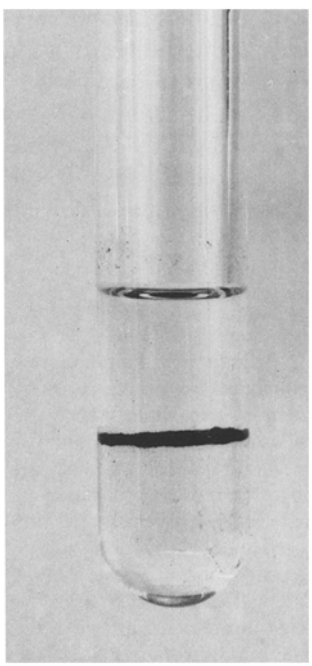

Fig. 1. Result of the ammonium sulfate-chloroform shaking procedure after centrifugation: clear, colorless supernatant containing peroxidase *(above)*; precipitated layer with hemoglobin *(middle)*; chloroform layer *(below)*

To test the influence of the hemoglobin concentration in the enzyme purification procedure, myeloperoxidase was extracted by 0.5% cetyltrimethylammoniumbromide from the rat peritoneal granulocytes gathered after glycogen stimulation [6]. Different amounts of rat hemoglobin, obtained by hemolysis of the rat red cells, were mixed with the myeloperoxidase extract to obtain hemoglobin concentrations of 5–500 mg/100 ml in the enzyme-hemoglobin mixture. Hemoglobin was removed from the enzyme-hemoglobin mixture by the ammonium sulfate-chloroform shaking described above; and the enzyme activity was measured by the same method as used for the tissue specimens. The activities obtained were expressed as percentages of the activity of the same enzyme concentration without hemoglobin, treated by the same purification procedure. The myeloperoxidase activity of rat peritoneal granulocytes was also measured by the same procedure as that used for tissue specimens, and the activity expressed as units per 10^7 granulocytes.

Using the hemoglobin content of the specimens, rough estimations were also made of the granulocyte content of the contaminating blood in the specimens. The average values for hemoglobin content, leukocyte number, and granulocyte percentages in the blood of adult rats were used in these calculations [7]. Based on the assumption that the myeloperoxidase content of the rat blood granulocytes was the same as that of the granulocytes obtained after peritoneal glycogen stimulation, the amount of peroxidase activity that might have resulted from the blood granulocytes in the specimen was also estimated. Using the hemoglobin concentrations observed in the specimens, the calculated mean peroxidase activity values of the contaminating blood granulocytes were subtracted from the total enzyme activity of the tissue specimens. The total enzyme activities and those obtained after subtraction were expressed as units per gram of dry weight of the tissue; for this purpose a sufficient aliquot of the homogenate was dried in an oven at 120°C for at least 24 h and weighed. The statistical analysis of the results was performed using Student's *t*-test.

Results

With increasing hemoglobin concentration in the peroxidase-hemoglobin mixture, an increasing amount of the peroxidase activity was lost in the purification procedure (Fig. 2). If the hemoglobin concentration was 25 mg/100 ml or less, an average of about 90% of the activity persisted. With a hemoglobin concentration of 250 mg/100 ml, about half the activity was lost; and with a concentration of 500 mg/100 ml, barely one-third of the activity persisted. It was also observed that the same hemoglobin concentration tended to result in a somewhat

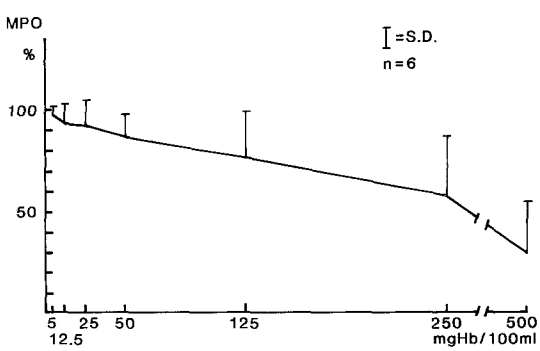

Fig. 2. Influence of the hemoglobin concentration on the recovery of the myeloperoxidase *(MPO)* activity (percentages) in the ammonium sulfate-chloroform shaking, hemoglobin removal procedure. Activity of the enzyme extract without hemoglobin, treated by the same procedure, = 100%. The activity levels of the enzyme used in these experiments, expressed as △ A/min, varied from 0.060 to 0.450

Table 1. Estimated peroxidase activity based on the granulocytes of the contaminating blood in the vital wounds aged from 5 min to 24 h, expressed as units per gram of dry weight and as percentages of the total peroxidase activity in each period of vital time. Means and standard deviations are given ($n = 12$)

	5 min	15 min	30 min	60 min	2 h	4 h	8 h	12 h	24 h
Units per gram of dry weight	0.05 ± 0.04	0.12 ± 0.09	0.13 ± 0.10	0.16 ± 0.15	0.12 ± 0.13	0.15 ± 0.09	0.12 ± 0.10	0.25 ± 0.35	0.16 ± 0.10
Percentages	45.8 ± 34.7	63.5 ± 38.9	22.6 ± 16.1	16.4 ± 22.3	1.4 ± 1.5	0.4 ± 0.3	0.1 ± 0.1	0.3 ± 0.6	0.04 ± 0.03

greater loss of peroxidase activity at low enzyme activity levels than at higher levels.

