AN INVESTIGATION INTO THE REMOVAL OF ENZYMES FROM PAPER FOLLOWING CONSERVATION TREATMENT*

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ABSTRACT--The efficiency of rinsing two different α–amylases out of paper after conservation treatment was measured using radioactively labeled enzymes. The radioactive enzymes were used to treat small samples of Whatman and Japanese papers. The papers were subjected to various rinsing procedures and the radioactivity remaining in the paper samples was determined. Buffers were used to maintain the pH of the enzyme solutions and their effect in the removability of the enzymes was analyzed. Two water rinses were shown to be sufficient in removing more than 99% of both enzymes tested under optimal conditions. It was also found that increasing the concentration of the enzyme solution resulted in an increase in retention of enzymes in the paper.

Enzymes are often used in paper conservation to aid in the removal of adhesive residues from previous repairs or to facilitate removal of secondary backing supports and mats. It is often necessary to remove these adhesive materials from paper artifacts because they may lead to deterioration of the paper as they age. The enzyme amylase is used to remove starch-based adhesives and proteases are used to remove protein-based adhesives such as animal glues.

The major thrust of research previously undertaken on enzymes has been on the application and use of various types of enzymes for treatment of artifacts in paper and painting conservation. The recommended procedure following an enzyme treatment has been to rinse the paper with room temperature water. In addition, some conservators employ an alcohol or hot water rinse to denature any residual enzymes. Quantitative analysis has not yet been undertaken to determine the extent to which enzymes are removed from paper artifacts after rinsing alone.

The aim of this research was to establish whether enzymes are in fact removed from paper after rinsing with water. This was done by radioactively tagging the enzymes with the radioactive iodine isotope, iodine 125, and determining if any radioactive enzyme was left in the paper after rinsing.

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MATERIALS AND METHODS

We chose to concentrate on two α–amylases used on two types of papers, a dense, wove Whatman paper from 1891 [1] and *Kizukishi* [2], a Japanese paper. The paper samples were coated on one side with a dilute solution of wheat starch paste [3], air dried and then artificially aged for a period of seven days in the Blue M Environmental Chamber at a temperature of 182° F and 59% relative humidity. The outer edges of the paper samples were trimmed and discarded and then the samples were cut into 1 cm square pieces.

Because radioactive materials were being used, large sheets of paper which would more realistically resemble an artifact could not be used. Maneuvering radioactive papers of such a large size would have been impossible behind the lead shields and difficult to dispose of. The ratio of surface area of the sample to the volume of enzyme solutions and rinses used were estimated using a large sheet of paper. The ratio used in the experiments was 1 milliliter of solution per square centimeter of paper.

The two amylases for the study were purchased from Sigma Chemical Co. They were a highly purified bacterial enzyme from a *Bacillus* species (A6380) [4] and a cruder fungal enzyme preparation from *Aspergillus oryzae* (A0273) [5].

The enzymes were iodinated by the chloramine-T method (a procedure not related to chloramine-T bleaching), illustrated in Figure 1 below [6,7]. The details of the procedure are as follows: 0.5 millicurie (mCi) of $Na^{125}I$ (5 µl) was added to 5 µl of deionized H_2O and 10 µl of 0.25 M sodium phosphate, pH 7.5. This solution was stirred in a polypropylene test tube and the following solutions were added in sequence: 10 µl of 0.5 mg protein/ml amylase in 0.05 M sodium phosphate pH 7.5; 10 µl of 5 mg/ml chloramine-T in 0.05 M sodium phosphate pH 7.5; and 100 µl of 1.2 mg/ml sodium metabisulfite in the same phosphate buffer. Buffers were used to control the pH of the solution for optimum efficiency of the enzymes. To this solution was added 100 µl of a 10 mg protein/ml solution of the amylase.

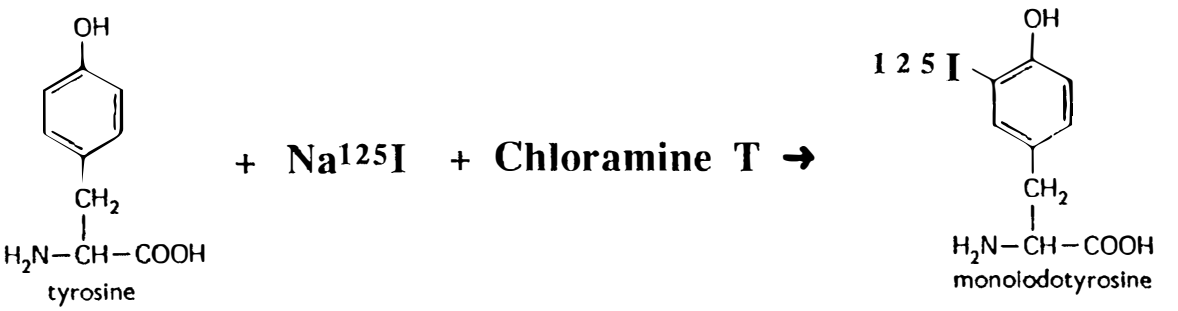

Figure 1 -- Iodination of tyrosine by the chloramine-T method

To separate unincorporated ^{125}I from the iodinated protein a centrifuge column packed with Sephadex G-25 fine was used after the method described by Penefsky [8]. Briefly, a 3 ml syringe was plugged with glass wool and filled with a slurry of Sephadex. This was allowed to drain and the column was equilibrated with 10 ml of 0.05 M sodium phosphate, pH 7.5. The syringe column was then spun for 2 minutes in a swinging bucket clinical centrifuge to remove excess buffer from the column. The iodination mixture (0.24 ml) was applied to the top of the Sephadex bed and the column was again spun for 2 minutes at the same rpm. The eluate from the column contained the radioactive enzyme while the unincorporated ^{125}I was retained in the Sephadex bed [9]. A small sample of the radioactive enzyme was added to 5 μg of the nonradioactive enzyme and was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel [10]. The gel was stained for protein by Coomassie blue, dried and exposed to x-ray film overnight.

Exposure of the paper samples to the radioactive enzyme was performed as follows: a nonradioactive solution of the enzyme was prepared, 0.1% (w/v) for the A6380 amylase and 1% (w/v) for the less pure A0273 amylase. The radioactive enzyme was added to this solution to make 100,000-700,000 cpm (counts per minute) per ml (generally 1 μl of the radioactive enzyme was added per ml of enzyme solution). This solution was thoroughly mixed and 1 ml was dispensed onto each paper sample (one square centimeter). The samples were exposed to this solution for 45 minutes, removed, touched briefly to filter paper and put into the first rinse. Rinses were 2 ml each and rinse time was 5 minutes for each rinse. The samples were briefly touched to filter paper between rinses. Two controls were routinely performed: first, a paper sample which was not rinsed and second, a sample which was rinsed on a Millipore suction device with 2 ml of buffer followed by 5 ml of deionized water. This latter control was considered to represent the most thorough rinsing possible. After rinsing, the samples were dried and counted in a Beckmann gamma counter for ^{125}I. A portion of the original enzyme solution was also counted. The percentage of the radioactivity remaining on the paper was obtained by dividing the cpm on the paper after rinsing by the initial cpm per ml of enzyme solution and are listed in Tables 1, 2 and 3.

RESULTS AND DISCUSSION

Iodination of α–amylases--The SDS-PAGE gel of the iodinated amylases is shown in Figure 2. This technique separates proteins on the basis of their molecular weight. The left hand lane contains the molecular weight standards, lane one contains the iodinated A6380 amylase and lane two contains the A0273 amylase. Both of the enzymes gave a major protein band upon staining. The less pure amylase contains other fainter bands, indicating the presence of additional proteins in this preparation. According to the manufacturer only about 25% of the less pure amylase is protein. An autoradiograph of the same gel can be seen in Figure 3. The exposed dark bands on the autoradiograph indicate the presence of material which has been labeled by the radioactive iodine. Comparison of the stained gel with the autoradiograph shows that the proteins, rather than other materials, in both preparations are being specifically iodinated.

Treatment of the paper samples with iodinated amylases-- A total of eight experiments were conducted involving various rinsing procedures and various enzyme buffer solutions. Data from these experiments are compiled in Tables 1 - 3.

α-amylase A6380:

Table 1 contains data gathered on the more pure α–amylase, A6380. Initially the enzyme was used in a 10 mM sodium acetate buffer, pH 5. Sodium acetate, pH 5 was selected because this pH was recommended for α-amylase A0273 in conservation literature [11]. The no rinse samples show that between 1-3% of the total cpm is retained in the paper samples. After two water rinses less than 1% of the total cpm remained on the Whatman paper whereas more than 2% of the total cpm remained on the Japanese paper. This was an unexpected result since the Japanese paper is much less dense than the Whatman paper and was expected to rinse more thoroughly. Further water rinses did not remove appreciably more enzyme from either paper type. It was reasoned that rinsing the paper first with the same buffer in which it was treated might help to keep the enzyme soluble during the rinsing procedure and, thus, more completely remove the enzyme. Rinsing with a buffer was also suggested by an enzymologist at Sigma Corportion. However, it was found that rinsing with the pH 5 sodium acetate buffer first, followed by water rinses was no more effective than water rinses alone. The addition of a final ethanol rinse to denature the residual enzyme did not remove more enzyme. The suction rinse using one buffer rinse, followed by two water rinses, was comparable to the four water rinses.