In the extracts prepared from the experimental wounds as described above in the Methods section, an average of about 16 mg hemoglobin/100 ml was observed. The variation between different wounds was very great, so that the highest values of hemoglobin observed were about 130 mg/100 ml, and the lowest ones only 1 mg/100 ml.

Table 1 shows the estimated peroxidase activities based on the graunulocytes of the contaminating blood in the vital wounds. The basis for these estimations was the mean activity of peritoneal granulocytes; the value obtained was 12.05 ± 1.47 units/10^7 granulocytes ($n = 6$). According to our calculation, the contaminating peroxidase activity varied from 0.05 to 0.25 units/g dry weight. When this contaminating activity was expressed as a percentage of the total peroxidase activity of the experimental wounds, values of 16.4%–63.5% of the total analysis value were obtained in wounds aged 60 min or less. In 2-h-old wounds, the mean percentage of this contamination was only about 1.4%, and in older wounds less than 1% of the total activity observed.

When the enzyme purification procedure described was used on the experimental incision wounds, the results were as follows:

In most of the control specimens the peroxidase activity was below the detection limit of the methods used. In about 34.4% of the control specimens peroxidase activity was observed: the mean activity ± SD was 0.087 ± 0.078 (0.085 ± 0.079) U/g dry weight, the first value being the total analysis result and the value in parenthesis the value obtained after subtraction of the estimated activity of the contaminating blood granulocytes from the analysis result. In 5-min-old, and in 15-min-old, vital wounds the activities were at the same level as in the positive controls, the values being respectively 0.14 ± 0.08 (0.09 ± 0.08) and 0.19 ± 0.13 (0.09 ± 0.09) units/g dry weight. In 30-min-old wounds, the mean activity was 0.64 ± 0.50 (0.51 ± 0.46) units/g dry weight. Activity then increased slowly over the next 30 min, and a more rapid increase began after 1 h. Compared with the total activity level observed in 30-min-old wounds, the average total activity was about 60 times higher in 4-h-old wounds, about 300 times higher in 12-h-old wounds, and about 600 times higher in 24-h-old wounds (Figs. 3, 4).

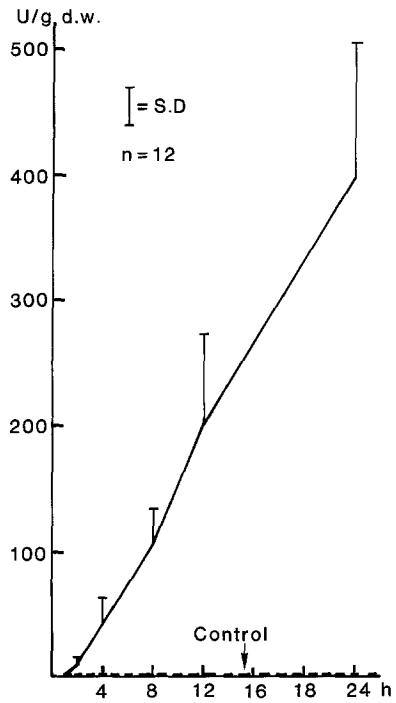

Fig. 3. Total peroxidase activities expressed as units/gram of dry weight in vital wounds 5 min to 24 h after infliction. The extracts of the specimens have been treated with the ammonium sulfate-chloroform shaking procedure. The mean values for wounds 2–24 h old are statistically significantly different from the mean of 30-min-old wounds ($P < 0.001$)

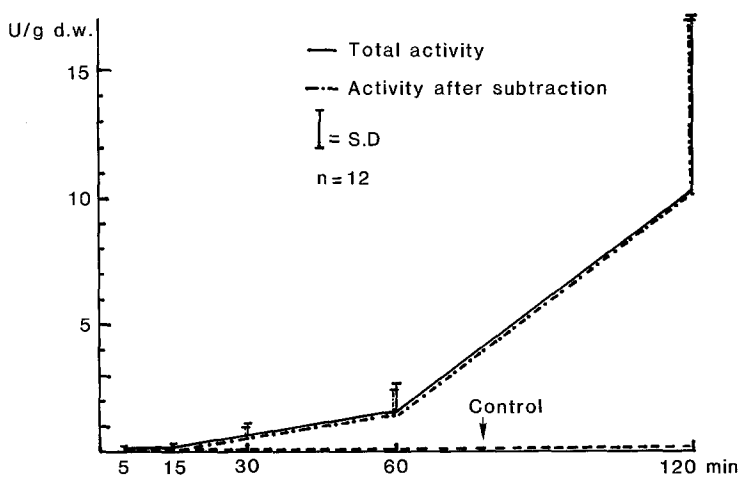

Fig. 4. Detail of the same curve as in Fig. 3 but on a larger scale. A slow increase in the activity seems to begin between 15 and 30 min. Total activities are shown, as well as the values obtained after the subtraction of the activity caused by the contaminating blood granulocytes

Among the wounds inflicted 1 min post mortem, activity was detected in only 42%. The mean values of the positive specimens were 0.036 ± 0.005 (0.026 ± 0.005) units/g dry weight, $n = 12$.