A search of the biochemical literature on this amylase revealed its pH optimum to be 6 [12, 13] instead of 5 so it was decided to try treating the paper with the enzyme at pH 6 in both a sodium acetate and a sodium phosphate buffer. Phosphate is a more effective buffer than acetate at this pH. The two buffers were tested at both 10 mM and 50 mM. A reduction in the amount of enzyme retained after rinsing was seen at this pH in both paper types. This reduction was particularly dramatic in the case of the Japanese paper, reducing the counts retained by almost twentyfold. Use of either the 50 mM sodium phosphate or the 50 mM sodium acetate buffer at pH 6 resulted in the least amount of counts retained by either of the paper types.

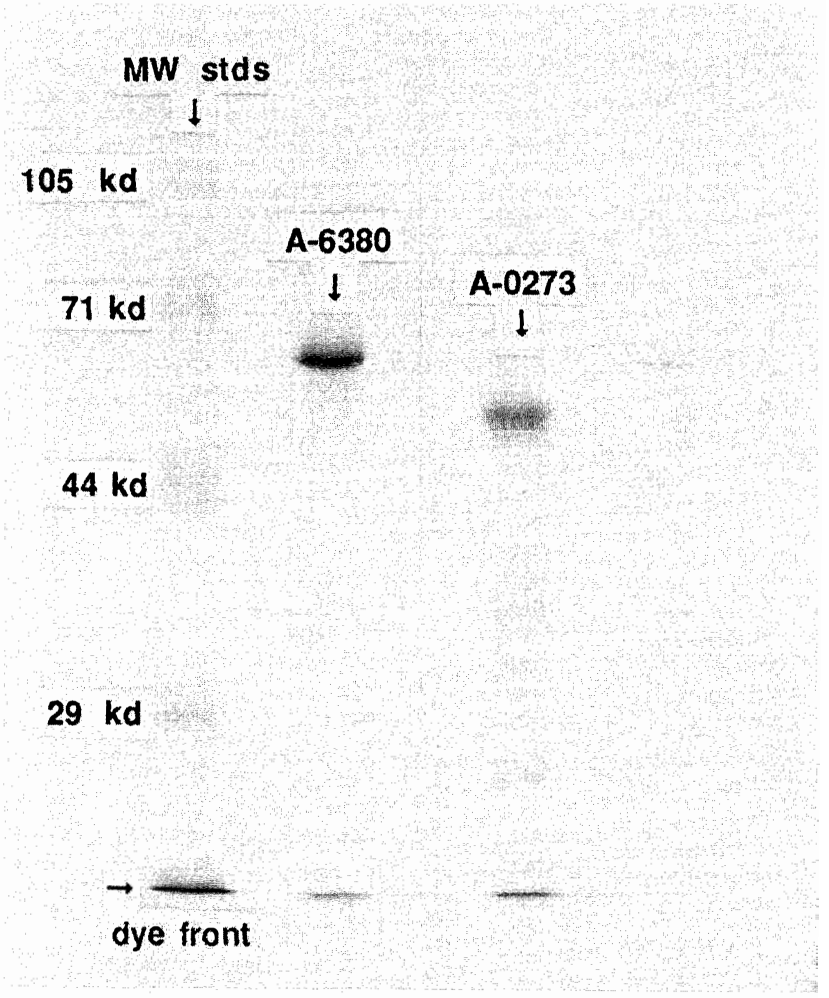

Figure 2 -- SDS-PAGE of two α-amylases

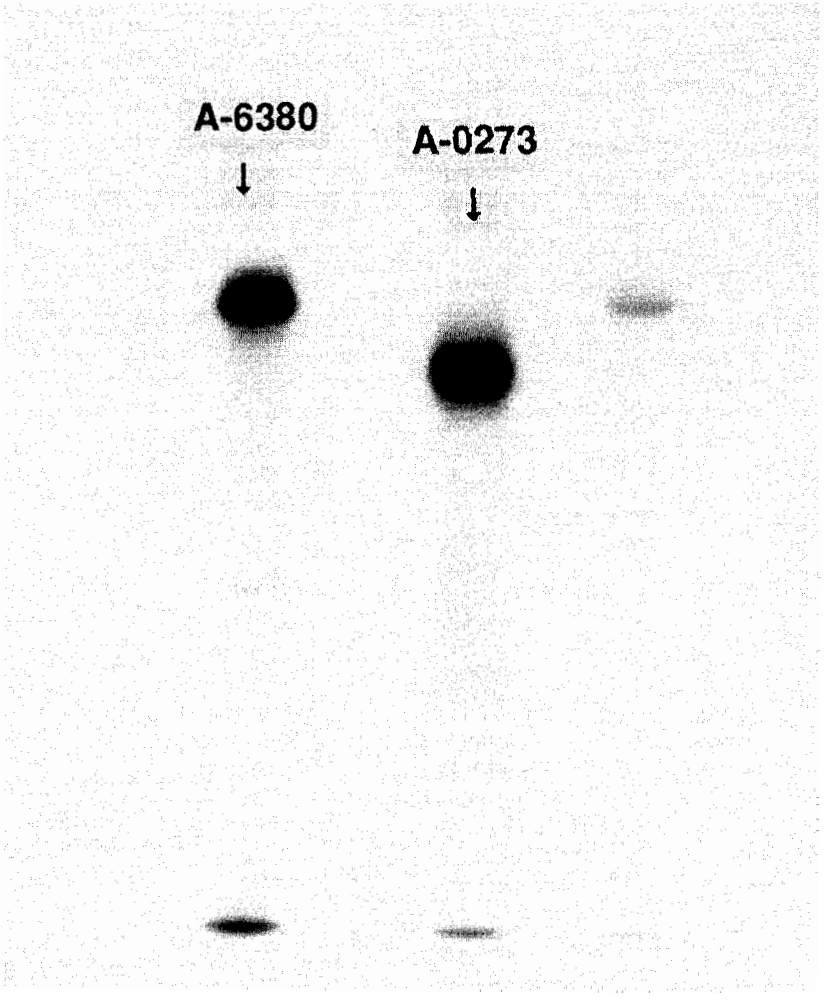

Figure 3 -- Autoradiograph of gel in Fig. 2

TABLE 1

α-AMYLASE A6380

Enzyme Buffer	Rinse	Average % of cpm retained (number samples tested)	
		Whatman	Japanese
10 mM acetate pH 5	no rinse	1.5 (3)	2.2 (2)
	2 water	.71 (4)	2.5 (4)
	3 water	.69 (4)	2.4 (4)
	4 water	.59 (4)	2.2 (4)
	1 buffer* 2 water	.68 (6)	2.7 (5)
	2 buffer* 1 water	.51 (2)	3.3 (2)
	1 buffer* 2 water 1 ethanol	.53 (2)	3.3 (2)
	suction 1 buffer* 2 water	.56 (1)	2.6 (1)
10 mM acetate pH 6	3 water	.30 (2)	.14 (2)
50 mM acetate pH 6	3 water	.22 (2)	.08 (2)
10 mM phosphate pH 6	3 water	.22 (2)	.14 (2)
50 mM phosphate pH 6	3 water	.11 (2)	.08 (2)
	1 buffer* 2 water	.06 (2)	.04 (2)

* rinse buffer is same as the enzyme buffer in each case

An additional experiment was performed with α-amylase A6380 to determine whether or not the binding of enzyme to the paper was due to the presence of substrate (starch) or an inherent property of the paper. This was tested by treating the Whatman and Japanese papers, with and without a paste substrate, with the enzyme in a 10 mM sodium acetate buffer, pH 5. The results of this experiment are presented in Table 2. The presence of the paste substrate on the papers did not significantly affect their retention of radioactivity, indicating that retention was an inherent property of the paper and not caused by the substrate. In this experiment the concentration of the enzyme was also varied to determine the effect of enzyme concentration on retention of radioactivity. It is apparent from the data that increasing the concentration of the enzyme also results in an increase in the retention of radioactivity on the paper.

α-amylase A0273:

The data obtained with less pure α–amylase A0273 is compiled in Table 3. The published pH optimum is 5 [14], so all experiments were performed at this pH. The results for this enzyme confirm that four water rinses were no more efficient than two rinses in removing enzyme from the paper. The use of acetate buffer rinses followed by water rinses was again no more effective than rinsing with water alone. Again, adding a final ethanol rinse or using a suction rinse were no more effective than water rinses alone. Raising the acetate concentration in the enzyme solution to 50 mM did not appreciably reduce the amount of enzyme bound to the Whatman paper. However, changing the buffer solution to 50 mM sodium phosphate reduced the amount of enzyme bound by 60%.

It should be noted with this less pure enzyme that approximately 75% of the solid weight of the preparation is not protein, at least according to the label. Since the iodination labeled only the protein, the other 75% of the preparation is not labeled and therefore not detectable by these experiments.