Discussion

The ammonium sulfate-chloroform shaking procedure was developed on the basis of several preliminary experiments. Neither half-saturation with ammonium sulfate alone nor chloroform shaking alone precipitated hemoglobin from the solution of 0.5% cetyltrimethylammoniumbromide. It also appeared that the higher the ammonium sulfate concentration in the shaking procedure, the more enzyme activity was lost. On the other hand, if the ammonium sulfate concentration was somewhat lower than the approximately half-saturation used in the procedure presented, it was not possible to remove all hemoglobin from the extract by this method. The addition of ammonium sulfate at 754 mg to 2 ml of extract resulted in a roughly 1.2-fold volume increase in the extract.

The ammonium sulfate in the purified enzyme extract often created a turbidity in the cuvette during analysis, leading to an artificial increase in absorption if the same reaction mixture was used as in our earlier work [2]. The turbidity did not appear when the reaction mixture contained the added amount of cetyltrimethylammoniumbromide, as described in the Methods section. In the method used in our earlier study [2], the cetyltrimethylammoniumbromideconcentration was about 0.07% in the final reaction; in the method described in this paper, the final reaction concentration of this compound was c. 0.1%. Removal of the ammonium sulfate by dialysis was also tried, but the procedure was time-consuming and resulted in a greater loss of enzyme activity.

The ammonium sulfate-chloroform shaking increased the activity values somewhat in those peroxidase extracts which did not contain hemoglobin. The reason for this was unknown. The increased salt concentration may have something to do with the activity increase, because the other salts, e.g., sodium chloride, added to the extracts in the preliminary experiments also seemed to increase the enzyme activity.

On the other hand, as the concentration of contaminating hemoglobin increased, increasing amounts of the enzyme activity were lost during the purification procedure. The reason for this is unclear. It could be speculated that the peroxidase enzyme possibly had some affinity to the precipitating hemoglobin. It is interesting that here the loss of the enzyme activity had nearly the same relationship to the hemoglobin concentration as did the inhibition of the enzyme activity by hemoglobin in the assay reaction [3]. If the enzyme extract with contaminating hemoglobin was sufficiently dilute, e.g., to a hemoglobin concentration of 25 mg/100 ml or below, then most of the enzyme activity was retained after the purification procedure.

Despite some enzyme activity loss in the hemoglobin precipitation procedure, the results obtained from the experimental wounds were at the same level as, or even higher than, in our earlier study [2], in which removal of the contaminating hemoglobin was not attempted. The reason for this may be the

higher enzyme reaction value obtained after the ammonium sulfate-chloroform shaking. In addition, the extraction procedure used was more thorough, as it included the sonification steps and an extra freeze-thaw cycle.

One benefit of the purification method described here was that the supernatants were clear and colorless. The procedure used in our earlier study [2] could not abolish the absorbance caused by hemoglobin; and, particularly in the extracts from the autopsy specimens, turbidity often appeared. The ammonium sulfate-chloroform shaking was preliminary tested with several autopsy specimens from traumatic skin lesions: it resulted in all cases in a clear, colorless supernatant with persistent enzyme activity.

In the estimation of the hemoglobin content of the extracts from the specimens, hemin analysis was used. In that method, absorbance is measured both before and after the addition of sodium dithionite; and the amount of hemin is related to the change in absorbance. In this way it was possible to eliminate most of the harmful effects of the turbidity in the extracts. Although myeloperoxidase is also a hemoprotein, the maximum absorption of the myeloperoxidase pyridine hemochromogen (586 mμ) is different from that of the reduced hemin pyridine hemochromogen (558 mμ) [8]. Therefore the presence of myeloperoxidase should not result in errors in the hemin analysis. Knowledge of the hemoglobin level in the specimens was necessary because of the need to dilute the extracts before the hemoglobin precipitation in some cases, and also in order to estimate the average values of the contaminating blood leukocytes in the specimens. The subtraction of the contaminating blood leukocyte activity from the analysis result seems to be necessary if the activity level in the specimens is very low, as in the experimental wounds during first hour, or if the amount of blood is very large, as it could be in some traumatic autopsy lesions. If the activity level in the specimens is higher, the contaminating activity is only about 1% or less of the analysis result, which is negligible.

The hemoglobin removal method described here also appeared to be applicable to the autopsy material, according to our preliminary observations. The hemin analysis can also be used with the autopsy specimens. However, the enzyme activity values of rat blood are naturally not applicable for use in autopsies, because in human blood the granulocyte number is about twice that in rat blood [7, 9]. Further, there could also be differences in the peroxidase activity between human and rat granulocytes. Thus, in human samples the corresponding values recorded in human blood must be used. It might also be as well take the activity in blood monocytes into consideration, although it is known that, at least in human blood, the myeloperoxidase activity caused by monocytes is very low compared with that caused by granulocytes [10].

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