It was not necessarily a goal of these experiments to remove all of the starch substrate from the paper samples. However, when an iodine reagent test [15] was performed on the paper samples following treatment, it was found for both enzymes that using the 50 mM phosphate buffer rather than acetate buffer with the enzyme was the most effective method for removing all of the substrate within the given 45 minute period.

TABLE 2
α-AMYLASE A6380

Comparison of different % enzyme solutions (w/v) in 10 mM sodium acetate buffer, pH 5, with and without paste substrate on the paper. Two samples of each type paper were treated with the enzyme and rinsed with 1 sodium acetate buffer followed by 2 water rinses.

% Enzyme Solution	% cpm retained in sample			
	Whatman w/paste	Whatman w/o paste	Japanese w/paste	Japanese w/o paste
0.05	.28	.26	1.1	1.1
0.1	.40	.44	1.5	1.7
0.5	1.1	1.4	2.6	2.8

TABLE 3
α-AMYLASE A0273

Enzyme Buffer	Rinses	Average % cpm retained (number of samples tested)	
		Whatman	Japanese
10 mM acetate pH 5	no rinse	1.12 (2)	.41 (2)
	2 water	.38 (5)	.05 (5)
	4 water	.38 (2)	.02 (2)
	1 buffer* 2 water	.49 (2)	.02 (2)
	2 buffer* 1 water	.43 (2)	.02 (2)
	1 buffer* 2 water 1 ethanol	.34 (2)	.01 (2)
	suction 1 buffer* 1 water	.46 (1)	.02 (1)
50 mM acetate pH 5	3 water	.36 (6)	.01 (6)
10 mM phosphate pH 5	3 water	.21 (6)	.02 (6)
50 mM phosphate pH 5	3 water	.16 (6)	.01 (6)

* rinse buffer is same as the enzyme buffer in each case

CONCLUSIONS

It is clear from the data that residual enzyme does remain in the paper, however, in extremely small amounts, on the order of 0.5 microgram enzyme/sq. cm of paper for the more pure amylase and 5 micrograms enzyme/sq. cm for the less pure amylase on the Whatman paper. Whether this residual enzyme is detrimental to the paper is not known and requires further experimentation. The retention of enzyme by the paper appears to be an inherent property of the paper and not necessarily caused by the presence of substrate on the paper. In addition, the amount of enzyme retained by the paper increased as its concentration in the treatment solution was increased. This suggests that the minimum amount of enzyme should be used when treating a paper artifact, although in practical situations this amount may be difficult to determine.

The data further suggests that sodium phosphate buffer, not sodium acetate as mentioned in some conservation literature, is a more effective buffer for these enzymes both in the removal of substrate from the paper and the subsequent removal of the enzymes from the paper. Two water washes appear to be sufficient for rinsing out both enzymes. It should be noted that all enzymes used in this investigation were used in buffered solutions. The effect of using the enzymes in unbuffered water was not investigated. It should also be noted, however, that the effect of buffer choice on the future stability of paper artifacts was not a focus of this investigation. Further experimentation is needed to ensure that the use of phosphate buffers is safe with paper artifacts.

It is not known whether this data will be generally applicable to all enzymes, such as proteases. Similar experiments using these enzymes should be performed to determine the preferred buffer for these enzymes.

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NOTE: This research was carried out by Theresa Meyer Andrews as a partial requirement for the degree of Master of Arts and Certificate of Advanced Study in Art Conservation expected in 1991 from the Art Conservation Department of the State University College at Buffalo.

ENDNOTES

[1] Paper with following watermark: J Whatman 1891. Cotton fiber, gelatin and alum present.

[2] *Kizukishi,* 100% *kozo,* Japanese paper, distributed by Conservation Materials Ltd., Sparks, NV.

[3] *Zin shofu,* precipitated wheat starch, distributed by Conservation Materials Ltd., Sparks, NV. 15% paste after cooking, used diluted to the consistency of skim milk.

[4] α–amylase, [From *Bacillus* species, A6380, Type II-A], Sigma Chemical Co., St. Louis, MO.

[5] α–amylase, [Fungal; Crude, From *Aspergillus oryzae*, A0273, Type X-A], Sigma Chemical Co., St. Louis, MO.

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[9] NOTE: The correct rpm setting for any particular centrifuge can be ascertained by spinning trial columns with a mixture of blue dextran and potassium ferricyanide. The optimal rpm is the minimum which allows the dextran (blue) to elute from the column while retaining the ferricyanide (yellow) on the column. In general 1000 rpm is sufficient.

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